Universidade de Aveiro
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FÁBIO ANDRÉ MARQUES DE CAMPOS

HARMONIZING METHODS TO ASSESS THE ECOTOXICITY OF LAYERED DOUBLE HYDROXIDES NANOMATERIALS IN A FRESHWATER MICROALGAE

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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 da Professora Doutora Susana Patrícia Mendes Loureiro, Professora Auxiliar com Agregação do Departamento de Biologia e do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro, e co-orientação do Doutor Roberto Carlos Domingues Martins, Investigador Auxiliar do Departamento de Biologia e Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro.

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palavras-chave

nanomateriais; nanoargilas; metodologias; regulamentação; ecotoxicologia aquática

resumo

A nanotecnologia tem sofrido um crescimento sem precedentes, marcado pelo desenvolvimento de nanomateriais inovadores com características físico-químicas únicas para uma ampla gama de aplicações. Os hidróxidos duplos-lamelares (LDHs) são um excelente exemplo dessa inovação. Atualmente, as metodologias de avaliação de risco são baseadas em metodologias de avaliação desenvolvidas para substâncias convencionais e não têm em conta as especificidades exibidas pelos nanomateriais. Os nanomateriais estão enquadrados como substâncias nos regulamentos europeus REACH (Registo, Avaliação, Autorização e Restrição de Químicos) e CRE (Classificação, Registo e Embalagem), sendo-lhes aplicáveis as disposições constantes em ambos os documentos. Assim, a avaliação do risco dos nanomateriais é crucial para o seu registo e posterior comercialização. No entanto, a avaliação de risco dos nanomateriais no âmbito REACH é baseada em metodologias para compostos convencionais o que pode originar resultados com baixa relevância e confiabilidade. Por isso, o presente estudo visou avaliar o impacto de duas metodologias de exposição recomendadas pela OCDE (diluições em série a partir de uma solução stock vs. adição direta de nanomaterial a cada concentração individual), no crescimento da microalga de água doce *Raphidocelis subcapitata* após a exposição a uma gama de concentrações de Zn-Al LDH e Cu-Al LDH. Adicionalmente, pós com diferentes tamanhos de grão (heterogéneo; <25, 25-63, 63-125, 125-250 e, >250 µm) de ambos os LDHs foram utilizados na exposição à mesma microalga, considerando uma possível toxicidade dependente do tamanho do grão.

Relativamente às metodologias, foram observadas diferenças na toxicidade obtida entre ambas as metodologias de exposição para Zn-Al LDH, contudo, nenhuma diferença foi detetada no caso do Cu-Al LDH. Relativamente aos tamanhos de grão nos pós de LDHs, a toxicidade do Zn-Al LDH foi similar para todos os tamanhos, contudo, os 3 tamanhos maiores do Cu-Al LDH produziram uma menor toxicidade comparando com a amostra heterogénea. Assim sendo, a separação do Cu-Al LDH por tamanhos granulométricos poderá ser uma vantagem para a formulação de uma nanomaterial mais amigo do ambiente. Os resultados da metodologia de exposição constituem um bom ponto de partida para a elaboração de adaptações das metodologias a realizar em testes ecotoxicológicos com algas envolvendo LDHs. Estudos futuros com outras metodologias de exposição, meios de exposição e diferentes nanomateriais permitirão atingir uma padronização mais precisa das metodologias de teste para nanomateriais.

keywords

nanomaterials; nanoclays; test metodologies; regulamentation; aquatic ecotoxicology

abstract

Nanotechnology has experienced an unprecedent growth, marked by the development of innovative nanomaterials with unique physicochemical characteristics for a wide range of applications. Layered double hydroxides (LDH) are a great example of such innovation. Currently, risk assessment methodologies are based on test methods developed for conventional chemical's characteristics and do not account for the specificities that nanomaterials exhibit. Under REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) and CLP (Classification, Labelling and Packaging of substances) EU regulations, nanomaterials are regarded as substances. The chemical safety assessment of nanomaterials is thus crucial to meet the needs for their registration and posterior commercialization. The safety assessment performed for REACH, based on the current non nano-specific ecotoxicological test methodologies might produce data with low relevance and reliability. Thus, the present study aimed to assess the impact of two different exposure methodologies, recommended by OECD (serial dilutions from a stock dispersion vs direct nanomaterial addition to each individual concentration), in the growth of the freshwater microalgae *Raphidocelis subcapitata* exposed to Zn-Al and Cu-Al LDHs. Moreover, different powder grain sizes (bulk, <25. 25-63, 63-125, 125-250 and, >250 µm) of both LDHs were used for the exposure of the same microalgae regarding a possible grain size-dependent toxicity.

Regarding exposure methodologies, differences were observed in the toxicity of Zn-Al LDH, however, there is no methodological difference in the toxicity output for Cu-Al LDH exposure. Zn-Al LDH toxicity was similar for all grain sizes, however, the 3 highest grain sizes of Cu-Al LDH produced lower toxicity when comparing to the bulk counterpart. Thus, a grain size separation for Cu-Al LDH might be of great advantage towards the formulation of an eco-friendlier nanomaterial.

Exposure methodology results pose as a good starting point for ecotoxicological testing involving LDHs. Additionally, other studies with exposure methodologies and media, and different nanomaterials are needed, in order to achieve better standardization procedures for nano-specific test methodologies.

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Abbreviations

Listed alphabetically

AFM	Atomic Force Microscopy
Ag	Silver
Al	Aluminium
Al_2O_3	Aluminium oxide
ANOVA	Analysis of Variance
ASTM	American Society for Testing and Materials
Au	Gold
Cr	Chromium
Cu	Copper
CuO	Copper oxide
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic acid
EC_{50}	Median Effective Concentrations
EDTA	Ethylenediamine Tetraacetic Acid
EU	European Union
Fe	Iron
GD	Guidance Document
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
ISO	International Standardisation Organisation
LDH	Layered Double Hydroxide
LOEC	Lowest Observable Effect Concentration
MBT	Mercaptobenzothiazole
Mg	Magnesium
Ni	Nickel
NM	Nanomaterial
NOEC	No Observable Effect Concentration
OECD	Organisation for Economic Co-operation and Development
PBT	Persistent, Bioaccumulative, and Toxic

PEC	Predicted Environmental Concentration
PID	Proportional-Integral-Derivative
PNEC	Predicted No-Effect Concentration
REACH	Registration, Evaluation, and Authorization of Chemicals
ROS	Reactive Oxygen Species
SPM	Scanning Probes Microscope
STM	Scanning Tunnel Microscope
TEM	Transmission Electron Microscopy
TG	Test Guideline
USFDA	United States Food and Drug Administration
UV	Ultraviolet
vPvB	Very Persistent, very Bioaccumulative
Zn	Zinc
ZnO	Zinc oxide

Chapter I

General Introduction

1. General Introduction

1.1. Nanotechnology History: A Brief Overview

In 1959, at the California Institute of Technology (Caltech), during the annual winter meeting of the American Physical Society, the Nobel prize laureate and American physicist Richard Feynman yielded a talk that would change the way that most researchers view and perceive space and their notion of dimensions and scales (Feynman, 1960). In the talk entitled "There's Plenty of Room at the Bottom", Feynman envisioned the possibility of manipulating materials at individual atomic and molecular scales, creating large products from the "bottom-up", atom-by-atom or molecule-by-molecule, virtually equal at the atomic level (Feynman, 1960; Iqbal et al., 2012; Peterson, 2004). Moreover, Feynman explained that, according to the laws of quantum physics, the "plenty of room" that he uncovered could become even greater if researchers could manage to decrease even more the size of the items that inhabit within the nanoscale, and consequentially leaving more "room" to be populated (Feynman, 1960).

According to Drexler (2004), Feynman's revolutionary vision "launched the global nanotechnological race", laying the foundations for the development of the nanotechnological field of science (Toumey, 2008; Wickson, 2008). Such impactful vision lead several authors to name Feynman as the creator of the nanotechnological science field (e.g. Gleick, 1993; Kurzweil, 2005; Wickson, 2008).

However, and despite of Feynman's discovery, nanostructures already existed since the Earth's dawn. Some authors refer that the very personification of such existence is the simple molecules of DNA (deoxyribonucleic acid), a nature assembled nanomaterial which codes for the very life's existence (Gnach et al., 2015; Wickson, 2008). Such idea lead to the creation of the DNA nanotechnology, a research area which intensively studies all of DNA's potential to self-assembly programable and predictable nanostructures, exhibited by DNA's hydrogen bonding between nitrogenous base pairs of adenine with thymine and guanine with cytosine (Gnach et al., 2015; Seeman, 2010), to aid in the manufacturing of several recent dynamic structures with nanometer-scale sized features (e.g. biosensors, nanowires) (Aldaye et al., 2008; Pinheiro et al., 2011; Shih and Lin, 2010). Moreover, humans have been using nanostructures since the fourth century, as Romans showed one of the most interesting achievements of nanotechnology used in the ancient glass industry, the Lycurgus cup. This relic is considered to be one of the oldest examples of synthetic nanomaterials and resembles a dichroic glass, which changes colour under certain specific light conditions: under direct light it appears green, and red-purple if light shines through it from behind (Figure 1.1) (Bayda et al., 2020; Freestone et al., 2007; Mansoori and Fauzi Soelaiman, 2005). In 1990, a team of scientists analysed the cup using a transmission electron microscope (TEM) and determined that the changing colours are the result of the presence of nanoparticles of silver-gold (Ag-Au) alloy, with a diameter of 50-10 nm, additionally containing 10% copper (Cu) scattered in the glass matrix (Barber and Freestone, 1990; Freestone et al., 2007; Wagner et al., 2000). A similar effect, derived from the use of Ag and Au nanoparticles, is also observed in late medieval church stained glass windows (Mulfinger et al., 2007). Other examples of ancient nanotechnology, to which medieval artisans and forgers were unaware, can include: i) the use of nanoparticles by Italian craftsmen to create Renaissance pottery in the 16th century (Nasrollahzadeh et al., 2019), ii) the inclusion of cementite nanowires and carbon nanotubes in the forging process of “Damascus” sabres by Ottomans, which provided strength and resilience to the blade’s edge (Reibold et al., 2006), or iii) the carbon nanoparticles presence in the ink spread across the Ajanta cave paintings (Ochekpe et al., 2009).

Figure 1.1 - The Lycurgus cup, stored in the British National Museum. The cup appears mate green in direct light (A) and red if light shines through it (B). Picture adapted from Bayda et al. (2020).

Overtime and after Feynman’s ideas, the human awareness of the nanoworld grew, as more recent nanotechnological events unfolded. In modern nanotechnological history, the development the scanning tunnel microscope (STM) in 1991 by IBM

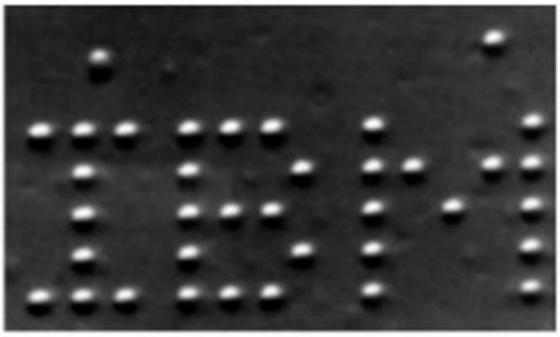

Figure 1.2 - Nanoscale IBM logo manufactured in a nickel substrate by manipulating 35 Xenon atoms. Reproduced from Eigler and Schweizer (1990).

researchers Binnig and Rohrer, became one of the first and most representative events (Binnig et al., 1982a, 1982b). This discovery was further publicised to the scientific community, as IBM researcher Don Eigler applied the STM into the manufacturing of a nanoscale IBM logo on a nickel surface by manipulating precisely 35 individual xenon atoms (Figure 1.2) (Eigler and Schweizer, 1990). Due to these events, other discoveries were possible, such as, the creation of the atomic force microscope (AFM) and scanning probes microscopes (SPM), which are nowadays instruments of choice for nanotechnological research (Bayda et al., 2020; Binnig et al., 1986). Later, in 1985, Robert Curl, Harold Kroto, and Richard Smalley found that carbon can also occur in the form of stable spheres, the fullerenes (Kroto et al., 1985); six years later, Iijima (1991) discovered the carbon nanotubes, other fullerene family member, which is currently very used as nanocomposite fibres in cement and concrete to improve mechanical and thermal stability, or in the energy storage and electronics industries (Bayda et al., 2020). With the course of time, new discoveries of promising nanomaterials, with a wide set of applications for different areas, varying from nano-oncology, biomedicine and diagnostics to environmental engineering, pharmaceutics, and electronics (Bayda et al., 2020; Kinnear et al., 2017; Yan Lee and K.Y. Wong, 2011; Yuan et al., 2019).

Currently, nanotechnology is overviewed as an emerging and promising modern science which is present and already impacts the human quotidian life (Hulla et al., 2015). In the future, the development of novel nanomaterials has to be performed in tandem with proper environmental and human risk assessment in order to ensure their safety (Seaton and Donaldson, 2005).

1.2. Nanotechnology, nanoscience, and nanomaterials

Nowadays, the definition of nanotechnology and nanoscience, and how to distinguish the two concepts, has been a matter of debate by the scientific community (Mulvaney, 2015). This matter gains great importance and relevance as the nano-field of research is experiencing an outstanding unprecedented growth, producing not only valuable nanomaterials (NMs) and other nanoproducts, but also precious knowledge that can be extended to a great number of other scientific fields (e.g. quantum physics) (Gleiter, 2009; Inshakova and Inshakov, 2017).

Many researchers defend that Feynman was the first to define “nanotechnology” during the scientist’s famous 1959 presentation “There’s Plenty of Room at the Bottom” (see section 1.1 for more detailed presentation overview). However Feynman did not exactly use the term, instead, he used terms such as “small scale”, “miniaturization” or "small things" (Feynman, 1960; Ochekpe et al., 2009; Smith, 2006). The first-ever report on the term "nanotechnology" dates back to 1974, belonging its authorship to the Japanese scientist Norio Taniguchi, which defined it as the "processes of separation, consolidation, and deformation of materials by one atom or one molecule" (Mulvaney, 2015; Taniguchi et al., 1974). However, such a definition is extremely vague and outdated and does not define the current state and main purpose of the nanotechnology field. Currently, one of the most accepted definitions for nanotechnology is given by the Organisation for Economic Co-operation and Development (OECD), which defines it as a "set of technologies that enables the manipulation, study or exploitation of very small (typically less than 100 nm) structures and systems" (Cheng et al., 2016). This OECD definition suggests the existence of two conditions: i) the scale of the materials, as nanotechnology deals with shapes and sizes of materials at a nanometer scale, and ii) the NMs' novelty and applications, as nanotechnology aims to take advantage of the NMs' properties by applying them in numerous science and industrial fields (Allhoff, 2009; Bayda et al., 2020; Cheng et al., 2016; Mansoori and Fauzi Soelaiman, 2005). The latter condition (i.e. novelty and applications) is the main difference to distinguish between nanotechnology and nanoscience, as the American Society for Testing and Materials (ASTM) defines nanoscience as the study of the properties of materials and molecules on the nanometric scale, ranging between 1 and 100 nm. In the nanoscience field, no application must derive from such study, whereas in the nanotechnology field the existence of applications for the studied NMs is mandatory (Mansoori and Fauzi Soelaiman, 2005; Mulvaney, 2015).

The definition of "nanomaterial" is also not consensual across the scientific community and regulatory entities/agencies, such as the European Union (EU) agencies, the United States Food and Drug Administration (USFDA) or the International Organization for Standardization (ISO) (Jeevanandam et al., 2018). An internationally accepted definition for the term "nanomaterial" does not exist yet (Jeevanandam et al., 2018). In the present work, the EU Commission definition will be adopted. Since 2011, the EU Commission defines "nanomaterial" as "a natural, incidental, or manufactured material containing particles, in an unbound state or as an aggregate or as an 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 1–100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %" (2011/696/EU). Currently, NMs are further classified into different groups and classes based on different criteria. Commonly, NMs are classified according to: i) dimensionality, ii) morphology, iii) state of agglomeration and uniformity and, iv) chemical composition, as firstly purposed by Gleiter (2000).

Regarding the dimensionality, NMs can be classified into four categories: i) zero-dimensional nanomaterials (0D), having all their dimensions in the nanoscale, i.e., below 100 nm (e.g. spherical or quasi-spherical nanoparticles, hollow sphere or core-shell nanomaterials); ii) one-dimensional nanomaterials (1D), having one dimension outside of the nanoscale while the other two dimensions are inside the nanoscale (e.g. ceramic nanotubes, nanowires, nanofibers, nanorods); iii) two-dimensional nanomaterials (2D), with only one dimension in the nanoscale whereas the other two are not (e.g. layered double hydroxides and other nanoclays, single- or multi-layered nanoplates, nanocoatings); iv) three-dimensional (3D) nanomaterials, possessing various dimensions past the 100 nm scale, as they combine multiple nanocrystal structures in different directions (e.g. graphite, carbon nanobuds, nanotubes, liposomes) (Aversa et al., 2018; Pokropivny and Skorokhod, 2007).

Concerning their morphology, NMs are classified in low- and high aspect ratio particles. High aspect ratio particles may have different shapes, such as nanohelices or nanotubes. Low aspect ratio particles also may have different shapes and include nanomaterials such as nanospheres (Buzea and Pacheco, 2017; Pokropivny and Skorokhod, 2007).

As per uniformity and agglomeration state, NMs are categorized in isometric and inhomogeneous, regarding their uniformity, or dispersed and agglomerate, concerning the aggregation state. Furthermore, agglomeration state relies on the electromagnetic properties, magnetism, and surface charge of NMs. In liquids, the NM agglomeration also depends on the surface morphology and functionalization, which confers either hydrophilicity or hydrophobicity (Buzea and Pacheco, 2017; Saleh, 2020).

Based on their chemical composition, NMs can be grouped into 3 categories: i) single constituent nanoparticles (NPs), ii) nanocomposites, iii) nanoheterostructures, iv) organic or inorganic nanomaterials, under which exist further subcategories, a) carbonaceous NMs, b) metallic and metal oxide NMs, c) branched dendrimers, d) quantum dots, e) ceramic NMs, f) semiconductor NMs, g) polymeric NMs, h) nanogels, and v) core-shell NMs (Azevedo et al., 2017; Buzea and Pacheco, 2017; Gleiter, 2000; Saleh, 2020).

Due to their extraordinary physicochemical properties, nanomaterials have a plethora of applications throughout various scientific and industrial fields. For example, different metallic and metal oxide nanoparticles (e.g. silver (Marx and Barillo, 2014), zinc oxide (Saliani et al., 2015), palladium (Adams et al., 2014), or carbon (Dizaj et al., 2015)) have been studied for their antimicrobial properties against a multitude of microorganisms, such as *Staphylococcus aureus* or *Escherichia coli* (Adams et al., 2014; Seil and Webster, 2012, respectively). Industrial applications may include the use of NMs in the construction industry in cement, coatings, paintings, and insulating materials (Bahadar et al., 2016; Stark et al., 2015), as electrocatalysts for energy storage and conversion (Dai et al., 2012), in textile and food industries as antifungal clothes (Anghel et al., 2012) or food packaging solutions (Hannon et al., 2015), respectively, or as treatments for photoaging and hyperpigmentation in the cosmetic industry (Lohani et al., 2014).

1.3. Layered double hydroxides: old nanomaterials with renewed potential

1.3.1 Brief historical overview and main physicochemical characteristics

Layered Double Hydroxides (LDHs), also known as anionic clays, are a class of promising lamellar NMs which are attracting great attention of the scientific community and industry (Cavani et al., 1991; Evans and Slade, 2005).

LDHs were discovered in 1842, in Sweden, as naturally occurring materials, being hydrotalcite the first identified LDH. However, only in 1915, the exact formula for hydrotalcite ($[Mg_6Al_2(OH)_{16}]CO_3{\cdot}4H_2O$) was discovered by Manasse (1915). The interest in LDHs' chemical characteristics increased in 1942 as Feitknecht published a series of papers on LDHs, naming them as “doppelschichtstrukturen” (double sheet structures). Feitknecht described that these materials were structurally made up of layers of one metal, intercalated with layers of a second metal (Feitknecht, 1942; Khan and O'Hare, 2002a). This model was later disproved by Allmann, after performing cristal X-ray diffraction on pyroaurite, confirming that both metal cations were present in the same layer (Allmann, 1968).

Generally, LDHs are represented by the formula $[M_{1-x}^{2+}M_x^{3+}(OH)_2]^{x+}[A^{n-}]_{x/n}{\cdot}mH_2O$, where M^{2+} (e.g. Mg^{2+},Ni^{2+}) and M^{3+} (e.g. Al^{3+},Fe^{3+},Cr^{3+}) are the divalent and trivalent cations, respectively, x is the ratio of $M^{3+}/(M^{2+}+M^{3+})$, and A^{n-} in the interlamellar layer is the exchangeable n-valent anion (e.g. CO_3^{2-}, NO_3^-, Cl^-, HPO_4^{2-}, SO_4^{2-}, $SiO(OH)_3^{4-}$) (Cavani et al., 1991; Khan and O'Hare, 2002a; Mishra et al., 2018). The general structure of LDHs is represented in Figure 1.3.

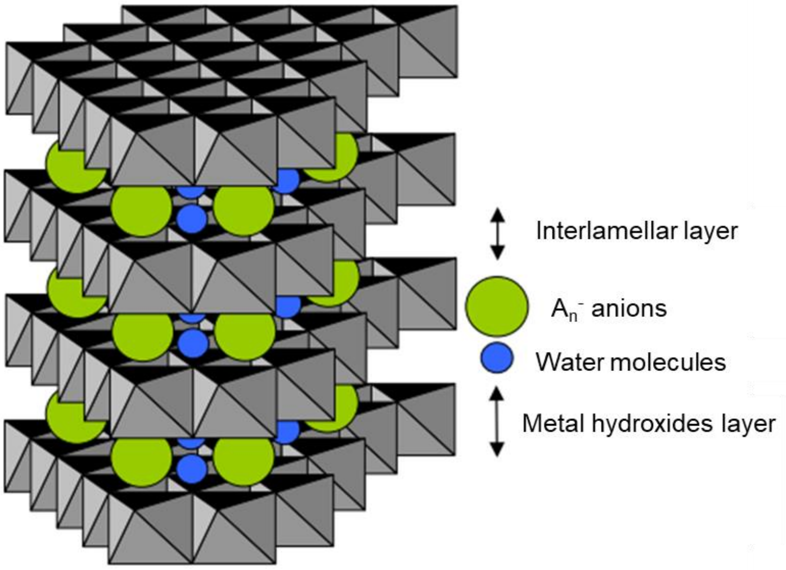

Figure 1.3 - Schematic representation of the general of an LDH's crystalline structure. Adapted from Goh et al. (2008).

There is a great variety of synthesis methods for obtaining LDHs, such as sol-gel (Panda et al., 2011), urea hydrolysis (Jitianu et al., 2013), and hydrothermal synthesis (Huang et al., 2015), being the most conventional the coprecipitation method (Ogawa and Kaiho, 2002). The coprecipitation has numerous advantages. It allows obtaining LDHs with a high level of purity and a high crystallinity (Bukhtiyarova, 2019). Moreover, the coprecipitation method is more suitable to be applied for large-scale production. (Pang et al., 2019). Generally, coprecipitation of LDHs can be deemed as the joint precipitation of $M(OH)_2$ and $M(OH)_3$, where metal salts (with divalent and trivalent metals in their composition) are employed as precursors to the nanomaterial's formation (Bukhtiyarova, 2019). Different chemical compounds can be used as precipitating agents: i) ammonia (Olanrewaju et al., 2000), ii) combination of alkali and the corresponding metallic carbonate (Sharma et al., 2008), or even iii) alkali itself (Álvarez et al., 2012). In order to LDH stabilized by nitrates (i.e., nitrate anions in the interlamellar layer) the precipitation has to be performed with metal nitrates, however, if the precipitation is performed in the presence of CO_2, a mixture of nitrate and carbonate forms will be synthesized, compromising a desirable batch of pure LDH in its nitrate form. In order to obtain pure LDH-nitrates, the methodology must be performed in a CO_2-free environment (Rezvani et al., 2014; Tamura et al., 2004). Other anions might be intercalated in the interlamellar region, such as halides (e.g. SO^{4-} or ClO^{3-}) (Brindley, 1980; Miyata, 1983), or silicates (e.g. $SiO(OH)_3^-$) (Khan and O'Hare, 2002b).

1.3.2 Layered double hydroxides applications

Recently, LDHs have deserved a lot of attention due to their wide range of possible applications in various fields and controllable physicochemical properties (Khan and O'Hare, 2002b; Rives, 2001). One of the advantages of LDHs amongst layered materials is a large number of possible compositions and metal-anion combinations that can be synthesized. Apart from that, LDHs have unique physicochemical characteristics like good biocompatibility, pH-dependent solubility, high chemical stability, ion adsorption, and anionic exchangeability, the latter standing out as the most extraordinary characteristic (Kuthati et al., 2015; Mishra et al., 2018). The LDHs anionic exchangeability mainly consists in exchanging the stabilizing anions intercalated in the interlamellar layer by anions present in the surrounding environment. This property allows controlling the release of intercalated active compounds in the anionic form (e.g. biocides, corrosion inhibitors, perfumes, dyes) upon the presence of specific triggers (e.g. water, pH changes). Due to weak hydrogen bonding between the hydroxyl groups of the metal hydroxide layer and the anion present in the interlayer, such process occurs with great success, as the LDH becomes chemically more stable with the exchange of

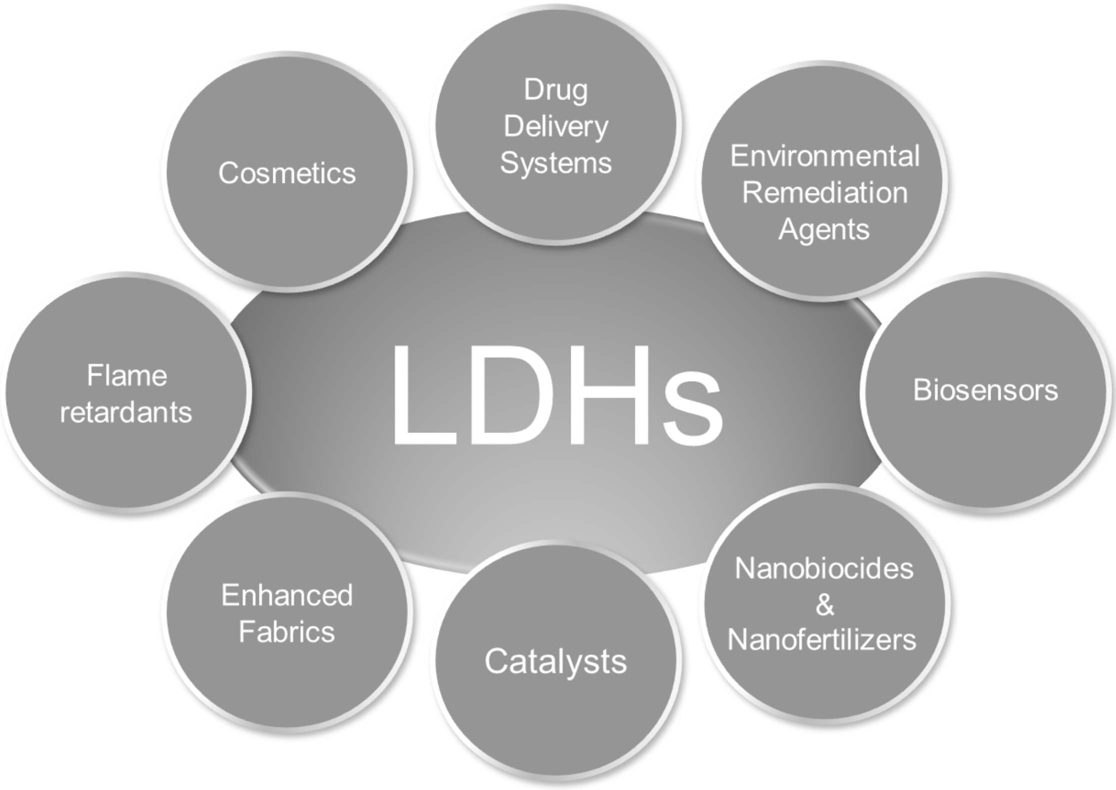

Figure 1.4 - Schematic representation of the most common LDHs' applications

the interlamellar layer native anions by environment present anions (Radha et al., 2005; Taylor, 1973).

Such flexible functional diversity qualifies LDHs to a great number of applications, e.g. halogen-free flame retardants, ion exchangers, adsorbents, antacids, drug delivery systems, biosensors, nanocarriers with anti-biocidal or anti-corrosion properties (depending on the entrapped active compound), among many others (Mishra et al., 2018; Yin et al., 2010). A general overview of the LDHs' applications is schematized in Figure 1.4.

1.3.2.1 Environmental applications

Recently, LDHs have been extensively studied as promising nanomaterials for a wide range of environmental applications.

A great number of studies found in the literature, depict the use of LDHs to increase the air quality, as adsorbents for airborne oxides and dust. For example, Yang and co-workers reported the ability of Mg-Al LDH to be a very promising nanomaterial to remove nitrate oxide species and soot, generated by the fuel combustion of motorized vehicles (Yang et al., 2014).

Moreover, LDHs are also promising nanomaterials to be applied in wastewater treatment, as they can remove organic and inorganic toxic chemicals from wastewaters, through ion adsorption or anionic exchange. The removal of metals, metalloids, and non-metals, such as arsenic, chromium, mercury, selenium, radioactive uranium has already been demonstrated (Asiabi et al., 2018; Pshinko, 2013; You et al., 2001a). A study conducted by You et al. (2001) reported that calcinated Mg-Al LDH significantly removed, by ion adsorption, the arsenic present in an aqueous solution. In another study, chromium was strongly absorbed by calcinated Mg-Al, Ni-Al and Zn-Cr LDHs (Goswamee et al., 1998). LDHs show a remarkable potential to remove organic compounds, such as nitrogen species (Kameda et al., 2009; Liu et al., 2020), 2-chlorophenol and other phenolic compounds (Chuang et al., 2008). Khenifi and colleagues showed the removal of organophosphate and organophosphate herbicides (glyphosate and glufosinate, respectively) from laboratory prepared aqueous solutions using a Ni-Al LDH (Khenifi et al., 2010). Moreover, LDHs have also the capacity to remove herbicides and pesticides from soil and wastewater coming out of agricultural fields. Chaara et al. (2010) and Legrouri et al. (2005) have demonstrated such capacity by using Mg-Al and Zn/Al-Cl LDHs to remove 2,4-dinitrophenol and 2-methyl-4,6-dinitrophenol pesticides, and 2,4-chlorophenoxyacetate, which is one of the most widely used herbicides for weed control in wheat and rice plantations, respectively. Similarly, freshwater and saltwater algal blooms, which may pose a harmful risk to the aquatic ecosystem, were controlled by LDHs application, by removing phosphate and nitrate species, due to LDHs' high affinity to HPO_4^{2-} (Chitrakar et al., 2006; Peng et al., 2009; Sengco and Anderson, 2004).

Recently, the use of LDHs as carriers for the slow and control release of fertilizers, pesticides, and herbicides by anionic exchange has also been studied and deemed as a promising future application in a great number of studies (Benício et al., 2017; Cardoso et al., 2006; Everaert et al., 2018).

1.3.2.2 Industrial and medical applications

Due to the capacity to intercalate different compounds into the interlamellar layer, LDHs exhibit great potential applications across various industrial fields, as carriers for such compounds.

In the coating and paints industry, LDHs have already been studied as host nanomaterials for anti-corrosives to increase the resistance of alloy materials, such as aluminium, to external agents (e.g. O_2, H_2SO_4, weathering) (Mallakpour et al., 2020; Yao et al., 2018). For example, in a study conducted by Stimpfling and colleagues the anticorrosion properties of a Zn-Al-Cl LDH loaded with EDTA (Ethylenediamine Tetraacetic Acid) in aluminium alloy 2024 were investigated (Stimpfling et al., 2013). They reported that when the LDHs entered in contact with the alloy, the EDTA molecule was liberated, covering the alloy's surface enhancing its corrosion capacity. Moreover, Li et al (2019) reported that a Li-Al LDH intercalated with 2-guanidinosuccinic acid greatly improved the resistance to corrosion of A6N01-T5 aluminium alloy, also inhibiting the growth of bacterial biofilms and conferring a self-repairing property. Furthermore, in the maritime industry, the application of biocide-loaded LDHs in ships' paints has been studied for its potential applications for preventing biofouling events (Avelelas et al., 2017). A promising study by Martins et al. (2017) highlighted the potential of Zn-Al LDH loaded with 2-mercaptobenzothiazole (MBT) to address the corrosion problem in maritime coatings.

Recently, LDHs have also been considered promising catalyst supporters in the chemical industry. The main advantages of LDHs are owned to their small pollution and waste generation, and also, they are recoverable after the reaction ends (Kerchiche et al., 2017; Singha et al., 2011). For example, the use of LDHs in the oxidation of primary alcohol was already reported in the literature (Singha et al., 2011), as well as in the methylation of phenolic compounds (Subramanian et al., 2013). Moreover, Mori et al. (2017) reported a high catalytic activity of a Mg-Al LDH in the CO_2 hydrogenation process. Such finding increases the knowledge of CO_2 as a hydrogen storage system, which may, in the future, result in a practical application.

In the textile industry, experiments involving the application of LDHs in the manufacturing of cotton or polyester fabrics showed an increment on their mechanic resistance, flame retardant, and ultraviolet (UV) protection (Aladpoosh and Montazer, 2019; Barik et al., 2017). Also, the use of Mg-Al LDHs to remove dye mixtures used by the textile industry during the fabrics' dyeing process has already been reported by Aşçı (2017).

Furthermore, in the cosmetic industry, the application of LDHs loaded UV-blocking compounds into the manufacturing of sunscreens is being also explored as an eco-friendlier solution, as conventional sunscreens may release toxic compounds (e.g. zinc nanoparticles) into the environment (Li et al., 2017; Silva et al., 2019). The use of LDHs in topical creams as carriers for anti-aging or skincare ingredients is also another auspicious application (Patel and Bajaj, 2010).

Regarding medical applications, LDHs pose as promising drug delivery systems (e.g. for antibiotics (Ryu et al., 2010), anti-inflammatory (Rives et al., 2013), antidiabetic (Ambrogi et al., 2009) or cardiovascular drugs (Yeganeh Ghotbi and Bin Hussein, 2010)) due to their low toxicity, high biocompatibility, pH-dependent and controlled drug release (Hussein Al Ali et al., 2013; Kura et al., 2014). For example, Wang and colleagues (Wang et al., 2012) managed to intercalate several antibiotics anions (benzoate, succinate, benzylpenicillin and ticarcillin) in a Mg-Al LDH. Similarly, Wang et al. (2009) managed to use a Zn-Al LDH as carrier for amoxicillin, one of the most worldwide used antibiotics. Moreover, the anti-inflammatory drug fenbrufen has already been intercalated into the interlamellar layer on various types of LDH (Mg-Al, Zn-Al, Fe-Al, and Li-Al) by Li et al. (2004), being reported a slow and controlled release, indicating that such LDHs might be excellent drug delivery system.

1.4. Ecotoxicological Risk Assessment of Nanomaterials: Nanoecotoxicology and the need for standardized ecotoxicological test methodologies of nanomaterials

The fast and relentless development of the nanotechnological field has led to the development of a plethora of NMs (Polonini and Brayner, 2015). Manufactured NMs are devised to exhibit specific unique physicochemical properties, qualifying them for various purposes and applications (Cullen et al., 2011; Handy et al., 2008b). These may include applications in electronics, textiles, medical devices and diagnostics, biosensors, agents for environmental remediation, food packaging, coatings (e.g. Avelelas et al., 2017; Freitas, 2005; Kuswandi, 2017; Legrouri et al., 2005; Roco, 2003; Singha et al., 2011).

The NMs' wide range of applications is leading to an exponential increase in the production and an environmental release particularly from industrial sources, agriproducts and consumer products. Additionally, NMs' behaviour and toxicity differ from their bulk counterpart, as highlighted in several studies (e.g. Hao et al., 2013; Jośko et al., 2016; Xiong et al., 2011). Therefore, proper environmental risk assessment of NMs performed with nano-specific test methodologies is crucial (Hartmann et al., 2017; Inshakova and Inshakov, 2017).

This environmental evaluation falls under the scope of the ecotoxicology field of science, which was firstly coined by Truhaut, in 1969, as "the branch of toxicology concerned with the study of toxic effects, caused by natural or synthetic pollutants, to the constituents of ecosystems, animal (including human), vegetable and microbial, in an integral context" (Truhaut, 1977). However, nowadays, ecotoxicology is considered as a total independent field of science, rather than a branch of toxicology (Calow and Forbes, 2014). However, the high number of NMs which arise in the environment as possible pollutants, lead to the development of a specialized subfield within ecotoxicology dedicated to study and predict the environmental fate and potential ecotoxicological effects of NMs and nanoscale structures, and also to classify the conditions that lead to such ecotoxicological effects, planning ways to prevent and treat them - the nanoecotoxicology (Kahru and Dubourguier, 2010; Liu et al., 2011; Singh et al., 2019). Several harmful effects of NMs have already been described in the literature, such as DNA damage, reactive oxygen species generation, malformations, reproductive alterations, bioaccumulation, and mortality (e.g. Avelelas et al., 2017; Hansen and Baun, 2012; Martins et al., 2017; Maurer-Jones et al., 2013).

The environmental risk assessment of NMs faces great challenges, mostly related to the physicochemical properties that NMs display, which will define their environmental behaviour, fate, bioavailability, and toxicity (Grasso et al., 2002; Lead et al., 2018; Lead and Wilkinson, 2006). Due to their shape, size, chemical composition, surface area, and charge, NMs may alter their behaviour depending on i) the environmental compartment they occur (e.g. soil, water or biota), ii) the environment's abiotic characteristics (e.g. pH, conductivity, water hardness, or organic matter content), aggregating, agglomerating or adsorbing to other external components, such as organic/inorganic matter or iii) the digestive system properties of organisms (Handy et al., 2008a, 2008b). Generally, it is expected that NMs show higher toxicity comparing to other particles of larger sizes, as their high surface-to-volume ratio makes them to be generally more highly reactive. Such a small size facilitates the NM penetration through biological membranes and barriers, enabling the interaction between the NMs and biological material (e.g. DNA) (Brayner et al., 2010; Gidwani and Singh, 2014; Moreno-Horn and Gebel, 2014). Also, the chemical composition of NMs (e.g. silver NPs, copper NPs) influences their toxicity, as ionic dissociation and chemical speciation events might occur (Brinch et al., 2016; Handy et al., 2008a).

Currently, NMs fall under the Regulation No 1907/2006 of the European Parliament and the Council of the European Union “REACH” framework (Registration, Evaluation, and Authorization of Chemicals) (Gavelin et al., 2007; Hansen and Baun, 2012) and of the Regulation No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures (Schwirn et al., 2020). In the European Union, the registration process dossier is currently mandatory for nanoforms produced or imported over 1 tonne per year, leaving however many others in the market without regulation (European Parliament and the Council of the European Union, 2006).

However, the REACH legislation is limited when considering the (eco)toxicological and physicochemical properties of NMs comparing to their bulk counterparts and whether the material in its nanoform is considered to be the same or a different substance from the bulk material. Until 2008, if a NM was considered to be a different substance, REACH required the registration and consequent risk evaluation (i.e. chemical safety assessment) for such substance if produced over 1 ton a year; if the NM was considered to be equal to the bulk material, the discussion for the suitability of the existent hazard information would be a matter of discussion, previous to the NM’s commercialization (Chaudhry et al., 2006; Hartmann et al., 2017). In 2008, following the publication of a review the Commission of European Communities argued that the terminology for “chemical substance” covers NMs and further stated that: “When an existing chemical substance, already placed on the market as bulk substance, is introduced on the market in a nanomaterial form (nanoform), the registration dossier will have to be updated to include specific properties of the nanoform of that substance. The additional information, including different classification and labelling of the nanoform and additional risk management measures, will need to be included in the registration dossier” (Commission of the European Communities, 2008).

The REACH’s chemical safety assessment is comprised of data gathered following: i) the physicochemical properties of substances, along with the persistence potential of the substance, ii) a dose-response evaluation (considering in some cases mutagenicity or carcinogenicity) ; and iii) the bioaccumulation potential of substances. This assessment leads to the substance's classification and labelling as persistent, bioaccumulative, and toxic (PBT) or very persistent and very bioaccumulative (vPvB) (European Chemicals Agency, 2008; European Commission, 2008). Additionally, the predicted environmental concentration (PEC), derived from an exposure assessment, and the predicted non-effect concentrations (PNEC), derived from the dose-response assessment, will enable the risk characterization. Currently, all ecotoxicological testing involving NMs is performed using test guidelines (TGs) developed by international organisations (e.g. OECD) for conventional chemicals (Aitken et al., 2011). However, NMs behave differently from conventional chemicals, being the testing procedure not straight-forward (Aitken et al., 2011). Available TGs do not account for the specific physicochemical characteristics of NMs, as they may transform, dissolve, aggregate/agglomerate, sink, interact with biomolecules and/or organic matter, among others processes, during the ecotoxicological test period changing the exposure conditions. Consequently, current TGs have to be double checked and if needed re-designed and adapted to be suitable to NM ecotoxicological testing (Hund-Rinke et al., 2016; Kühnel and Nickel, 2014; Sayre et al., 2017). Some authors even refer that some of the already existing ecotoxicological data on NMs, obtained using the current TGs, may not be sufficiently sensitive, hence affecting the environmental relevance and reliability of the test (e.g. Hund-Rinke et al., 2016; Khan et al., 2017; Skjolding et al., 2016). Moreover, the harmonization of exposure methodologies is critical as inter-laboratory data comparison regarding a specific NM sometimes might not be possible as different laboratories have created customized specific exposure protocols to meet their research objectives and endpoints (Handy et al., 2012a).

The OECD has a key role in the development and, although current OECD Test Guidelines for ecotoxicity are in principle considered to be applicable (until a certain point) to NMs, nano-specific TGs are currently being evaluated and adapted (Hansen et al., 2017; Petersen et al., 2015). The nano-specific test guidelines or amendments (e.g. as annexes) should account for NMs specificities, as previously mentioned. In literature some suggestions to testing procedures have already been reported, such as (but not limited to):

i) include an appropriate physicochemical characterization of the NMs in the test media (that resemble biological matrices),

ii) measure concentrations of the NM in different metrics, e.g. mass and particle number;

iii) account for mechanical effects that may be toxic, not only chemical effects derived from the NM's presence,

iv) ensure a homogenous NM dispersion, achieved by sonication or addition of stabilizing agents, or different dispersion methodologies,

v) select conventional or non-conventional test organisms and/or organism's life stages, based on predicted exposure scenarios,

vi) chose reference materials. (Handy et al., 2008a, 2008b; Hartmann et al., 2017; Schwirn et al., 2020).

Most recently, the OECD published the guidance document 317 regarding NM testing and addressing specific issues of the testing procedure (OECD, 2020). The document is comprised of a series of recommendations and mandatory guidelines to be applied when conducting a testing procedure involving NMs. Some of those were taken from the pre-existing literature such as the mandatory physicochemical characterization of the NM not only in the test media, but also as an as-produced material. Moreover, considerations to the preparation of the NM test dispersions are addressed and procedural modifications to already existing standardized OECD TGs, based on the NM behaviour, are covered by the guidance document (e.g. TG No. 201 or TG No. 221) (OECD, 2020). The publication of such a guidance document lays solid foundations for a more reliable and sensitive environmental risk assessment also enabling inter-laboratory data comparisons. Although, as newer knowledge regarding NM testing is gained, the update on such guidance documents and TGs has to be considered.

1.5 Thesis aims

The main aims of this thesis are:

1. To evaluate the output of two different OECD-recommended exposure methodologies (serial dilutions from a stock dispersion and individual NM addition to each exposure concentration) in the toxicological profile of two commercially available nanomaterials (Zn-Al and Cu-Al LDH) towards the freshwater microalgae *R. subcapitata*;
2. To assess the toxicological profile of different Zn-Al and Cu-Al LDH powder grain sizes (bulk, < 25, 25 - 63, 63 - 125, 125 - 250, and > 250 µm) in the growth inhibition of *R. subcapitata*.

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Chapter II

Harmonising nanomaterial exposure methodologies in ecotoxicology: the effects of two innovative nanoclays in the freshwater microalgae *Raphidocelis subcapitata*.

2. Harmonising nanomaterial exposure methodologies in ecotoxicology: the effects of two innovative nanoclays in the freshwater microalgae ***Raphidocelis subcapitata***.

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2.1 Abstract

The nanotechnology field has witnessed an unprecedented growth marked by the development of new innovative nanomaterials with unique physicochemical characteristics for a wide range of applications. Layered-double hydroxides (LDH) are hexagonal nanoclays with 20 to 40 nm height standing as an innovative nanomaterial (NM) that can act as a carrier of active compounds (e.g. Zn/Cu-Al LDHs) and/or exhibit antifouling properties (e.g. Cu-Al LDH). These properties and applications raise some environmental awareness, leading to the need of developing strategies to accurately assess inherent nanohazards. Available standard guidelines to evaluate the ecotoxicity of conventional substances do not account for the specificities of nanomaterials, leading sometimes to inaccuracies and an increase in uncertainty. Herein, the present study aimed to assess the output of two different exposure methodologies (serial dilutions of the stock dispersion vs. direct addition of NM powder to each concentration) on the toxicological profile of different powder grain sizes of Cu-Al and Zn-Al LDHs (bulk, <25, 25-63, 63-125, 125-250 and > 250 µm) in the growth of the freshwater microalgae *Raphidocelis subcapitata*. In the direct addition of NM powder methodology, the nanomaterial was weighted individually for all exposure concentrations (60, 90, 120, 150, 180 and 210 mg/L and 1, 2, 3, 5, 7, 9 mg/L, for the Zn-Al and Cu-Al LDHs, respectively). In the serial dilutions methodology, a stock dispersion was made and used as the highest concentration tested, with the remaining concentrations being achieved by serial dilutions.

Results revealed that the serial dilutions methodology was preferable for Zn-Al LDH, whereas for Cu-Al LDH both methodologies were suitable for experimental

execution and reproducibility purposes. Thus, the serial dilutions methodology was selected as the preferred methodology to carry out the remaining growth inhibition tests with the other sizes, for both LDHs. Regarding the different grain size toxicity assessment, a size-dependent response was observed. Powders with low grain size caused high effects on microalgae growth for Cu-Al, whereas all Zn-Al grain sizes yielded similar toxicity. Considering the differences found between exposure methodologies for the Zn-Al LDH, further research involving other nanomaterials and key species have to be carried out to achieve standardization and validation for inter-laboratory comparison. Moreover, the Cu-Al LDH separation by grain sizes might be advantageous.

2.2 Introduction

Nanotechnology is overviewed as one of the most promising fields of the 21st century. Numerous nanomaterials (NMs) have been designed to exhibit unique desirable physicochemical characteristics for a wide set of applications throughout many fields of science and industry (e.g. electronics, energy storage, cosmetics, environmental remediation, coatings, wastewater treatment, biomedicine, pharmaceutics, optics, and physics) (Brinch et al., 2016; Gnach et al., 2015; Gollavelli and Ling, 2012; Iftekhar et al., 2018; Sayre et al., 2017; Silva et al., 2019; Zhang et al., 2010) .

Considering their specific and different characteristics when compared to conventional substances, some attention has to be devoted to approaches and methodologies in the risk characterization phase of such NMs. In the literature, a great number of reviews highlight the crescent necessity of developing nano-specific test methodologies (e.g. Handy et al., 2012, 2008a; Hartmann et al., 2017; Hund-Rinke et al., 2016). Currently, test methodologies are designed for conventional chemicals which do not fully account for these inherent physicochemical characteristics of NMs (Hund-Rinke et al., 2016; Khan et al., 2017; Skjolding et al., 2016). These methodologies have to account for NMs' characteristics which will dictate their behaviour in different environmental compartments and by extent their ecotoxicological profile, fate, and bioaccumulation (Handy et al., 2008a). Throughout the years, different methodological approaches have been developed and reported in the literature with the aim of covering all these existing knowledge gaps, regarding exposure characterization, effects evaluation, among others (Handy et al., 2008a, 2008b; Hartmann et al., 2017; Schwirn et al., 2020).

The Organisation for Economic Co-operation and Development (OECD) has a key role in the standardization of test methodologies, being those “nano-specific” related still under development (Petersen et al., 2015; Rasmussen et al., 2019). Very recently, the OECD has published a guideline document (GD number 317) addressing nano-specific testing issues and recommendations on aquatic and sediment ecotoxicological bioassays (OECD, 2020). Some of these include: i) a mandatory NM physicochemical characterization in relevant test mediums and as-produced material, ii) appropriate exposure and dispersion methodologies to ensure homogeneous NM dispersion, and iii) alterations to already published and widely used OECD Test Guidelines (TG) for environmental hazard assessment (e.g. OECD TG 201, OECD TG 211), to account for possible outcome scenarios obtained from the NM exposure behaviour (OECD, 2020). The guidance document poses itself as a promising starting point for the standardization of test methodologies, however, the update on the guidance document will be of need as more knowledge is gained from NM testing. The OECD TG 201 (OECD, 2006) - Freshwater Alga and Cyanobacteria, Growth Inhibition Test - is one of the most commonly used TGs in hazard assessment studies of chemical substances (e.g. Croce et al., 2017; Heinlaan et al., 2020; Pereira et al., 2018; Reynolds et al., 2021; Sohn et al., 2015), being under a current updated to account for the specificities of NMs. The assessment of the toxicity of chemicals towards freshwater algae is of utmost importance since algae's play an important role in dynamics of the freshwater ecosystems and of the global biogeochemical cycles, as primary producers and carbon dioxide fixators (Missirian et al., 2019; Nava and Leoni, 2021; Ramaraj et al., 2014). A possible impairment in their function will have consequences to the whole ecosystem extent, possibly leading to its collapse (Chapman, 2013).

Some NMs present characteristics that make them hard to test in terms of hazard, considering that they are not stable in testing media, presenting upon testing low reproducibility in results. Besides, some artefacts may arise while testing NMs, like the presence of impurities, dissolution, Reactive Oxygen Species (ROS) formation during dispersion, interaction with test reagents, or difficulties to measure endpoints or characterize in organisms' tissues (Petersen et al., 2015). One of those NMs that can provide some difficulty in testing are layered double hydroxides (LDHs), nanoclays with 20-40 nm in height, composed of divalent (M^{2+}) and trivalent (M^{3+}) metallic cations intercalated with an exchangeable anion (Evans and Slade, 2005; Forano et al., 2006). LDHs exhibit peculiar characteristics, such as good biocompatibility, high chemical stability, and pH-dependent solubility (Mishra et al., 2018). However, the functional and compositional flexibility (e.g. numerous metal-anion combinations), and the anionic exchangeability pose as the two primary advantages of LDHs (Kuthati et al., 2015; Mishra et al., 2018). Due to their characteristics, these nanoclays are regarded as very promising NMs for numerous applications, namely anti-fouling or anti-corrosion coatings, wastewater treatment, nanocarriers for drugs, pesticides, or fertilizers, cosmetic products, flame-retardants, textiles (Aşçı, 2017; Barik et al., 2017; Chitrakar et al., 2006; Commission of the European Communities, 2008; Eisentraeger et al., 2003; European Chemicals Agency, 2008; Grasso et al., 2002; Lead et al., 2018; Lead and Wilkinson, 2006; Liu et al., 2011; Maurer-Jones et al., 2013; Sayre et al., 2017; Singh et al., 2019).

Considering the above mentioned, this study aimed at: i) first assessing the output of different exposure methodologies in the toxicological profile of two commercially available LDHs (Zn-Al and Cu-Al LDH, stabilized with an NO_3^- anion) to the green microalgae *R. subcapitata*, and ii) finally assess toxicity profile of different powder grain sizes (Bulk, < 25, 25-63, 63-125, 125-250 and > 250 µm) from these two LDHs, using the best methodology provided previously in (i), in the same algae species. The information generated in this work will be of utmost importance to the update of the OECD TG 201 for nanomaterial testing but will also be reported to the nanomaterials manufacturer (Smallmatek. Lda., Aveiro, Portugal). This will provide the industry stakeholder information on the toxicity of the different grain sizes and if the separation by grain sizes represents an advantageous manufacturing procedure to achieve an eco-friendlier LDH based on a specific grain size.

2.3 Materials and Methods

2.3.1 Tested nanomaterials

In the present study, two different LDHs were tested, i) zinc-aluminium (Zn-Al) LDH and ii) copper-aluminium (Cu-Al) LDH. All LDHs were synthetized as described in Gomes et al. (2020) by the Portuguese company Smallmatek, Lda. (Aveiro, Portugal). Briefly, LDHs were manufactured by co-precipitation of the respective hydroxide metallic salts in a solution oversaturated with sodium nitrate ($NaNO_3$), being the pH value kept constant (pH =10 ± 0.5) with the addition of sodium hydroxide (NaOH). All process was performed at room temperature in a pilot-scale reactor (developed by BTL-Indústrias Metalúrgicas, S.A., Oliveira de Azeméis, Portugal) equipped with a proportional-integral-derivative (PID) control and peristaltic pumps, to ensure optimal pH and temperature condition and accurate addition of all chemicals, respectively. A slurry was obtained and washed in deionized water, being then dried using an industrial spray dryer to ensure the formation of homogeneous and fine powders of each bulk material. This bulk material is a heterogenous powder with different grain sizes. Lastly, these powders were separated into 5 different grain size groups (< 25, 25-63, 63-125, 125-250, and > 250 µm) using a vibratory sieve shaker (Retsch, Haan, Germany).

All test concentrations, for each nanomaterial type and grain size, were achieved by ultrasonic bath sonication (Selecta; 40 kHz) for 1 hour to guarantee maximum particle dispersion in Woods Hole MBL medium.

2.3.2 Microalgae growth inhibition testing

2.3.2.1 Freshwater microalgae *Raphidocelis subcapitata* cultures

Cultures of *R. subcapitata* were maintained at 20°C ± 1°C under continuous PAR light (photosynthetic active radiation). In order to obtain cells in the exponential growth phase, inoculates were prepared 3 to 5 days prior to the bioassays, being the cultures renewed after 7 days by inoculation in new culture medium (OECD, 2011). Woods Hole MBL culture medium (pH = 7.2) was prepared according to Stein et al. (1973) and used for algae culturing and bioassays. All the material was previously sterilized in an autoclave at 121°C and 1 bar, for 20 min.

2.3.2.2 Growth inhibition tests

Algae growth inhibition tests with *R. subcapitata* followed the OECD TG 201 adapted by Eisentraeger et al. (2003) and with different exposure conditions (see sections below). Exponential growth cells were collected from the algae culture and their absorbance measured in a microplate reader at 440 nm, being the concentration calculated using the following Eq. (1):

$$C = -17107.5 + ABS \times 7925350$$

$$R^2 = 0.998$$

where C represents the algae concentration in cells/mL and ABS the absorbance recorded at 440 nm. The test was assembled in 24-well microplates for 72 hours. Each treatment's replicate was obtained, by incubating, in a well, 100 µL of a *R. subcapitata* cell suspension (5×10^5 cells/mL) with 900 µL of LDH test dispersion.

The microplates were incubated under recommended TG conditions (temperature: 23ºC ± 1ºC; continuous PAR light; constant agitation) for 72 hours. Every 24 hours, absorbance measurements were recorded in a microplate reader at 440 nm, and the concentration of algae calculated using Eq. (1). The average specific growth rate for each concentration was calculated as a logarithmic increase in the algal biomass using the following Eq. (2):

$$\mu_{i-j} = \frac{lnCj - lnCi}{Tj - Ti}$$

where, μ_{i-j} represents the average specific growth rate from time i to time j; Ti is the initial exposure time; Tj is the end time of exposure; Ci and Cj the cellular concentration at time i and time j, respectively. Moreover, the inhibition percentage in average specific growth rate was also accessed by using the following Eq. (3):

$$\%Ir = \frac{\mu_C - \mu_T}{\mu_C} \times 100$$

where, $\%Ir$ is the inhibition percentage in average specific growth rate; μ_C is the mean value for the average specific growth rate in the negative control, and μ_T is the mean value for the average specific growth rate in each treatment.

2.3.2.2.1 Exposure methodology-dependent toxicity tests

Two NM exposure methodologies were preliminary tested based on the recommendations from the OECD GD 317: i) serial dilutions from the stock dispersion, previously prepared in Woods Hole MBL medium, to build a range of concentrations and ii) each concentration was made individually by direct addition of NM powder to MBL media (OECD, 2020). In addition, two independent growth algae inhibition tests, each with one of the exposure methodologies, were performed with 2 replicates per treatment, and as previously described (see section 2.3.3.2), to evaluate reproducibility of results.

Tests were only performed with the bulk powder for the Zn-Al LDH, and < 25 µm grain size for the Cu-Al LDH, as according to the manufacturer provided information, LDHs behaviour in ultra-pure water is similar and can be used as a starting point for testing the contamination procedure. Tested concentrations were 10, 100 and 200 mg Zn-Al LDH/L and 1.75, 3.5, and 7 mg Cu-Al LDH/L; a negative control with culture medium and algae cells only was also added to the experimental setup. The test concentrations were achieved by adding the LDH powder to each concentration individually, or by serial diluting from a stock solution (the highest test concentration used for each LDH type in MBL). Blanks for the negative control and for the tested concentrations (culture medium and dispersed LDHs with no algae) were also prepared.

This provided information on potential artefacts on the spectrophotometry measurements created by the NMs presence. The average specific growth rate in each treatment was recorded every 24 hours.

2.3.2.2.2 Grain size-dependent toxicity tests

The toxicity of the grain sizes of the LDH powder was also investigated. A growth inhibition test was performed as previously described (cf. section 2.3.3.2) for each grain size (i.e. < 25, 25-63, 63-125, 125-250, and > 250 µm) and bulk LDH powder, for both LDHs. Based on the results gained from the exposure methodology tests, the serial dilutions methodology was selected. The test concentrations used were: 0, 60, 90, 120, 150, 180 and 210 mg/L and 0, 1, 2, 3, 5, 7 and 9 mg/L for Zn-Al LDH and Cu-Al LDH, respectively. Further, blanks for the negative and for the tested concentrations (culture medium and dispersed LDHs with no algae) were also prepared for artefacts prevention. The average specific growth rate in each treatment, for each grain size from each LDH was recorded every 24 hours.

2.3.3 Stability of LDH nanomaterials in MBL media

2.3.3.1 Physicochemical characterization of LDHs

Dynamic light scattering (DLS), and zeta potential (ζP) measurements were performed in the Woods Hole MBL culture medium used for the microalgae growth inhibition tests, using a Zetasizer Nano-ZS (Malvern Analytical, Malvern, UK). One concentration (10 mg/L), for each LDH and grain size fraction, was prepared and submitted to sonication in an ultrasonic bath (Selecta; 40 kHz) for 1 hour (see subsection 2.3.1), prior to the measurements. All measurements were performed in triplicate, at constant room temperature, in five time points (0, 6, 24, 48 and 72 h). The pH and conductivity were also measured at the same time points, using a pH and conductivity probes (WTW, Weilheim, Germany), respectively.

2.3.3.2 Nitrate release measurement

Both LHDs have interlamellar galleries with intercalated nitrate anions, where anionic exchangeability (i.e. the release of nitrates) occurs. Therefore, nitrates released were measured in MBL media using a HACH DR/2000 field spectrophotometer (Hach Company, Colorado, CA, USA). The method chosen (nr. 8039) for nitrate species quantification was based on the cadmium reduction and performed as described in supplied HACH official manual. Dispersions for both LDHs and respective grain sizes were prepared in MBL as previously described (i.e. ultrasonication bath for 1 hour), being the test concentrations achieved by serial diluting from a stock solution. The measurements performed at 0 (immediately after ultrasonication) and 72 hours (end of the algae growth inhibition test), with only one replicate. Between measurements, the prepared dispersions were maintained under the same exposure conditions used for the algae growth inhibition tests, in order to mimic the test scenario. Only 3 concentrations were selected for both LDH types and grain sizes. The concentrations used were 60, 120 and 210 mg/L, and 1, 5 and 9 mg/L for the Zn-Al and Cu-Al LDHs, respectively, which correspond to the lowest, intermediate, and highest concentrations used during the algae growth inhibition tests with each type of LDH and grain size.

2.3.3.3 Dissolution of Zn, Cu and Al from LDH nanomaterials

LDH nanoclays are one of the NMs with constriction to obtained real exposure concentrations in ecotoxicity and fate testing, and usually only nominal concentrations are reported. In the present study, in order to partially overtake this issue, measurements for the concentration of Zn, Al and Cu forms, particulate and ionic, were performed for all grain sizes of both LDHs (Zn-Al and Cu-Al), by inductively coupled plasma mass spectrometry (ICP-MS), for the lowest, intermediate, and highest test concentrations used in the algae growth inhibition tests from both LDHs (Zn-Al LDH: 60, 120 and 210 mg/L; Cu-Al: 1, 5 and 9 mg/L), at time 0 h (immediately after ultrasonication) and 72 h (end of the growth inhibition test) (n=1). A stock dispersion of each grain size, from both LDHs, was prepared in Woods Hole MBL medium as previously described (see subsection 2.3.1), being the remaining test concentrations achieved by serial dilution from the mentioned stock dispersion. These samples were then immediately centrifuged at 15,000 *g*, for 5 minutes, to enable the separation between the particulate fraction (present in the pellet) and the ionic fraction (present in the supernatant). Both fraction samples were acidified to a final concentration of 2% HNO_3 (v/v). Between sampling times (0 and 72 h), test dispersions were maintained under the same exposure conditions as the growth inhibition tests. Finally, samples were readily stored at 4ºC until ICP-MS analysis. All analyses were carried out at the Central Laboratory of Analysis of the University of Aveiro, Portugal (certified laboratory).

2.3.4 Statistical analysis

Ecotoxicity data was analysed for normality and homogeneity of variances using the Shapiro-Wilk and Levene’s tests, respectively. If data followed a normal distribution and homoscedasticity, a one-way analysis of variance (ANOVA) was performed to assess for differences between treatments ($p \leq 0.05$), followed by a post-hoc multiple comparisons using the Dunnet’s method to compare each treatment with the control whenever differences were attained. When data failed normality distribution and homoscedastic, a Kruskal-Wallis ANOVA on Ranks was performed, followed by a post-hoc multiple comparisons Dunn’s method when significant differences were obtained ($p \leq 0.05$). Moreover, median effective concentrations (EC_{50}) values for the average specific growth rate for all bioassays were calculated using a non-linear regression three-parameter (3P) logistic model. Statistical treatment was performed on SigmaPlot software, version 12.0 (SystatSoftwareInc, San Jose, CA, USA). Additionally, the slopes obtained from the non-linear regression and the EC_{50} values from the exposure methodology-related assays and from the grain size-dependent toxicity assays were compared by a generalized likelihood ratio test (Chi-Square (χ^2) test).

2.4 Results

2.4.1 Nanomaterials stability in MBL media

The OECD GD 317 (OECD, 2020) refers that a NM dispersion is considered stable if “the mass concentration does not derivate more than 20% from the initial value due to sedimentation within a relevant time scale”. However, in this study the mass concentration variation overtime was not assessed as LDH nanomaterials are not possible to quantify in terms of mass. Nonetheless, the same OECD document states that “other manufactured nanomaterial exposure metrics (e.g., the extent of agglomeration, dissolution, or change in other manufactured nanomaterial characteristics, zeta potential) in addition to total concentration may also be evaluated relative to the ± 20% variability target, where practicable”. Considering this, the zeta-potential and hydrodynamic size, nitrate release and Zn, Cu and Al dissolution (and as particles) were also quantified.

2.4.1.1 Physicochemical characterisation

Tables 2.1 and 2.2 show the results of the tested nanomaterials behaviour in Woods Hole MBL medium over time, including pH, conductivity, zeta potential and hydrodynamic size data for each grain size class and time point (0, 6, 24, 48 and 72 h).

Regarding Zn-Al LDH dispersions (Table 2.1), pH and conductivity showed low variation for the different sizes over 72 h. Zeta potential results did not follow a clear tendency for all grain sizes over time although finer-sized powders (< 25 µm, 25-63 µm and 63-125 µm) tended to increase over time. Despite zeta potential values for all Zn-Al LDH grain sizes increase from time 0 to 72 h such values are still within the range to classify the colloidal dispersion as unstable (cf. Table 2.1). The hydrodynamic size results were also very variable for all grain sizes over time, as no clear grain size-dependent or time-dependent effect was observed.

The results for Cu-Al LDH parameters are presented in Table 2.2. The pH and conductivity values did not show any remarkable variations for the different grain sizes over the 72 hours. Concerning the zeta potential values, the results for this nanomaterial showed an overall inclination to increase over time. However, no grain size-dependent effect was observed. Similarly, to the zeta-potential, the hydrodynamic size of Cu-Al LDH showed a general tendency to increase across all different grain sizes over time, however no grain-size related effect was observed. The zeta-potential and hydrodynamic size measurements performed for all grain sizes of both LDHs, were used to assess the dispersion's stability by calculating the variation between the first and last timepoints (i.e. 0 and 72 h, respectively). The results for the Zn-Al and Cu-Al LDH are presented in Table 2.3 and 2.4, respectively.

Regarding the Zn-Al LDH, results show that when considering the zeta-potential values, only 3 grain sizes were stable when dispersed in Woods Hole MBL medium (i.e. 25-63, 125-250 and > 250 µm), since their variation between timepoints was lower than ± 20 %. Considering the hydrodynamic size measurements, the grain size fractions that produced stable dispersions are the bulk powder, 125-250 and > 250 µm. Considering all variation values from both parameters, all dispersions regardless of grain size are considered to be stable, either considering the zeta-potential or hydrodynamic size variations overtime. Only the < 25 µm grain size dispersion is considered to be not stable by either parameter.

The Cu-Al LDH results (Table 2.4) show that all grain size fractions produce stable dispersions, except for the 25-63 µm grains, considering the zeta-potential parameter. However, considering the hydrodynamic size, only 3 grain size fractions produced stable dispersions (i.e. < 25, 25-63 and 63-125 µm). However, and differently from the Zn-Al LDH, the results show that all grain size fractions produced stable dispersions, depending on the type of parameter being evaluated. For example, the 25-63 µm grains dispersion is considered not stable, attending to the zeta-potential parameter, however, attending to the hydrodynamic size such dispersion is deemed stable.

Table 2.1 – Behaviour of Zn-AL LDH nanomaterials in Woods Hole MBL medium. Different grain size fractions were tested and data was shown regarding pH, conductivity, hydrodynamic size and zeta potential in time: 0, 6, 24, 48, and 72 hours. N=1; Bulk refers to a heterogeneous mixture of different grain sizes.

				ζP (mV)		**Size (nm)**	
	Time (h)	**pH**	**Conductivity (µS/cm)**	**Mean**	**SD**	**Mean**	**SD**
Bulk	**0**	7.81	448	-20.90	0.71	451.57	105.24
	6	7.79	446	-14.63	0.46	509.50	118.18
	24	7.81	449	-15.73	0.39	647.37	157.22
	48	7.83	447	-13.20	0.08	391.53	45.29
	72	7.81	448	-15.03	0.40	487.03	69.91
< 25 µm	**0**	7.81	451	-19.20	0.90	429.27	58.36
	6	7.81	452	-18.60	0.29	765.20	220.79
	24	7.82	449	-18.90	0.73	595.23	70.45
	48	7.81	450	-15.77	0.31	671.43	84.28
	72	7.82	449	-12.03	0.76	739.80	9.42
25 - 63 µm	**0**	7.8	448	-20.73	0.40	411.27	42.05
	6	7.8	447	-18.37	0.73	571.63	155.76
	24	7.8	448	-19.83	0.60	619.93	112.05
	48	7.82	449	-18.30	0.88	906.70	148.96
	72	7.8	447	-18.90	0.92	587.23	73.36
63-125 µm	**0**	7.8	449	-19.60	0.71	484.50	76.44
	6	7.81	449	-19.18	0.71	560.73	68.45
	24	7.81	448	-19.73	0.25	634.33	134.50
	48	7.82	449	-17.63	0.48	736.80	75.39
	72	7.81	448	-16.03	1.45	666.27	122.36
125-250 µm	**0**	7.82	449	-19.67	0.90	641.20	99.22
	6	7.83	447	-19.07	0.34	649.07	117.02
	24	7.82	448	-20.70	0.62	577.23	138.18
	48	7.8	448	-19.20	0.73	603.00	163.23
	72	7.81	449	-18.33	0.76	747.93	71.53
> 250 µm	**0**	7.81	450	-18.73	0.65	849.50	110.29
	6	7.81	449	-17.50	0.71	586.37	89.09
	24	7.81	451	-21.20	0.24	581.40	231.39
	48	7.79	448	-20.47	0.66	546.53	124.06
	72	7.8	449	-17.97	0.78	750.07	242.83

Table 2.2 - Behaviour of Cu-AL LDH nanomaterials in Woods Hole MBL medium. Different grain size fractions were tested and data was shown regarding pH, conductivity, hydrodynamic size and zeta potential in time: 0, 6, 24, 48, and 72 hours. N=1; Bulk refers to a heterogeneous mixture of different grain sizes.

				ζP (mV)		**Size (nm)**	
	Time (h)	**pH**	**Conductivity (µS/cm)**	**Mean**	**SD**	**Mean**	**SD**
Bulk	**0**	7.78	448	-20.13	0.40	251.13	31.06
	6	7.79	450	-19.83	0.17	472.20	42.18
	24	7.78	447	-17.83	0.47	636.77	97.62
	48	7.81	449	-18.60	0.82	821.87	3.95
	72	7.81	448	-18.83	0.61	818.43	66.20
< 25 µm	**0**	7.79	450	-17.83	1.04	312.07	62.84
	6	7.81	447	-16.17	0.69	471.47	36.81
	24	7.78	448	-17.87	0.31	560.87	90.48
	48	7.79	449	-16.60	0.64	578.67	52.78
	72	7.81	448	-17.23	0.97	580.00	17.66
25 - 63 µm	**0**	7.8	449	-17.30	0.37	439.03	87.75
	6	7.8	450	-14.00	0.29	616.23	183.06
	24	7.76	448	-9.41	0.89	717.67	203.82
	48	7.82	448	-7.76	3.22	716.37	39.46
	72	7.8	449	-8.77	0.08	439.23	100.02
63-125 µm	**0**	7.81	451	-17.27	1.11	414.97	12.39
	6	7.81	447	-14.57	0.46	576.93	107.41
	24	7.8	449	-14.47	0.53	672.60	82.14
	48	7.82	448	-16.80	0.64	635.73	82.47
	72	7.81	448	-15.33	0.71	585.63	22.53
125-250 µm	**0**	7.8	450	-18.67	1.07	425.23	90.63
	6	7.83	449	-16.97	0.45	611.17	89.04
	24	7.82	450	-19.30	0.91	614.83	124.56
	48	7.8	448	-16.10	1.16	599.90	72.06
	72	7.81	449	-17.87	0.61	495.53	113.03
> 250 µm	**0**	7.82	449	-17.37	0.78	496.30	56.94
	6	7.81	448	-18.23	0.53	462.80	110.79
	24	7.81	450	-16.23	0.33	610.00	182.31
	48	7.79	451	-18.23	0.41	638.70	156.67
	72	7.8	449	-17.83	0.97	572.10	70.80

Table 2.3 - Stability of each Zn-Al LDH grain size dispersion (10 mg/L). The % variation between timepoints (0 - 72h) was calculated, for both ζP and hydrodynamic size parameters.

	ζP (mV)			**Size (nm)**		
	Time (h)		**% Variation**	**Time (h)**		**% Variation**
	0	**72**		**0**	**72**	
Bulk	-20.90	-15.03	-28.07	451.57	487.03	7.85
< 25 µm	-19.20	-12.03	-37.33	429.27	739.80	72.34
25 - 63 µm	-20.73	-18.90	-8.84	411.27	587.23	42.79
63-125 µm	-19.60	-16.03	-18.23	484.50	666.27	37.52
125-250 µm	-19.67	-18.33	-6.78	641.20	747.93	16.65
> 250 µm	-18.73	-17.97	-4.09	849.50	750.07	-11.70

Table 2.4 - Stability of each Cu-Al LDH grain size dispersion (10 mg/L). The % variation between timepoints (0 - 72h) was calculated, for both ζP and hydrodynamic size parameters.

	ζP (mV)			**Size (nm)**		
	Time (h)		**% Variation**	**Time (h)**		**% Variation**
	0	**72**		**0**	**72**	
Bulk	-20.13	-18.83	-6.44	251.13	818.43	-225.90
< 25 µm	-17.83	-17.23	-3.36	312.07	580.00	-85.86
25 - 63 µm	-17.30	-8.77	-49.33	439.03	439.23	-0.05
63-125 µm	-17.27	-15.33	-11.20	414.97	585.63	-41.13
125-250 µm	-18.67	-17.87	-4.29	425.23	495.53	-16.53
> 250 µm	-17.37	-17.83	2.69	496.30	572.10	-15.27

2.4.1.2 Nitrate release measurement

Nitrate concentrations were measured to assess the anionic exchange release, for all Zn-Al and Cu-Al LDH grain size fractions, and are presented in Table 2.5 and 2.6, respectively.

For the Zn-Al LDHs, the release of nitrate was LDH concentration dependent, and only small increasing variations were observed from time 0 h to time 72 h (except for 60 mg/L of 125-250 µm grain size fraction, with a small decrease and for 210 mg/L of 25-65 µm grain size fraction, which was maintained). For the Cu-Al LDH, the concentration dependency was not so marked, and in some cases at 0h nitrate concentrations were similar to the negative control. Regarding time, there were also small increases at 72 h, except for the 9 mg/L of the > 250 µm grain size fraction.

However, the dispersions' stability evaluation based on this endpoint was not performed, as previously mentioned. The nitrate release occurs very rapidly in Woods Hole MBL medium (see Table 2.5 and 2.6), as a possible anionic-exchange plateau is also rapidly reached, hence no variation between this study's timepoints would be observed. Such

Table 2.5 - Nitrate content release for all Zn-Al LDH grain size fractions. Measurements were performed at 0 and 72 hours.

			Nitrate Content (mg/L NO_3^-)	
			0h	**72h**
Control (MBL)			82.7	84.9
Zn-Al LDH	**Bulk**	**60 mg/L**	95.9	93.7
		120 mg/L	113.5	115.7
		210 mg/L	137.7	137.7
	< 25 µm	**60 mg/L**	95.9	98.1
		120 mg/L	113.5	120.1
		210 mg/L	137.7	139.9
	25-63 µm	**60 mg/L**	91.5	95.9
		120 mg/L	115.7	122.3
		210 mg/L	139.9	139.9
	63-125 µm	**60 mg/L**	93.7	95.9
		120 mg/L	109.1	117.9
		210 mg/L	137.7	142.1
	125-250 µm	**60 mg/L**	95.9	93.7
		120 mg/L	111.3	115.7
		210 mg/L	137.7	139.9
	> 250 µm	**60 mg/L**	91.5	98.1
		120 mg/L	111.3	117.9
		210 mg/L	135.5	139.9

fast anionic-exchange of nitrates observed in high ionic strength media was also reported by Kotlar et al. (2020).

Table 2.6 - Nitrate content release for all Cu-Al LDH grain size fractions. Measurements were performed at 0 and 72 hours.

			Nitrate Content ($mg/L\ NO_3^-$)	
			0h	**72h**
Control (MBL)			82.7	84.9
Cu-Al LDH	**Bulk**	**1 mg/L**	82.7	87.1
		5 mg/L	82.7	91.5
		9 mg/L	89.3	95.9
	< 25 µm	**1 mg/L**	82.7	84.9
		5 mg/L	84.9	91.5
		9 mg/L	87.1	93.7
	25-63 µm	**1 mg/L**	82.7	89.3
		5 mg/L	84.9	91.5
		9 mg/L	89.3	95.9
	63-125 µm	**1 mg/L**	82.7	84.9
		5 mg/L	84.9	93.7
		9 mg/L	89.3	95.9
	125-250 µm	**1 mg/L**	84.9	87.1
		5 mg/L	84.9	91.5
		9 mg/L	87.1	93.7
	> 250 µm	**1 mg/L**	82.7	84.9
		5 mg/L	87.1	89.3
		9 mg/L	89.3	89.3

2.4.1.3 Dissolution of Zn, Cu and Al from LDH nanomaterials

The concentrations of the particulate and ionic forms of Zn and Al, and Cu and Al measured on Zn-Al and Cu-Al LDHs dispersions, respectively, are displayed in Tables 2.7 (Zn-Al LDH) and 2.8 (Cu-Al LDH).

Regarding the Zn-Al LDH, both Zn and Al concentrations (particulate and ionic) showed an overall tendency to increase in a concentration-dependent manner, being the increase more pronounced for the particulate form. However, when considering a time-related effect, an opposite tendency is observed between forms for both Zn and Al concentrations. An overall decrease for the particulate form was observed for both elements and an increase observed for the ionic form, with minor exceptions (e.g. Zn concentration for the particulate form, increases between timepoints, for the two highest test concentrations in the > 250 µm grain size). Moreover, the concentration values for both elements and respective forms are not related with grain size, as concentrations slightly increase or decrease, between grain sizes.

As per the Cu-Al LDH, a similar behaviour was observed. An overall increase in the Cu and Al concentrations for both forms (i.e. particulate and ionic) in a concentration-dependent manner is noted (with minor exceptions). Regarding the Cu and Al concentrations, an opposite tendency was observed in a time-dependent manner. For the particulate form of both elements (Cu and Al), a decrease in the concentration was observed overtime, whereas the concentration of both Cu and Al ionic forms increases over time. Moreover, a grain size-dependent decrease is observed for the Cu ionic form. The Cu's ionic form concentration is higher in the < 25 µm grain size, and decreases with the increasing grain size, being the lowest value obtained for the larger grain size (i.e. > 250 µm).

Considering the values obtained for both LDH types and their respective grain size fractions, an overall good stability is observed for all dispersions in MBL (variations of ±20%), however with few minor exceptions.

Table 2.7 - Measured concentrations of particulate and ionic Zn and Al, at 0h and 72h in MBL for all grain size fractions of Zn-Al LDHs at concentrations of 60, 120 and 210 mg/L. N=1.

	Nominal Test Concentrations (mg/L)	Particulated Fraction (mg/L)				Dissolved Fraction (mg/L)			
		Al		Zn		Al		Zn	
		0 h	72 h	0 h	72 h	0 h	72 h	0 h	72 h
MBL (Control)		< 0.050	< 0.050	< 0.050	< 0.050	< 0.050	< 0.050	< 0.050	< 0.050
Bulk	60	3.389	3.008	15.418	13.461	0.301	0.425	2.030	3.101
	120	7.199	6.908	34.586	32.957	0.275	0.398	2.125	3.142
	210	14.544	13.769	77.559	74.895	0.404	0.462	3.111	3.695
< 25 µm	60	2.053	4.357	9.400	20.447	0.209	0.199	1.419	1.942
	120	7.337	6.786	35.165	31.734	0.282	0.559	2.092	3.995
	210	14.404	14.936	77.532	78.705	0.322	0.552	2.569	4.184
25 - 63 µm	60	3.287	3.368	14.719	15.016	0.154	0.711	1.360	4.616
	120	7.228	7.674	34.256	36.073	0.501	0.401	3.491	3.177
	210	13.443	14.273	71.317	76.265	0.427	0.580	3.307	4.367
63 - 125 µm	60	3.608	3.212	15.924	14.286	0.553	0.654	3.781	4.577
	120	7.381	5.470	35.840	26.674	0.671	2.432	4.594	13.972
	210	14.293	13.770	75.398	73.789	0.549	0.911	4.129	6.424
125 - 250 µm	60	2.116	3.991	9.629	18.655	0.570	0.538	3.940	3.896
	120	8.137	7.447	38.493	35.831	0.489	0.607	3.636	4.455
	210	14.962	14.162	78.758	75.326	0.652	0.361	4.721	3.469
> 250 µm	60	3.003	3.366	13.771	15.099	0.421	0.610	3.102	4.355
	120	7.252	7.490	35.596	35.969	0.158	0.773	1.889	5.640
	210	10.834	15.728	59.114	84.151	0.232	0.866	2.420	6.566

Table 2.8 - Measured concentrations of particulate and ionic Cu and Al, at 0h and 72h in MBL for all grain size fractions of Cu-Al LDHs at concentrations of 1, 5 and 9 mg/L. N=1.

	Nominal Test Concentrations (mg/L)	**Particulated Fraction (mg/L)**				**Dissolved Fraction (mg/L)**			
		Al		Cu		Al		Cu	
		0 h	72 h	0 h	72 h	0 h	72 h	0 h	72 h
MBL (Control)		< 0.050	< 0.050	< 0.050	< 0.050	< 0.050	< 0.050	< 0.050	< 0.050
Bulk	1	< 0.050	< 0.050	0.188	0.061	< 0.050	< 0.050	< 0.050	0.198
	5	0.148	0.102	0.942	0.498	< 0.050	0.078	0.160	0.839
	9	0.419	0.527	2.873	2.924	< 0.050	< 0.050	0.310	1.484
< 25 µm	1	0.054	< 0.050	0.270	< 0.050	< 0.050	0.053	0.155	0.421
	5	0.211	0.143	1.391	0.592	0.080	0.149	0.674	1.540
	9	0.373	0.411	2.524	2.088	0.168	0.193	1.387	2.534
25 - 63 µm	1	0.051	< 0.050	0.255	0.105	< 0.050	< 0.050	< 0.050	0.324
	5	0.262	0.180	1.737	0.912	< 0.050	0.098	0.316	1.151
	9	0.514	0.499	3.526	2.792	< 0.050	0.123	0.485	2.018
63 - 125 µm	1	< 0.050	< 0.050	0.100	0.174	< 0.050	< 0.050	< 0.050	0.110
	5	0.091	0.128	0.565	0.765	< 0.050	< 0.050	0.133	0.507
	9	0.502	0.617	3.482	3.866	< 0.050	0.075	0.236	1.506
125 - 250 µm	1	< 0.050	0.084	0.066	0.505	< 0.050	< 0.050	0.068	0.213
	5	0.092	0.109	0.519	0.619	< 0.050	0.078	0.365	0.755
	9	0.312	0.369	2.104	2.346	0.061	0.124	0.593	1.554
> 250 µm	1	< 0.050	< 0.050	< 0.050	< 0.050	< 0.050	< 0.050	< 0.050	< 0.050
	5	< 0.050	0.197	0.059	1.193	< 0.050	< 0.050	< 0.050	0.144
	9	< 0.050	< 0.050	< 0.050	< 0.050	< 0.050	< 0.050	0.063	0.940

2.4.2 Exposure methodology test

In the exposure methodology approach, the algae growth inhibition tests assessed the differences between exposures upon serial dilutions from the stock dispersion prepared in Woods Hole MBL and those from the direct addition of NM powder to each concentration (Figures 2.1 and Figures 2.2 for Zn-Al and Cu-Al LDH, respectively).

Tests were done twice in order to evaluate their reproducibility. Regarding the Zn-Al LDH, both exposure methodologies exhibit similar toxicological profiles, with a concentration-dependent decrease in the specific growth rate of *R. subcapitata.* For the 1st independent test (Fig. 2.1, (1)), significant differences ($p < 0.05$) were found on all tested concentrations, despite the exposure methodology, except for the 10 mg/L concentration. Moreover, a significant difference between exposure methodologies was found at the 100 mg/L test concentration. In the 2nd independent test (Fig. 2.1, (2)), statistically significant differences ($p < 0.05$) were also found for all test concentrations, regardless of the exposure methodology, except for the 10 mg/L treatment. Additionally, when comparing between methodologies for the same test concentration, significant differences were observed for the highest test concentrations (i.e. 200 mg/L). The calculated EC_{50} values for each exposure methodology of each independent growth inhibition test with Zn-Al LDH are summarized in Table 2.9 and are expressed in: i) total LDH mass and ii) Zn mass. For the individual direct addition of NM powder to each test

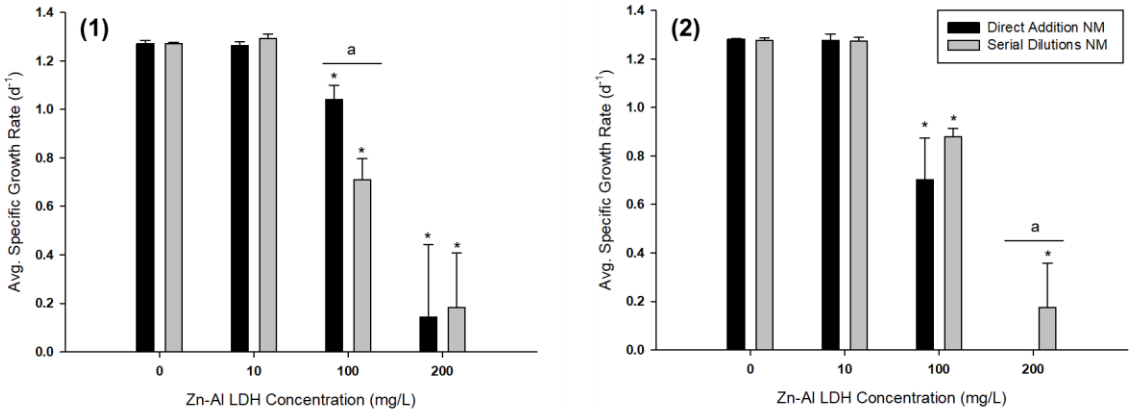

Figure 2.1 - Average specific growth rates for *Raphidocelis subcapitata* when exposed to Zn-Al LDH (bulk form) using two different exposure methodologies, in the (1) 1st independent assay and (2) 2nd independent assay. Asterisks (*) evidence significant differences ($p < 0.05$) in the average growth rate when compared with the control for a given exposure methodology, the bars show the standard error. The letter “a” represents statistical differences when comparing data for the same concentration between methodologies ($p < 0.05$). Caption: Direct Addition NM: direct addition of NM powder to each concentration methodology; Serial Dilutions NM: serial dilutions from a stock dispersion methodology.

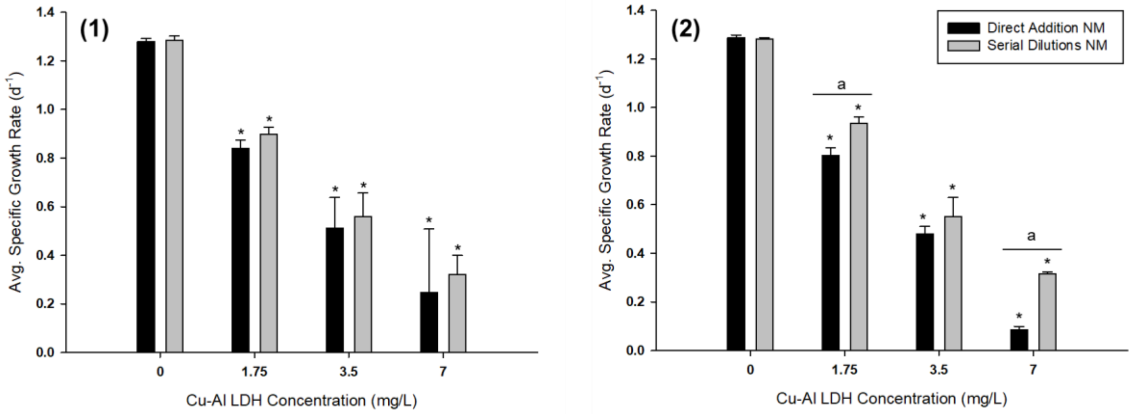

Figure 2.2 - Average specific growth rates for *Raphidocelis subcapitata* when exposed to Cu-Al LDH ($<$ 25 μm grain size) using two different exposure methodologies, (1) from the 1st independent assay and (2) from the 2nd independent assay. Asterisks (*) evidence significant differences ($p > 0.05$) in the average growth rate when compared with the control for a given exposure methodology, the bars show the standard error. The letter "a" represents statistical differences when comparing data for the same concentration between methodologies. Caption: Direct Addition NM: direct addition of NM powder to each concentration methodology; Serial Dilutions NM: serial dilutions from a stock dispersion methodology.

concentration methodology, EC$_{50}$ values are statistically different ($\chi^2 = 4.60$, $p < 0.05$). Differently from the individual addition methodology, for the serial dilution methodology the results from the comparison between the EC$_{50}$ values of each independent test showed no significant difference ($\chi^2 = 2.95$, $p > 0.05$).

Concerning the Cu-Al LDH exposure to *R. subcapitata*, concentration-dependent decreases in the specific growth of the microalgae were observed in both 1st and 2nd independent assays (Fig. 2.2, (1) and (2), respectively). Significant differences were noted for all concentrations in both independent assays, regardless of the used exposure methodology. When comparing between exposure methodologies for the same test concentration, only statistical differences were found for the 1.75 and 7 mg/L concentrations in the 2nd independent test. The calculated EC$_{50}$ values for each exposure methodology in each independent growth inhibition test with Cu-Al are summarized in Table 2.10 and are expressed in either i) total Cu-Al LDH mass per litre and ii) Cu mass per litre. The EC$_{50}$ values obtained for each exposure methodology in different independent assays were very similar, as no statistically significant differences between independent tests were reported for either exposure methodology ($\chi^2 = 0.46$, $p > 0.05$ for the direct addition methodology, and $\chi^2 = 0$, $p > 0.05$ for the serial dilutions methodology).

Table 2.9 - EC_{50} values with respective standard deviation (SD), calculated for the 1st and 2nd independent algae growth inhibition tests with *Raphidocelis subcapitata*, for both exposure methodologies, using Zn-Al LDH (bulk form). Chi-square (χ^2) values were also calculated to assess for differences between EC_{50} values from separate tests, for the same methodology. Asterisks (*) indicate for statistically significant differences ($p < 0.05$) between EC_{50} values of each independent test, for the same methodology, considering the calculated χ^2 value.

Exposure Methodology	1st Test				2nd Test				χ^2
	EC_{50} (mg Zn-Al LDH/L)	SD	EC_{50} (mg Zn/L)	SD	EC_{50} (mg Zn-Al LDH/L)	SD	EC_{50} (mg Zn/L)	SD	
Individual Addition	134.36	12.57	44.45	4.16	100.61	29.51	33.28	9.76	4.60*
Serial Dilution	107.67	8.97	35.62	2.97	123.47	6.83	40.84	2.26	2.95

Table 2.10 - EC_{50} values with respective standard deviation (SD), calculated for the 1st and 2nd independent algae growth inhibition tests with *Raphidocelis subcapitata*, for both exposure methodologies, using Cu-Al LDH (< 25 µm grain size). Chi-square (χ^2) value was also calculated to assess for differences between EC_{50} values from separate testes, for the same methodology. No statistically significant differences ($p < 0.05$) between EC_{50} values of each independent test, for the same methodology, were obtained considering the calculated χ^2 value.

Exposure Methodology	1st Test				2nd Test				χ^2
	EC_{50} (mg Cu-Al LDH/L)	SD	EC_{50} (mg Cu/L)	SD	EC_{50} (mg Cu-Al LDH/L)	SD	EC_{50} (mg Cu/L)	SD	
Individual Addition	2.69	0.46	0.35	0.06	2.42	0.16	0.31	0.02	0.46
Serial Dilution	3.06	0.26	0.40	0.03	3.13	0.20	0.41	0.03	0.00

2.4.3 Grain size-depended toxicity tests

The results for the average specific growth of *R. subcapitata* exposed to Cu-Al LDH are shown in Figure 2.3 and Table 2.1, being the EC_{50} values expressed as total Zn-Al LDH mass per litre or as Zn mass per litre. A similar toxicological profile, marked by a concentration-dependent decrease in the average growth of *R. subcapitata,* is observed for all grain size fractions. Moreover, statistically significant differences ($p < 0.05$) are also reported for all tested concentrations (60, 90, 120, 1250, 180 and 210 mg/L) regardless of the grain size. The average specific growth EC_{50} values do not highlight a possible grain size-dependent relation as they are very similar. Regarding the EC_{50} values obtained for the growth inhibition, a variation, similar to the one observed for the EC_{50} values for the average specific growth, was also noted. The average growth EC_{50} value statistical comparison between the bulk powder and the remaining grain sizes is summarized in Table 2.12. No significant differences ($p < 0.05$) in the chi-square values were observed for all comparisons.

The results for the average specific growth of *R. subcapitata* exposed to Cu-Al LDH are shown in Figure 2.4 and Table 2.13, being the EC_{50} values also expressed as total mass of Cu-Al LDH per litre and mass of Cu per litre. Differently from the Zn-Al LDH, a marked concentration-dependent decrease is only observed for the bulk powder, < 25, 25-63 and 63-125 µm grain size fractions. However, for the two coarsest grain size fractions such decrease was not observed. Moreover, the calculated EC_{50} values for the average specific growth and growth inhibition showed a noticeable grain-size dependent increase. Table 2.14 summarizes the results obtained for the comparison between the EC_{50} values for the average specific growth of the Cu-Al LDH bulk size with all other grain size fraction. Significant differences were found between the values of the bulk powder and both < 25 µm ($\chi 2 = 21.79$, $p < 0.05$), and 63 - 125 µm ($\chi 2 = 44.77$, $p < 0.05$) grain size fractions. Despite not being possible to compare the values of the bulk sizes with the 125-250 and > 200 µm grain size fractions, the authors consider that a significant difference for both comparisons is probably of case.

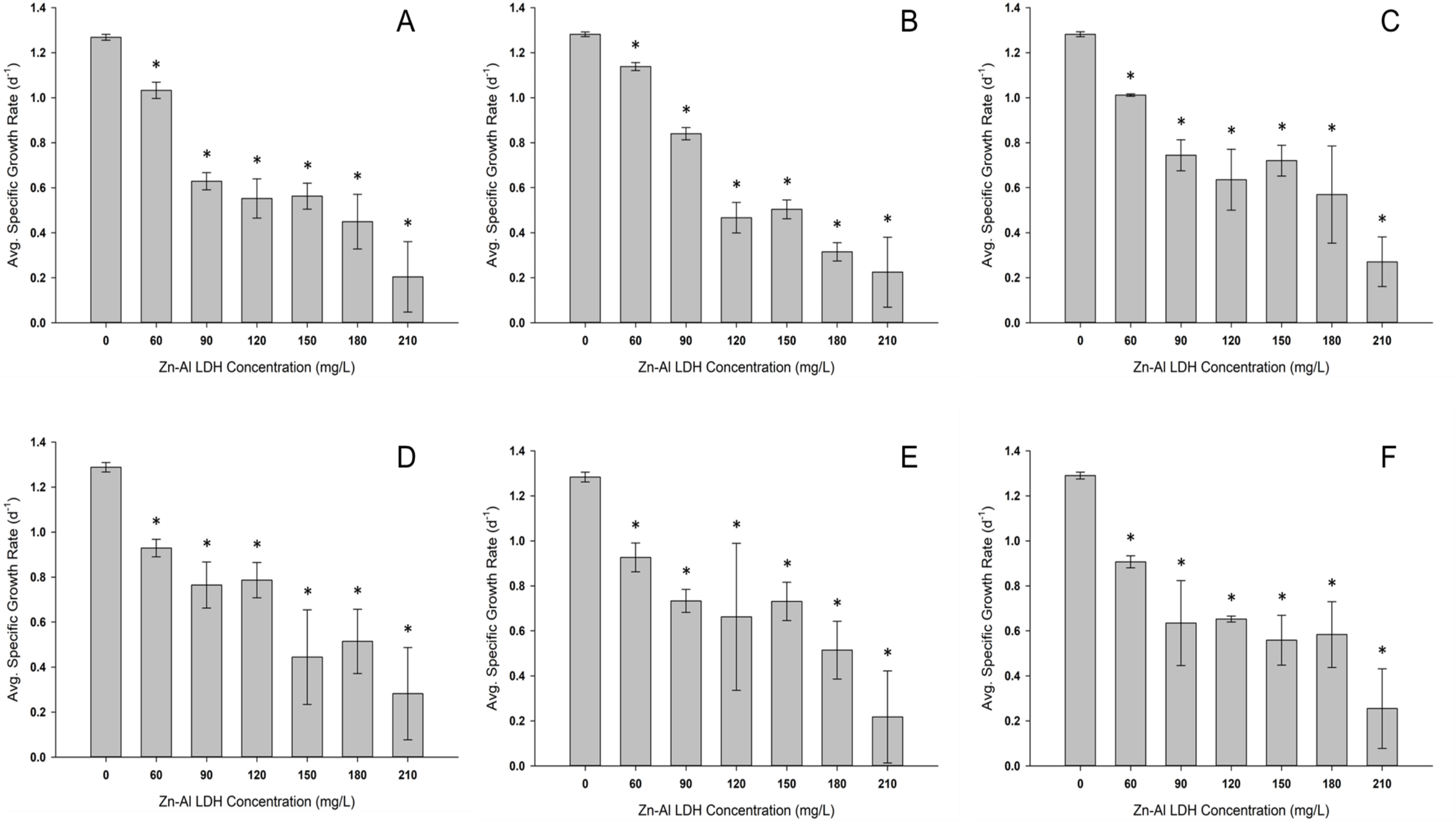

Figure 2.3 - *Raphidocelis subcapitata* average specific growth rates after 72h of exposure to different grain size fractions of Zn-Al LDH: A) Bulk; B) <25 µm; C) 25-63 µm; D) 63-125 µm; E) 125-250 µm; F) > 250 µm. Asterisks (*) evidence significant differences (p < 0.05) of the average growth rate when compared with the control, the bars show the standard deviation.

Table 2.11 - No Observed Effect concentration (NOEC), Lowest Observed Effect Concentration (LOEC), and EC_{50} values for the exposure of *Raphidocelis subcapitata* to Zn-Al LDH, for all grain size fractions. Values in parenthesis represent the upper and lower limits of the 95% confidence interval.

				EC_{50} (mg Zn-Al LDH/L)		**EC_{50}** (mg Zn/L)	
		NOEC (mg Zn-Al LDH/L)	**LOEC** (mg Zn-Al LDH/L)	Specific Avg. Growth Rate	% Inhibition	Specific Avg. Growth Rate	% Inhibition
Zn-Al LDH	Bulk	< 60	60	109.72 (93.90 - 125.54)	113.33 (8.00 - 218.66)	36.30 (31.06 - 41.53)	37.49 (2.65 - 72.33)
	< 25 µm	< 60	60	112.87 (103.43 - 122.31)	97.29 (86.72 - 107.85)	37.34 (34.21 - 40.46)	32.18 (28.69 - 35.68)
	25-63 µm	< 60	60	130.78 (106.46 - 155.11)	133.00 (-153.46 - 420.14)	43.26 (35.22 - 51.31)	44.00 (-50.75 - 138.98)
	63-125 µm	< 60	60	120.84 (98.37 - 143.31)	120.01 (-293.33 - 305.91)	39.97 (32.54 - 47.41)	39.70 (-97.03 - 101.20)
	125-250 µm	< 60	60	126.78 (94.31 - 159.25)	126.13 (-293.33 - 545.60)	41.94 (31.20 - 52.68)	41.72 (-97.03 - 180.48)
	> 250 µm	< 60	60	110.83 (85.76 - 135.90)	110.75 (-187.57 - 409.05)	36.66 (28.36 - 44.96)	36.63 (-62.05 - 135.31)

Table 2.12 - Chi-square (χ^2) values obtained for the average specific growth EC_{50} comparison between the bulk LDH powder and the remaining Zn-Al LDH grain size fractions. No statistically significant differences between EC_{50} values were found, considering the obtained χ^2 value.

$EC_{50}(1)$ / $EC_{50}(2)$	**χ^2**
Bulk / < 25 µm	0.12
Bulk / 25 - 63 µm	2.72
Bulk / 63 - 125 µm	0.83
Bulk / 125 - 250 µm	1.45
Bulk / > 250 µm	0

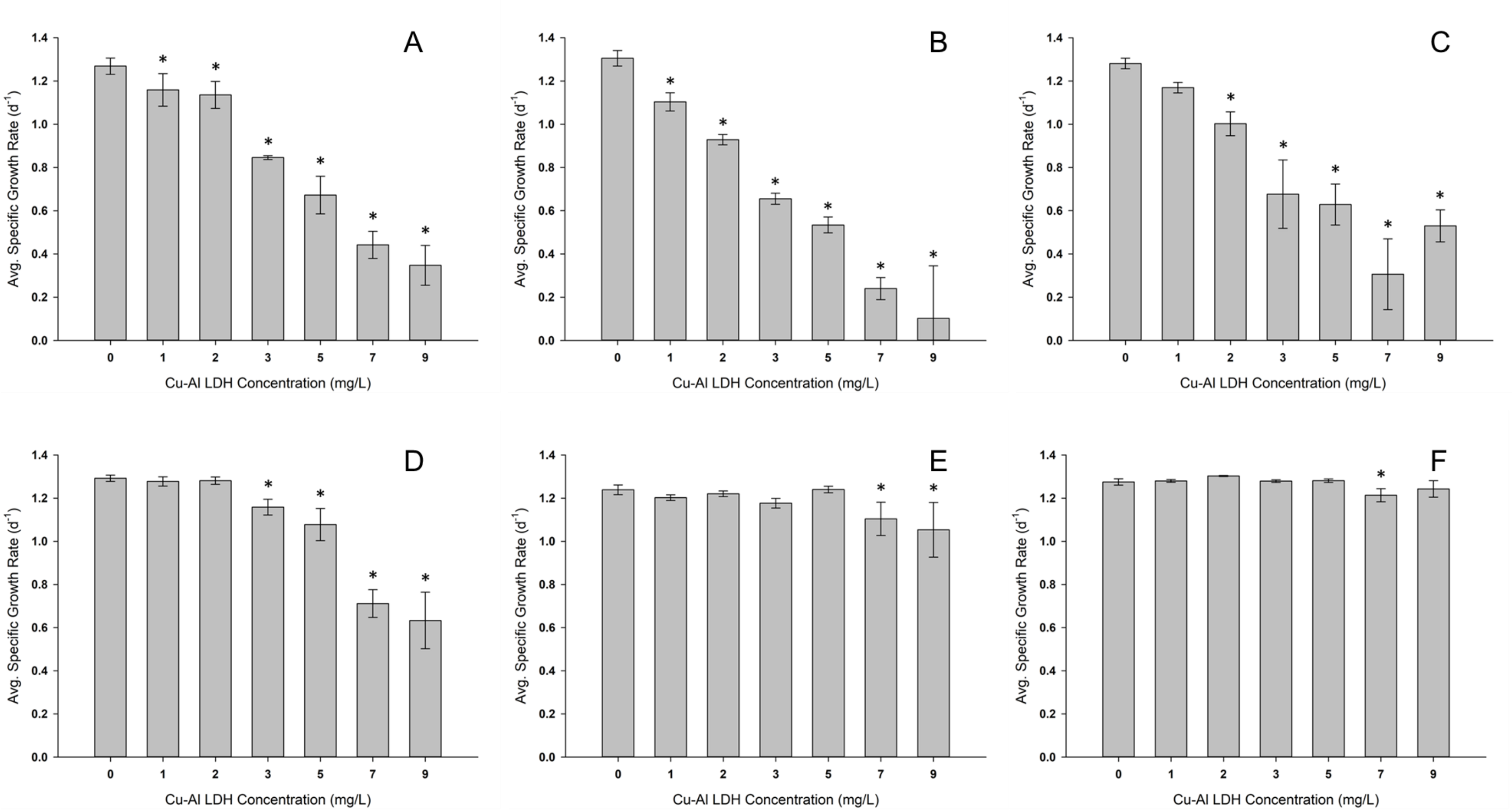

Figure 2.4 - *Raphidocelis subcapitata* average specific growth rates after 72h of exposure to different grain size fractions of Cu-Al LDH: A) Bulk; B) <25 µm; C) 25-63 µm; D) 63-125 µm; E) 125-250 µm; F) > 250 µm. Asterisks (*) evidence significant differences ($p < 0.05$) of the average growth rate when compared with the control, the bars show the standard deviation.

Table 2.13 - No Observed Effect concentration (NOEC), Lowest Observed Effect Concentration (LOEC), and EC_{50} values for the exposure of *Raphidocelis subcapitata* to Cu-Al LDH, for all grain size fractions. Values in parenthesis represent the upper and lower limits of the 95% confidence interval.

				EC_{50} (mg Cu-Al LDH/L)		**EC_{50}** (mg Cu/L)	
		NOEC (mg Cu-Al LDH/L)	**LOEC** (mg Cu-Al LDH/L)	Specific Avg. Gowth Rate	% Inhibition	Specific Avg. Gowth Rate	% Inhibition
Cu-Al LDH	Bulk	1	2	5.11 (4.58 - 5.63)	4.94 (2.35 - 7.53)	0.67 (0.60 - 0.73)	0.64 (0.31 - 0.98)
	< 25 µm	<1	1	3.27 (2.77 - 3.78)	3.22 (1.79 - 4.66)	0.42 (0.36 - 0.49)	0.42 (0.23 - 0.61)
	25-63 µm	1	2	4.31 (3.25 - 5.36)	2.44 (1.80 - 3.08)	0.56 (0.42 - 0.70)	0.32 (0.23 - 0.40)
	63-125 µm	2	3	8.43 (7.73 - 9.14)	6.29 (3.98 - 8.60)	1.09 (1.01 - 1.27)	0.82 (0.52 - 1.12)
	125-250 µm	5	7	> 9	> 9	> 1.17	> 1.17
	> 250 µm	5	7	> 9	> 9	> 1.17	> 1.17

Table 2.14 - Chi-square (χ^2) values obtained for the average specific growth EC_{50} comparison between the bulk LDH powder and the remaining Cu-Al LDH grain sizes fractions. The statistical comparison for the 125-250 and > 250 µm was not determined (n.d). Asterisks (*) evidence for statistically significant differences (*p* < 0.05) between EC_{50} values, considering the obtained χ^2 value.

EC_{50}(1) / EC_{50}(2)	**χ^2**
Bulk / < 25 µm	21.79*
Bulk / 25 - 63 µm	2.25
Bulk / 63 - 125 µm	44.77*
Bulk / 125 - 250 µm	n.d
Bulk / > 250 µm	n.d

2.5 Discussion

2.5.1 Zn-Al LDH and Cu-Al LDH behaviour in MBL media

The physicochemical parameters like pH and conductivity of Cu-Al LDH and Zn-Al LDH dispersions behave in time accordingly to the literature for other types of media (Mishra et al., 2018). A study conducted by Gomes et al. (2020) reported a high stability of dispersions containing Zn-Al LDH-nitrates dispersed in distilled water for one month in a wide pH range: between pH 3 and 12, only ~10% of the LDH were dissolved after one month of immersion. Such partial dissolution may help to form different metallic and non-metallic complexes in the test medium (Iftekhar et al., 2018), which may help to explain the slight alteration between the pH values of the initial test medium (pH = 7.2) and the ones obtained for the Zn-Al and Cu-Al LDHs. This partial LDHs dissolution also helps to explain the general increase in the Zn, Al and Cu ionic forms over time, obtained in the ICP-MS measurements, despite grain size fractions, as the dissolution of the LDH releases the divalent and trivalent metallic ions, present in the LDHs' dual-layer, to the test dispersion (Galvão et al., 2016). Moreover, in some cases, an 1% increase in the ionic form of the different metals over the 72 h is noted, which is in agreement LDHs dissolution rate over a month reported by Gomes et al. (2020).

When dispersed in ultra-pure water, LDHs powders hydrate and disperse getting a typical nanoclay conformation (e.g. Sun and Dey, 2015). In the present study, DLS and ζP values measured in all grain size fractions of both LDHs were variable. This might be explained based on the LDHs' trademark dual-layered geometry. DLS is considered to be the most used tool for size and zeta-potential characterization of NMs, however, it has already been shown to be unsuitable for non-spherical geometries (Levin et al., 2017, 2016), as many nano-specific behaviours (e.g. rotational diffusion) are not taken into account during the measurement (Khlebtsov and Khlebtsov, 2011; Liu and Xiao, 2012). However, and disregarding the possible DLS unsuitability to characterize non-spherical NMs, as LDHs powders possess an intrinsic ability to readily disperse and individualize themselves in ultra-pure water (Choy et al., 2000; Yan and Chen, 2013), a similar hydrodynamic size should be expected to be observed for all grain sizes of each LDH type (i.e. Zn-Al and Cu-Al), when comparing equivalent timepoints, which clearly was not the case in the present study. However, considering the average hydrodynamic size value obtained from all timepoint measurements, for all grain size fractions, of both LDHs, the great previously mentioned differences are softened. The differences between the mentioned average values, for the grain sizes of both LDHs might be easily explained considering the natural variance that may derive from: i) the LDHs' dynamics in high ionic test media, as the continuous anionic-exchange might affect the NMs dynamic and behaviour, or ii) from the LDHs drying process, which depending on its duration might affect the LDHs behaviour dynamics (Galvão et al., 2016; Xu et al., 2006). Despite the previously mentioned variations, the ζP measurements for both Zn-Al and Cu-Al LDH indicate a global tendency to increase over time, however, a grain size-dependent influence is not clear considering the reported results. The values obtained for all Zn-Al and Cu-Al grain sizes, regardless of the timepoint, are negative ranging between - 5 and - 30 mV. This finding indicates that both LDHs have incipient colloidal stability, which leads to some extent aggregation and sedimentation events (Kumar and Dixit, 2017; Riddick, 1966). Such behaviour has been observed in our study however this was to be expected. Dispersion in high ionic strength media (e.g. Wood Hole MBL) leads LDHs (with no specific functionalization towards an increase on the colloidal stability), to aggregate as reported by Pavlovic et al. (2017). The Derjaguin, Landau, Verwey and Overbeek (DLVO) theory helps to explain this behaviour (Evans and Wennerström, 1999; Pavlovic et al., 2016). In ionic solutions, colloids (e.g. NMs) tend to have their surface surrounded by dissolved ions, forming a repulsive double layer, granting them a certain surface charge. This layer's magnitude decreases with the increasing ionic strength of the media due to a screening effect of the surface charge triggered by the counterions present in the medium. As the magnitude decreases, the van der Wall's attraction forces between NMs become more dominant, ultimately leading to their aggregation, agglomeration and sedimentation (Elimelech et al., 2013; Pfeiffer et al., 2014).

The release of nitrate anions present in the interlamellar region was also assessed for all grain sizes of both LDHs. An overall concentration-dependent increase in the nitrate concentration was observed for both LDHs. This finding was to be expected, since the nitrate anion content available for anionic exchange increases with the increasing LDH concentrations. However, the lowest concentration of Cu-Al LDH (1 mg/L) yielded nitrate concentrations equivalent to the ones observed in the control (Woods Hole MBL), for both timepoints of all grain sizes. This suggests that such low LDH concentrations might not be suitable to be employed when using MBL as dispersion media to evaluate the fate and behaviour of LDHs. Moreover, an overall subtle increase in the nitrate concentration between time points for all grain size fractions is also noted. This minor increase between timepoints is consequently related to prompt and fast release of the intercalated nitrate anion which occurs within the first minutes after addition to an aqueous ionic solution, as reported in a study conducted Tedim et al. (2012) using a Zn-Al LDH intercalated with nitrate anions. A similar fast release of intercalated nitrate anions as also been reported in soils (Kotlar et al., 2020).

The ICP-MS analysis of the metals Zn, Al and Cu for both particulate and ionic forms and both LDHs revealed a general concentration-dependent increase regardless the tested grain size. This finding was expected as higher Zn and Al concentrations for both Zn and Al were to be expected with the increase in the Zn-Al LDH concentration. For the same Zn-Al LDH concentration, higher concentrations of Zn, in both particulate and ionic forms, are observed when comparing to the Al ones. Such discovery is easily justified given the LDH formation process which was performed according to Gomes et al. (2020). In the LDH formation process, the ratio between divalent (i.e. Zn^{2+}) and trivalent (i.e. Al^{3+}) cations is 3:1, meaning that more quantity of Zn is used in order form the LDH. Furthermore, this arithmetic concentration-dependent increase was observed for both metallic elements, at both time points, with a few major exceptions (e.g. for the Al, between the 120 and 210 mg/L test concentration, at the 0h, for the > 250 µm grain size). The mentioned few major exceptions might have occurred due to a possible natural experimental variance, or human error. When preparing all test concentrations by serial dilutions, more or less amount of LDH could be pipetted to the subsequent test concentration, possibly as a result of poor test dispersion homogenization, which should have been performed.

2.5.2 Algae growth inhibition assays

2.5.2.1 Exposure methodology-dependant toxicity

Taking into account the challenges that are often posed in NM ecotoxicological testing, the selection of an appropriate exposure methodology should consider the NMs stability in test media, based on the NMs behaviour (e.g. agglomeration, sedimentation; dissolution), which constitutes a very critical step to ensure a constant exposure, and ultimately, to get reproducible experimental results (Handy et al., 2012b).

In this part of the study, the impact of two different exposure methodologies (serial dilutions from a stock dispersion, and direct addition of the as-produced test NM to each exposure concentration) in the toxicological profile of both Zn-Al and Cu-Al LDHs exposed to the freshwater green microalgae *R. subcapitata,* was assessed. Unlike Cu-Al LDH, the results reported for Zn-Al LDH suggest that the serial dilutions methodology proved to be more suitable to carry out further testing with this particular NM, considering that showed reproducibility in the preliminary tests, and it was also confirmed by the similarity of EC_{50s} in the final grain size experiment. Such difference between exposure methodologies for Zn-Al LDH may be related to the bulk test dispersion stability over time, as no variation of more than ±20% in the hydrodynamic size parameter was observed for this grain size, over time, deeming the dispersion as stable. The OECD GD 317 reports that the serial dilutions from a stock dispersion methodology is considered to be more appropriate in cases where good stability of the NM is a test media is observed (OECD, 2020). Moreover, these findings suggest that further studies with Zn-Al LDH using the serial dilutions methodology should have good reproducibility, regardless of the number of independent assays, whereas, for the Cu-Al LDH, either methodology may produce similar related results. Ultimately, the results obtained in the grain-size dependent toxicity assays (recall section 2.5.2.2) for both tested grain sizes (i.e. bulk powder and < 25 µm grain size, for the Zn-Al and Cu-Al LDHs, respectively), revealed similar EC_{50} values to the ones obtained in this part of the study, hence, emphasizing the future tests' good reproducibility involving these particular LDH types.

Comparatively to other zinc-containing LDHs, the results obtained in our study highlight that the Zn-Al LDH employed in the present study yielded less toxicity upon exposure to *R. subcapitata*. However, some difficulties were found when comparing results among studies due to the differences in units expressing ecotoxicological endpoints. Koba-Ucun et al. (2021) reported a 72 h-EC_{50} inferior to 10 mg/L for the average growth of *R. subcapitata* exposed to Zn-Fe LDH, a commonly used catalyst. As the authors do not report the EC_{50} value based on Zn mass (of the Zn-Fe LDH), the previous comparison was performed considering the total LDH mass concentration, although the ratio of Zn in the NMs from both studies is similar. Furthermore, compared to other zinc or aluminium forms (e.g. nano-forms, as ZnO, or ionic forms) described in the literature, Zn-Al LDH is also less toxic. For example, in a recent study conducted by Samei et al. (2019), different shapes and sizes of ZnO nanoparticles (small and large spherical, and small and large rods) inhibited the growth rate of *R. subcapitata* at concentrations lower than 0.64 mg Zn/L. Andreani et al. (2021) reported a 72-h EC_{50} of 3.90 (3.86 - 3.94) mg Zn/L (values were converted to mg Zn/L for a more relevant and convenient comparison) for *R. subcapitata* average growth rate exposed to ZnO nanoparticles. Aruoja et al. (2009) also reported 72-h EC_{50} values of 0.042 (0.01 - 0.12) and 0.042 (0.04 - 0.05) mg Zn/L after exposing $ZnSO_4$ and nano-ZnO to *R. subcapitata*, respectively.

Similarly to what was found for Zn-Al LDH, there are few studies in the literature focusing on the effects of Cu-Al LDHs or chemically-similar LDHs. Nonetheless, Ding et al. (2018) reported a 72-h EC_{50} of 10.00 ± 2.28 mg/L when exposing the freshwater microalgae *Scenedesmus quadricauda* to Cu-Mg-Fe LDH. However, as the authors do not express the EC_{50} value in mg Cu/L or give the Cu-Mg-Fe LDH's elemental ratio, no possible reliable toxicity comparison can be withdraw. Comparatively to other Cu forms (e.g. nano-copper oxide (nano-CuO), or $CuSO_4$), the present study's Cu-Al LDH can be either more or less toxic to freshwater microalgae. Alho et al. (2020) assessed the toxicity of CuO NPs (< 50 nm) in the growth inhibition of *R. subcapitata,* reporting a 72-h EC_{50} of 0.64 (0.60 - 0.66) mg Cu/L, hence being less toxic comparatively to our Cu-Al LDH (EC_{50} $\cong$ 0.41 mg Cu/L). These findings are further supported by Aruoja et al. (2009), which reported an EC_{50} of 0.71 mg Cu/L following the exposure of nano-CuO to the same freshwater microalgae. However, in the same study Aruoja and co-workers also assessed the toxicity of the $CuSO_4$ (as ionic control) upon exposure to *R. subcapitata* and reported an EC_{50} of 0.02 mg Cu/L. This indicates that comparatively to other nano-forms, the present study's Cu-Al LDH yields slightly higher (almost insignificant) toxicity when exposed to freshwater microalgae, however less toxic than salt Cu forms like $CuSO_4$.

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2.5.2.2 Grain size-dependent toxicity

The influence of the particle size in the toxicological profile of a given NM has been a matter of constant debate amongst the scientific community. Considering the available literature, the data collected from numerous studies widely suggests that smaller NMs or nanoparticles (e.g. ZnO, TiO_2, Ag, or Au nanoparticles) have increased toxicity when comparing to larger particles (e.g. Clément et al., 2013; Hu et al., 2018; Ivask et al., 2014; Karlsson et al., 2009; Lopes et al., 2014; Pan et al., 2012; Silva et al., 2014; Xiong et al., 2013; Zhao and Wang, 2012). As particles' size decreases, the surface area increases exponentially, resulting in a heightened surface display of the particles' atoms or molecules. Hence, the NMs' surface becomes more reactive towards other NMs or other surrounding components (biological or not) with the decreasing size, as more chemical reactions might result from such enhanced surface area (Aillon et al., 2009; Powers et al., 2006; Sharifi et al., 2012).

Taking this into account, a range of different LDH grain size powders, of two LDH types (i.e. Zn-Al and Cu-Al) were exposed to the microalgae *R. subcapitata*, in order to assess for differences between the toxicological profile of the bulk LDHs comparatively to relevant grain size fractions (commercially and industrially relevant according to the manufacturer) in the growth inhibition of the microalgae. The results from the Zn-Al LDH suggest no differences between the bulk and other grain sizes from an ecotoxicological point of view, as no statistical differences were encountered between EC_{50} values. Differently from other NMs, LDHs rapidly disperse and individualize themselves upon presence in aqueous solutions, acquiring a very similar hydrodynamic size, regardless of the initial powder grain size (Choy et al., 2000; Sun and Dey, 2015; Yan and Chen, 2013). The present study's DLS results for Zn-Al LDH partially corroborate such behaviour, as no clear trend or a significant difference was observed between grain size fractions, hence, possibly justifying the lack of statistical difference between grain size fractions. As previously mentioned (pls. see section 2.5.1.1), Zn-Al LDH poses as a less toxic nano-solution to *R. subcapitata* comparing to other Zn-based LDHs (Koba-Ucun et al., 2021), or even to other Zn nanoforms (Andreani et al., 2021), regardless of the powder grain size.

Differently from Zn-Al LDH, toxicological differences are observed between the bulk and 4 grain size fractions of the Cu-Al LDH. The EC_{50} obtained for the bulk and < 25 µm grain size (5.11 and 3.27 mg Cu-Al LDH/L, respectively), suggests the < 25 µm grain size is more toxic to the tested microalgae. The different toxicity between the mentioned grain sizes might be due to the higher ionic Cu concentrations detected in the < 25 µm test dispersions, as depicted in the ICP-MS analysis, considered an extremely toxic metal to *R. subcapitata*, even at low concentrations (Al-Hasawi et al., 2020). The fractions 63 - 125, 125 - 250, and > 250 µm were less toxic comparatively to the bulk counterpart, possibly related to the lower Cu dissolution on those dispersions and not to the hydrodynamic size of the dispersed Cu-Al LDH (since the hydrodinamic size is very similar between grain size fractions). As the grain size increases, the ionic Cu concentration as well as the growth inhibition effects tend to decrease. These results will be reported back to the manufacturer Smallmatek, Lda., which may lead to the selection of these particular grain sizes for specific applications, where no toxicity must be observed.

Furthermore, both LDHs, regardless of grain sizes or exposure methodology, yielded toxicity towards the microalgae (except for the 125-250 and > 250 µm Cu-Al LDH grain size). The LDHs toxicity towards *R. subcapitata,* reported in our study, results from the combined effects of:

i) the exposure to the metallic elements' (majorly from the ionic form), present in both LDH types (Zn, Al and Cu), which may induce toxicity in the algae by generating reactive oxygen species (ROS) (Foyer and Shigeoka, 2011; Xia et al., 2015), impairing the photosynthetic system (Gunawan et al., 2013), or by increasing the lipid peroxidation of the algae's cell membrane (Ozkaleli and Erdem, 2018), and

ii) the interactions between LDHs and algae cells, and events that occurred during testing, such as, a) agglomeration and sedimentation of the LDH, mechanically impairing the algae growth, b) shading effects that affect the microalgae growth by reducing the amount of captured light, or c) adsorption of the LDHs to the algae's cell (Déniel et al., 2019; Figueiredo et al., 2019; Hartmann et al., 2013, 2010; Schwab et al., 2011).

Several new recent alterations to test methodologies addressing the previously mentioned interactions are starting to be reported in the literature, which will lead to less variability in the results, hence increasing the relevancy and reliability of the toxicological results (Hartmann et al., 2013; Hund-Rinke et al., 2016). Moreover, the nitrate anion concentration in test media does not explain or seems to affect the toxicological profile of both LDHs, regardless of the tested grain size or tested concentration, since the detected nitrates concentrations do not impair the green microalgae's growth, as a study by Jeanfils et al. (1993) suggests. In that study, only nitrate concentrations above 310 mg/L slightly decreased the growth of *C. vulgaris.*

Finally, Zn-Al LDH proved to be less toxic than Cu-Al LDH, considering all EC_{50} values obtained, as higher values were observed for the first. The ecotoxicological difference between LDHs results from their metallic composition. According to a study published by Al-Hasawi et al. (2020), Cu yields greater toxicity towards the growth of *R. subcapitata*, compared to Zn and Al, being the latter the least toxic. Moreover, similar results have been previously reported by Ouyang et al. (2012) for *C. vulgaris*.

2.6 Conclusion

The main findings encountered in this study suggest that for these particular LDHs (i.e. Zn-Al and Cu-Al), the serial dilutions exposure methodology seems to be suitable to carry future algae growth inhibition tests, although for functionalized LDHs (e.g. Zn-Al LDH loaded with biocides, as used in Avelelas et al., 2017; Martins et al., 2017; Gutner-Hoch et al., 2018, 2019) new exposure methodology studies have to be performed. Moreover, no grain size-dependent toxicity was observed for the Zn-Al LDH, however, for the Cu-Al LDH, 3 grain size fractions are of interest when considering future possible applications due to their lower toxicological profile comparatively to the bulk counterpart. Such applications include but are not limited to, anti-fouling or anti-corrosive paints and coatings, carriers for fertilizers, phosphorus removal from eutrophicated lakes or removal of toxicants from wastewater just to name a few, all in which freshwater microalgae could be negatively affect despite being non-target organisms. Additionally, based on the growth inhibition results obtained for the Zn-Al LDH and Cu-Al LDH, a clear conclusion is withdrawn: if only one was to be selected for a specific application, where both LDH types could employed, it had to be the Zn-Al LDH, due to their lower toxicity.

However, the Cu-Al LDH application to other highly specific usages cannot not be discarded.

Future studies should also consider using state-of-the-art techniques for the LDHs' physicochemical characterization (e.g. particle size distribution by electric birefringence-based methods), over traditionally employed ones (e.g. DLS) which do not account for inherit specificities (e.g. NM geometry, or shape), as reported in our study. Moreover, addressing the LDHs' behaviour, by sedimentation/agglomeration preliminary testing, in order to ensure constant NM test concentrations, is undoubtedly of advantage to derive a testing strategy for accurate exposure and derivation of effects. Further ecotoxicological testing with other ecologically relevant freshwater species (e.g. *Daphnia magna, Lemna minor)* should be also performed, to ensure a full hazard assessment of these NMs. Additionally, carrying future tests with organisms from different functional groups (e.g. bacteria) or trophic levels (e.g. fish) should also be considered regarding the generation of species sensitive distribution (SSD) curves, towards the hazard assessment of LDHs in the freshwater ecosystem. This information will be critical for regulatory purposes and will reinforce the evidences that Zn-Al LDH are low/no toxic for a wide-range of marine organisms, including microalgae (Avelelas et al., 2017) and invertebrates (Martins et al., 2017; Gutner-Hoch et al., 2018, 2019).

Ultimately, this study poses as a solid starting point, contributing with real experimental data, towards a most needed NM test methodology harmonization, which will enable a realistic inter-laboratory data comparison, and at the same time, granting more environmental relevance to the study.

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Chapter III

Final considerations, and future perspectives

3. Final considerations and future perspectives

Nowadays, the exponential growth of the nanotechnology industry and the NM manufacturing for various industrial and consumer applications leads to great concerns about the environmental hazard that may derive from such NM presence in the environment (Behra and Krug, 2008).

In order to predict the hazard of NMs, a proper evaluation of their ecotoxicological profile has to be carried out. However, the current test methodologies are designed for conventional chemicals and do not account for inherent NMs' physicochemical properties. Unlike conventional chemicals, NMs may exhibit different exposure test behaviour, due to such characteristics. Hence, nano-specific test methodologies involving dispersion and exposure methods are of great need (Behra and Krug, 2008; Dekkers et al., 2016; Hansen and Baun, 2012b; Hartmann et al., 2017; Vanhaecke, 2016). Recently, the OECD has developed and published a guidance document addressing some of the issues concerning NM risk assessment test methodologies (OECD, 2020).

The present study aimed to test two recommended exposure methodologies (serial dilutions from a stock dispersion or direct addition of NM to each test concentration) from the OECD guidance document 317 on the toxicological output of two commercially available promising LDHs (Zn-Al and Cu-Al) exposed to *R. subcapitata*. Differences between exposure methodologies were only found for Zn-Al LDH, with the serial dilutions being the one producing reproducible toxicity results. Due to that reason, the serial dilutions methodology was selected to carry the further algae growth inhibition tests aiming assess if different grain sizes (bulk, < 25, 25-63, 63-125, 125-250, and > 250 µm) of both LDHs yielded different toxicity in the growth of the microalgae. For Zn-Al LDH, only concentration-dependent toxicity was observed for each grain-size, but no size-dependent toxicity was depicted. However, the three bigger size fractions of Cu-Al LDH (63-125, 125-250, and > 250 µm) yielded lower toxicity on the algae's growth rate when compared to the small sized and the heterogeneous (bulk) LDH. Considering this result, these three grain sizes might be subjected to further industrial or consumer applications as a more environmentally friendly NM. These findings are also going to be reported back to the manufacturer as the grain size separation might be of potential advantage regarding nanosafety.

The study conducted poses as a solid starting point for further exposure methodology testing of NMs, and for more studies involving both LDHs as they seem to be very promising NMs (when comparing to other more toxic nano-forms, e.g. ZnO nanoparticles). In the future, more studies involving other methodologies and other NMs have to be carried out in order to standardize test methodologies depending on the NM type and physicochemical characteristics, to enable inter-laboratory data comparison.

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