TALLINN UNIVERSITY OF TECHNOLOGY
SCHOOL OF ENGINEERING
Department of Materials and Environmental Technology

TOXICITY OF ENGINEERED NANOCOMPOSITE PARTICLES OF VARIOUS METAL OXYHYDROXIDES TO CRUSTACEAN *DAPHNIA MAGNA*

METALLI-PÕHISTE NANOKOMPOSIITOSAKESTE TOKSILISUS VESIKIRPUDELE *DAPHNIA MAGNA*

MASTER THESIS

Student: Triin Vaimann

Student code: 204131KAKM

Supervisors: Asya Ivanova Drenkova-Tuhtan, PhD
Research Fellow
Irina Blinova, PhD
Senior Researcher

Tallinn, 2023

AUTHOR'S DECLARATION

Hereby I declare, that I have written this thesis independently.
No academic degree has been applied for based on this material. All works, major viewpoints and data of the other authors used in this thesis have been referenced.

"....." May 2023

Author: Triin Vaimann
/signature /

Thesis is in accordance with terms and requirements

"....." May 2023

Supervisor: Asya Ivanova Drenkova-Tuhtan
/signature/

"....." May 2023

Supervisor: Irina Blinova
/signature/

Accepted for defence

"....."....................2023 .

Chairman of theses defence commission: .....
/name and signature/

Non-exclusive Licence for Publication and Reproduction of Graduation Thesis[1]

I, Triin Vaimann hereby

1. grant Tallinn University of Technology (TalTech) a non-exclusive license for my thesis Toxicity of Engineered Nanocomposite Particles of Various Metal Oxyhydroxides to Crustacean *Daphnia Magna*,

(*title of the graduation thesis*)

supervised by Asya Ivanova Drenkova-Tuhtan and Irina Blinova,

(*Supervisor's name*)

1.1 reproduced for the purposes of preservation and electronic publication, incl. to be entered in the digital collection of TalTech library until expiry of the term of copyright;

1.2 published via the web of TalTech, incl. to be entered in the digital collection of TalTech library until expiry of the term of copyright.

1.3 I am aware that the author also retains the rights specified in clause 1 of this license.

2. I confirm that granting the non-exclusive license does not infringe third persons' intellectual property rights, the rights arising from the Personal Data Protection Act or rights arising from other legislation.

_____ (*date)*

[1] *The non-exclusive licence is not valid during the validity of access restriction indicated in the student's application for restriction on access to the graduation thesis that has been signed by the school's dean, except in case of the university's right to reproduce the thesis for preservation purposes only. If a graduation thesis is based on the joint creative activity of two or more persons and the co-author(s) has/have not granted, by the set deadline, the student defending his/her graduation thesis consent to reproduce and publish the graduation thesis in compliance with clauses 1.1 and 1.2 of the non-exclusive licence, the non-exclusive license shall not be valid for the period.*

Department of Materials and Environmental Technology

THESIS TASK

Student: Triin Vaimann, 204131KAKM

Study programme, KAKM02 Chemical and Environmental Technology

Supervisor(s):

Asya Drenkova-Tuhtan, PhD, Research Fellow, NICPB (asya.drenkova@kbfi.ee)

Irina Blinova, PhD, Senior Researcher, NICPB (irina.blinova@kbfi.ee)

Thesis topic:

Toxicity of Engineered Nanocomposite Particles (NPs) of Various Metal Oxyhydroxides to Crustacean *Daphnia Magna*

Metalli-põhiste nanokomposiitosakeste toksilisus vesikirpudele *Daphnia magna*

Thesis main objectives:

1. To assess the toxicity of 11 metal oxyhydroxides to *Daphnia magna*
2. To evaluate the potential hazard of the tested compounds to aquatic ecosystems.

Thesis tasks and time schedule:

No	Task description	Deadline
1.	Literature review and collection of available data on toxicity of metals (Zn, Fe, Zr) to aquatic microcrustaceans.	03.01.2022
2.	Analysis of the behaviour (sedimentation and solubility) of the 11 investigated NPs in the medium used for the toxicity tests.	07.02.2022
3.	Quantification of the total metal concentrations in the test medium.	21.02.2022
4.	Assessing the toxicity of the 11 nanocomposite engineered particles to aquatic microcrustacean *Daphnia magn*a.	07.03.2022
5.	Initial draft submission to the supervisors	16.04.2023

Language: English **Deadline for submission of thesis:** 26.05.2023

Student: Triin Vaimann ..... "....."..... 2023

/signature/

Supervisor: Asya Drenkova-Tuhtan ..... "....."..... 2023

/signature/

Supervisor: Irina Blinova ..... "....."..... 2023

/signature/

Head of study programme: Marina Kritševskaja "....."..... 2023

/signature/

Terms of thesis closed defence and/or thesis restricted access conditions to be formulated on the reverse side.

CONTENTS

PREFACE

The topic for this Thesis was suggested by the researcher and primary supervisor, Asya Ivanova Drenkova-Tuhtan. This work was performed in the frame of the "NanoPhosTox" project (Grant agreement ID: 867457), which is funded by the European Commission as part of the MSCA-IF-EF-ST action within the Horizon 2020 program H2020-EU.4 [1]. The practical research was conducted at the Laboratory of Environmental Toxicology (headed by Dr. Anne Kahru) at the National Institute of Chemical Physics and Biophysics (NICPB). Asya Drenkova-Tuhtan and Irina Blinova provided consultation, support, and guidance to the author throughout the study.

The objective of this thesis was to study toxicity of eleven nanocomposite sorbent materials designed for phosphorus recovery from wastewater to aquatic micro-crustacean *Daphnia magna* and evaluate environmental safety of tested materials. The research was divided into three sections:

1. Assessment of the toxicity of 11 metal oxyhydroxides to *Daphnia magna*.
2. Characterization of the test solutions using Picofox X-ray fluorescence spectroscopy.
3. Evaluation of the potential hazard of tested compounds to aquatic ecosystems.

I express my gratitude to my supervisors Asya Drenkova-Tuhtan and Irina Blinova for their invaluable guidance, generous allocation of time, and unwavering patience during the experimental work and thesis writing. I also extend my heartfelt appreciation to all the personnel at NICPB for their kindness and support while working at the Laboratory of Environmental Toxicology. Particularly Heiki Vija for his guidance in working with Picofox and Jelizaveta Richter for her collaboration in growing the algae. I would like to convey my special thanks to the head of the laboratory, Dr. Anne Kahru, for providing me with the opportunity to conduct this research. Last but not least, I am sincerely grateful for my family, without them it would have been impossible.

Key words: crustacean *Daphnia magna*, ecotoxicology, acute toxicity, nanocomposite sorbent materials, master thesis.

LIST OF ABBREVIATIONS AND SYMBOLS

ASTM - American Society for Testing and Materials

ECC - European Economic Community (EEC)

EC_{50} – the median effective concentration of the test substance that induces an adverse effect in 50% of the test organisms after a specified exposure time

ECHA - European Chemicals Agency

ICP-OES - Inductively Coupled Plasma Optical Emission spectroscopy

ISO - International Organization for Standardization

LC_{50} – the median lethal concentration of the test substance that induces mortality in 50% of the test organisms after a specified exposure time

LOD - Limit of detection

MPs - Magnetic particles

NC - Nanocomposite

NP - Nanoparticles

OECD - The Organization for Economic Cooperation and Development

P - Phosphorous

TXRF - Total Reflection X-ray Fluorescence Spectroscopy

USEPA - United States Environmental Protection Agency

WWTP - Wastewater treatment plant

INTRODUCTION

The global concern regarding phosphorus (P) deficiency is increasing, as it is a vital nutrient for all living beings and a crucial fertilizer component for crops growth and global food security [2], [3], [4]. Currently, phosphorus is mainly obtained through mining phosphate rock, which is a scarce and non-renewable resource that is geographically unevenly distributed. Regions with limited or no reserves of phosphate rock are heavily dependent on import of phosphate-based fertilizers, which poses a serious threat for geopolitical and economic reasons. Additionally, phosphate rock mining has a negative impact on the environment. Nevertheless, it is possible to recover phosphorus from secondary P-rich sources which are currently considered waste streams (e.g. wastewater, animal manure, food waste, etc.). Phosphorus, however, is not only an essential nutrient but also an environmental pollutant which must be removed from wastewater before discharging the effluent in order to prevent eutrophication problems, i.e. excessive growth of aquatic plants and algae blooms, leading to oxygen depletion in the receiving water bodies. The discharge limit values for total phosphorus in the treated effluent of wastewater treatment plants (WWTP) are established by the EU Urban Wastewater Treatment Directive (91/271/EEC) [5]. WWTPs with a population equivalent ranging from 10,000 to 100,000 must adhere to a limit of 2 mg-P/L, while WWTPs with a population equivalent exceeding 100,000 must not exceed 1 mg-P/L. Thus, before discharging the treated wastewater effluent, it is necessary to meet the regulatory discharge limit values for the maximum allowable phosphorus concentration. Therefore, there is a high demand to develop technologies which can simultaneously remove and recover phosphorus from wastewater. Moreover, phosphorus recovery from wastewater is a more sustainable approach than conventional phosphate rock mining what is in accordance with the principles of the circular economy model.

Previous research suggests that engineered nanocomposite particles are effective in recovering phosphorus from wastewater [6], [7], [8]. This thesis is part of the EU-funded project "NanoPhosTox" [1], which aims to assess the ecotoxicity potential of these particles, and the outcomes in this thesis contribute successfully to achieving some of the project tasks. It is essential to test the nanocomposite materials for toxicity as there is a risk of their unintentional discharge into surface waters (in case of inefficient harvesting and unsuccessful retaining within the engineering treatment facility) or possible leaching of their metal precursors into the effluent of the wastewater treatment plant and subsequently into the receiving water body. Thus, summing up the

above, the tested composites may enter environment as a result of application, storage or transportation (in the case of accidental contamination).

The nanocomposite materials selected for this research have several advantages, including highly efficient phosphorus recovery from wastewater through reversible sorption and the ability to reuse the particles multiple times after regeneration [7]. These benefits are achieved by coating magnetic particles with the nanostructured adsorbent materials [8] and using permanent magnets to collect the multi-component particles from the wastewater. Altering the pH allows for desorption of the phosphorus from the composite particles, which can be regenerated and reused in the next application. Such approach has become increasingly popular [9], [10].

The main hypothesis of the thesis is that zinc (Zn)-containing nanocomposites are more toxic to aquatic organisms than the ones not containing Zn due to release of toxic Zn ions. It was shown in the scientific literature that metal-based nanoparticles (NPs) induce toxicity mostly via bioavailable toxic metal ions [11]**,** [12]. In order to prove the hypothesis, the toxicity of 11 nanocomposite sorbent materials for phosphorus recovery from wastewater were evaluated using acute test with crustacean *Daphnia magna*. The nanocomposites used in this research were synthesized by the main supervisor, Asya Drenkova-Tuhtan, as part of the "NanoPhosTox" project [1]. Specifically, this work accomplishes three main tasks:

1. Assessment of the toxicity of 11 metal oxyhydroxides to *Daphnia magna*;
2. Characterization of the test solutions using total reflection X-ray fluorescence spectroscopy (TRXF);
3. Evaluation of the potential hazard of tested compounds to aquatic ecosystems.

This thesis consists of three main sections:

1. Literature review – this section provides an overview of the significance of phosphorus as an irreplaceable nutrient, technologies for its recovery from wastewater, and the use of crustaceans in cost-effective *in vitro* toxicity screening test;
2. Methods – this section presents characterisation of the 11 nanocomposite materials developed for phosphate adsorption from wastewater and outlines the methodologies (elemental analysis with Picofox and acute immobilisation test with *Daphnia magna*) used to characterize and assess their safety;

3. Results – This section displays the results of the experiments, analyzes the made hypotheses, interprets the outcomes, draws conclusions, and proposes further research directions.

1. LITERATURE REVIEW

1.1 Phosphorus in the environment

Phosphorus is an essential element for life, playing key roles in DNA, RNA, cell membrane structure and function, and energy metabolism [13], [14]. Phosphates are a class of compounds that contain the tetrahedral anion PO_4^{3-} ion. Phosphates can occur in various forms, including inorganic and organic phosphates. Inorganic phosphates are derived from minerals such as apatite and can be found in rocks and soils [15]. Organic phosphates are derived from living organisms and can be found in molecules such as nucleic acids and phospholipids [14].

In plants, phosphorus is a vital component of adenosine triphosphate (ATP), which is the primary energy carrier in the cells. The energy stored in the chemical bonds of adenosine triphosphate is used to power a wide range of biological processes, including protein synthesis, cell division, and muscle contraction [16]. Phosphorus is a vital nutrient for plant growth, but at the same time, a limited resource [14]. Overuse of phosphorus by anthropogenic activity can lead to different environmental problems. For example, excess phosphorus in freshwater bodies can lead to eutrophication, which can harm aquatic life and impair water quality [17]. The excessive and imbalanced amounts of phosphorus and nitrogen, both historically and presently, have caused eutrophication in over 97% of the Baltic Sea region [18].

Phosphorus is also widely used in agriculture as a fertilizer, helping to increase crop yields and improve food security [19], [20]. Around 80% of the natural phosphorus resources, which are non-renewable and obtained from phosphate rock, are utilized by the fertilizer production industry [21]. However, excess phosphorus in the environment can lead to negative impacts, such as eutrophication of water bodies. Eutrophication is the process by which an excess of nutrients, particularly phosphorus and nitrogen, leads to the overgrowth of aquatic plants and algae, which can deplete the oxygen levels in the water, killing fish and other aquatic life. [22]

Additionally, phosphorus is a crucial element in various industrial sectors, including electronics, automotive, pharmaceuticals, food, plastics, and more. Phosphorus is a finite and non-renewable resource [19], with most of the world's reserves located in a few countries [23]. The uneven distribution of phosphorus resources worldwide, mainly concentrated in Morocco and Western Sahara, and to a lesser extent, China, the USA, Russia, and other regions, coupled with unstable prices, may result in critical situations regarding food security and political tensions [24]. As demand for phosphorus is constantly increasing, there is growing concern about potential shortages in the future [25].

Recycling of phosphorus from human and animal waste and food production by-products can help to conserve this important resource [21], [26]. There are also alternative sources for phosphorus recovery including manure, slaughter waste and steelmaking slag [27]. In East Asian countries, including China, Korea and Japan, steelmaking slag is one of the most important secondary phosphorus resources [19]. Wastewater, sewage sludge, animal production residues, and to a lesser extent, waste from the agri-food sector [21] are the primary waste resources with significant amounts of phosphorus. Thus, the development of technologies for phosphorus recovery from secondary sources, such as municipal wastewater [8] holds immense importance.

1.1.1 Phosphorus recovery from wastewater

The prevailing technique for removing phosphate from wastewater is through chemical precipitation using metal salts to form insoluble metal phosphates, which are then removed with the sewage sludge. However, the metal phosphates that are formed through this process lack any fertilizer value because they are not directly available for the plants, and as a result, the phosphate must be extracted from the sludge in a more purified, plant-available form to be considered as a fertilizer. This process is expensive and stoichiometrically requires the use of large amounts of chemicals. [8]

Phosphorus can be recovered from wastewater in the form of struvite, a crystalline compound which is a mixture of magnesium, ammonium and phosphate (also known as MAP) and is a directly plant-available slow-release fertilizer. Struvite can be recovered through a struvite precipitation process, which involves adding magnesium and ammonium to wastewater (if not already present in the wastewater) under controlled conditions [21], [28]. The formed struvite crystals can be harvested through sedimentation or filtration.

The advantage of P-recovery through struvite precipitation is that it can be easily scaled up to meet the needs of large wastewater treatment plants, and that the precipitated struvite can be used directly as a fertilizer [28]. A disadvantage is that the process requires often times additional source of magnesium and ammonium (if not present in the wastewater). In case of sufficient magnesium and ammonium concentration in the wastewater (e.g. in sludge liquors, filtrates, centrates, etc.), struvite can also form naturally, usually in the pumps and pipes of the periphery equipment, which leads to blockages and higher operating costs for the treatment plant [29]. Thus, a controlled,

intentional precipitation of struvite in these treatment units, have the additional advantage to prevent such operational problems.

Another way to remove phosphorus from wastewater is through biological phosphorus removal with the help of anaerobic polyphosphate-accumulating bacteria [30]. This process is often applied in combination with chemical phosphorus removal, where chemicals, such as alum or ferric chlorides, are used to remove dissolved phosphorus [31].

Biological phosphorus removal has the advantage of being a relatively low-energy process [32], and it can also enhance the biogas production of the treatment plant [33]. However, the process can be sensitive to changes in wastewater composition, and it can be difficult to control the amount of phosphorus removed though it [32], [33].

There are various methods for phosphorus recovery from wastewater. The summary of phosphorus recovery methods is depicted in Figure 1.1. Methods are classified into four categories: physical - blue, thermal - red, biological - green, chemical - yellow. Each approach has its own strengths and weaknesses. [21]

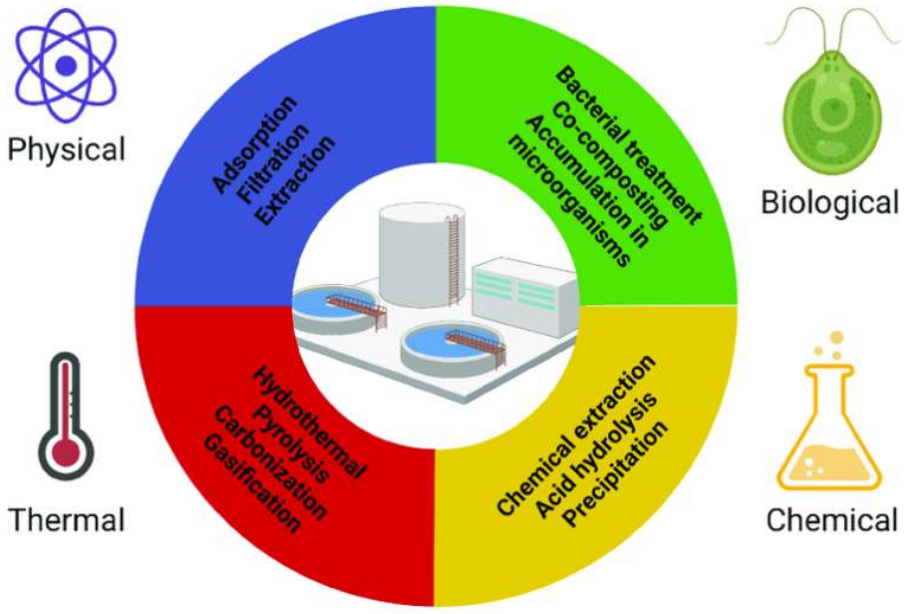

Figure 1.1. Summary of phosphorus recovery methods [21].

1.1.2 Nanocomposite engineered particles for phosphorus recovery from wastewater

If the wastewater has not undergone any targeted P-elimination at the treatment plant, phosphorus can alternatively be recovered directly from the treated effluent with a relatively low phosphorus concentration distributed over a higher hydraulic load. Adsorption is considered to be a highly effective method for removal of soluble phosphate, especially in the low concentration range (µg/L–mg/L) [8], [9]. By utilizing a suitable adsorber, it may also be possible to recover the phosphate in a pure form through desorption using an appropriate regeneration solution [8]. In recent times, several research groups have been working on creating magnetic nanocomposite materials that can effectively remove phosphorus. For instance, Fang et al. (2017) [34] developed a silica-free superparamagnetic ZrO_2@Fe_3O_4 composite that enhances phosphate recovery from sewage. Chen et al. (2020) [35] created $La(OH)_3$-modified magnetic $CoFe_2O_4$ nanocomposites for the same purpose, while Sürmeli et al. (2022) [36] developed superparamagnetic nanocomposite microparticles that were modified with various layered double hydroxides.

Due to its high efficiency, ease of operation, and cost-effectiveness, adsorption is an attractive method, particularly when dealing with low concentrations of phosphate [35].

Drenkova-Tuhtan (2018) [37] proposed the use of nanocomposite magnetic carrier particles (sized between 3-25 µm) modified with an engineered adsorbent material (ZnFeZr-based) for the selective and reversible sorption of phosphorus from wastewater. The composite micro-sorbents can be magnetically extracted from water, regenerated in an alkaline solution, where phosphorus desorption and enrichment occur, and then reused. The phosphorus rich solution can be used as a source for further phosphorus recovery. This technology offers a dual benefit: phosphorus removal down to ultra-low effluent concentrations < 5 µg/L PO_4-P and < 50 µg/L P total, which eliminates any eutrophication risk, and the option for subsequent recovery of the valuable nutrient. As such, it is a superior alternative to conventional phosphorus removal methods in wastewater treatment. While many natural and engineered materials are proposed in literature as phosphate adsorbents, for practical applications they must be easily harvestable, reusable, and inexpensive to manufacture using abundant non-toxic precursors, as is proposed in this work. Deposition of the adsorbent on magnetic carrier particles enables its selective harvesting via magnetic separators, subsequent regeneration, and reuse. [7], [37]

Nevertheless, successful commercialization of any new material and/or technology depends not only on the innovative value but also on health, safety and environmental aspects. Thus, environmental safety of new materials must be evaluated prior to their full-scale application.

In the given thesis, the nanocomposite materials proposed by Dr. Asya Drenkova-Tuhtan, were used. In a preceding MSc thesis authored by Kevin Uke (2022) [38], he used the same materials in his experimental work to test their toxicity potential to naturally bioluminescent marine bacteria *Vibrio fischeri*, which was the initial phase in testing the materials' safety. The current thesis present results of further investigation of the materials' toxicity using crustacean *Daphnia magna*.

1.2 Aquatic crustaceans as test organisms

Freshwater ecosystems are home to a diverse array of aquatic organisms, including crustaceans. Crustaceans such as crayfish, shrimp, freshwater crabs and many other species of planktonic crustaceans (including *Daphnia sp.* also called as water fleas) play important ecological role as both primary and secondary consumers [39]. These organisms may also be exposed to a wide range of contaminants in their aquatic environments, including heavy metals, pesticides, and other toxic substances. As such, it is important to assess the potential toxicity of these contaminants to crustaceans in order to protect and preserve these important aquatic species. [39], [40]

Crustaceans are used for assessment of chemicals safety both in the laboratory and field studies. In laboratory studies, crustaceans are typically exposed to a range of different concentrations of a particular contaminant, and the effects (e.g., survival, growth, reproduction, and behavioural changes) of this exposure are then monitored over a period of time to evaluate environmental safety of tested compounds. Field studies are mainly focus on the accumulation of potentially toxic compounds in the tissues of organisms collected in the natural aquatic ecosystems. [41]

A large number of studies have been conducted to assess the toxicity of various contaminants to freshwater crustaceans. For example, research has shown that exposure to heavy metals such as lead, cadmium, and copper can have significant negative effects on the survival and growth of crayfish and shrimp [42], [43]. Similarly, exposure to pesticides has been shown to have toxic effects on these organisms [44]. Some micro-crustacean species (*Daphnia magna*, *Daphnia pulex*, *Ceriodaphnia dubia*, and *Ceriodaphnia affinis*) are the preferred pelagic invertebrate taxon in REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) legislation [45].

1.2.1 *Daphnia magna*

Daphnia magna (Figure 1.2), also known as the water flea, is a small (size up to 5 mm), freshwater crustacean which belongs to the genus *Daphnia*. These organisms are commonly found in ponds, lakes, and other freshwater ecosystems. They are a crucial component of the aquatic food chain (serve as an important food source for fish). *Daphnia* species are considered to be important indicators of water quality, with *Daphnia magna* being one of the oldest models for this field of study [40]. According to several studies [39], [46], [47]. *Daphnia magna* have been used as bioindicators in freshwater ecosystems to assess the effects of pollution by a wide range of chemicals, including heavy metals, pesticides etc. *Daphnia magna* is a keystone species which plays a critical role in structuring aquatic communities, and its presence can have a significant impact on the diversity and abundance of other aquatic organisms [39], [47].

Figure 1.2. *Daphnia magna.*

One of the key characteristics of *Daphnia magna* is its ability to reproduce rapidly. Under optimal conditions, it can reproduce through a process called parthenogenesis, in which eggs are produced without fertilization. This allows populations to increase quickly, making them useful as a model organism for studying population dynamics [46].

Daphnia magna have also been used in a wide range of research studies. They have been used to study population dynamics, toxicology, ecotoxicology, and evolutionary biology [48], [49], [50]. *Daphnia magna* short generation time, ease of culture and handling make them an ideal model organism for these studies.

1.2.2 Using *Daphnia magna* in toxicity testing

For several decades, Daphnids have been widely used in aquatic toxicology, as they have been shown to be sensitive to a wide range of chemicals. In particular, a large database for toxicity of different chemicals (solvents, pesticides, heavy metals etc) for *Daphnia magna* exists in the Laboratory of Environmental Toxicology at the National Institute of Chemical Physics and Biophysics [11].

There are several reasons why *Daphnia*, and specifically *Daphnia magna*, are popular for experimental work: sensitivity to toxic substances, short life cycle, small size, high fecundity, wide spatial distribution, and the availability of omics-based tools [40]. Furthermore, *Daphnia magna* is recommended for use in aquatic environmental studies according to OECD chemical testing guidelines [51].

Daphnia magna has been widely utilized for the toxicity assessment of various chemicals in water and is recommended for this purpose by organizations such as ASTM (2014) [52], ISO International Standard 6341 (1996) [53], OECD 202 (2004) [51] and U.S. EPA (2002) [55]. Due to its extensive use in toxicity testing, *Daphnia magna* is considered a model organism for quantifying toxicity and is recognized as such in the legislation of many countries including Austria, Australia, Belgium, Canada, Denmark, France, Germany, Italy, New Zealand, the Netherlands, Norway, Portugal, Spain, Sweden, the United States, and the United Kingdom [39].

In toxicity testing, various test designs or formats have been developed to assess the effect of pollutants on *Daphnia magna*, including short-term acute toxicity tests (OECD202) [51] and long-term reproduction tests (OECD211) [54].

Acute toxicity tests are designed to assess the effects of a toxicant on *Daphnia magna* during short-term exposure and are typically conducted over a period of 24 and 48 hours. The endpoint of acute *D. magna* test is immobilisation of the test organisms. EC_{50} values (the concentration of the test substance that induces an adverse effect in 50% of the test organisms) are calculated for quantifying the impact of a substance on the tested organisms. In addition, EC_{10} (the concentration at which 10% of the organisms tested exhibit a statistically significant effect of the chemical) and NOEC (No-observed-effect concentration) are very common parameters. [56]

The most commonly used format for acute toxicity testing is the static renewal method, in which a known number of *Daphnia magna* are exposed to a range of concentrations of the toxicant, and the number of immobilised daphnids is counted at the end of the test period (Test No. 202: *Daphnia sp*. Acute Immobilisation Test) [51].

Daphnia magna reproduction test (no. 211) is designed to assess the effect of a toxicant on the reproduction of *Daphnia magna*. This assesses the long-term effects of exposure to a toxicant on *Daphnia magna* and are typically conducted over a period of a few weeks (21 days). The most commonly used method for reproduction testing is the reproduction rate method, in which a known number of *Daphnia magna* are exposed to a range of concentrations of the toxicant, and the number of offspring produced by each individual is monitored over the test. [54]

2. METHODS

2.1 Nanocomposite materials

Eleven previously developed, engineered nanocomposite materials (Table 2.1, Figure 2.1) were chosen for the tests, based on conclusions from earlier research work, demonstrating their high efficiency and selectivity for phosphate adsorption, and successful reusability without compromising P-adsorption efficiency [6], [7], [8], [10]. The first five were synthesized with different nominal molar ratios of the two-, three- and four-valent metals Zn^{2+}, Fe^{3+}, and Zr^{4+}, namely 18:5:1, 10:1:1, 6:1:1, 4:1:1, and 3.6:0.2:1 (Table 2.1). The other five nanocomposite materials contained different metal precursors for the two-valent metal, such as Ca^{2+} and Mg^{2+}, to either replace or reduce the toxic Zn^{2+}, namely CaFeZr 6:1:1, CaZnFeZr 3:3:1:1, MgFeZr 6:1:1, MgZnFe 1:1:1, and CaFe 2:1 [8]. Chemical 11, ZnFeZr 6:1:1 @ Magnetic Particles, was the first nanocomposite material combined with Fe_3O_4/SiO_2 magnetic carrier particles and successfully tested at pilot-scale for the reversible adsorption of phosphate over numerous cycles of adsorbent reuse [7]. The elements are presented as ions, as the precursor metal salts used for the synthesis of the nanocomposites were pre-dissolved before the precipitation reaction for the formation of the metal oxyhydroxides. And exactly the combination of two-, three- and four-valent metal ions is supposed to form the so-called Layered Double Hydroxides (LDH) which is a group of materials, known to be highly efficient for phosphate adsorption. All the materials were proven to be LDH or LDH-like formations.

The method for synthesizing metal hydroxide precipitates, such as layered double hydroxides and their related structures, follows a previously published procedure [6], [8], [57]. To prepare the precursor solution for each material, the desired metal salts were dissolved in 100 mL of deionized water. The resulting solutions were then slowly, drop-by-drop, added to a round flask containing 400 mL of 0.15 M NaOH, while stirring at 300 rpm for 10 minutes, maintaining pH > 10 to ensure complete precipitation of all metals to hydroxides. The mixture was stirred for additional 5 minutes at this high pH and then adjusted to pH 7 with hydrochloric acid to simulate the pH level found in wastewater. This step is crucial to prevent any potential dissolution of metal cations when the materials are added to wastewater, which typically has a pH between 6-8. By neutralizing the precipitate immediately after synthesis, the resulting solid remains stable under these conditions and will not undergo any further changes when added to wastewater. After this step, the samples were centrifuged, washed twice with deionized water, and dispersed in deionized water [8].

Table 2.1 Characteristics of the nanocomposite materials used in this study as water suspensions (photographed in Figure 2.1), including particle size, nominal (theoretical) metal molar ratios, stock concentrations, and stock pH values.

No	Nanocomposite materials	Nominal molar ratio	Stock concentration (g/L)	pH	Particle size D_{50} (µm)
01	ZnFeZr 6:1:1	$6Zn^{2+}$: $1Fe^{3+}$: $1Zr^{4+}$	16.8	6.7	4.5
02	CaFeZr 6:1:1	$6Ca^{2+}$: $1Fe^{3+}$: $1Zn^{2+}$	5.5	7.9	6.0
03	CaZnFeZr 3:3:1:1	$3Ca^{2+}$: $3Zn^{2+}$: $1Fe^{3+}$: $1Zr^{4+}$	10.5	6.5	5.9
04	MgFeZr 6:1:1	$6Mg^{2+}$: $1Fe^{3+}$: $1Zr^{4+}$	5.8	8.5	9.5
05	MgZnFe 1:1:1	$6Mg^{2+}$: $6Zn^{2+}$: $1Fe^{3+}$	9.7	7.2	3.2
06	CaFe 2:1	$2Ca^{2+}$: $1Fe^{3+}$	4.6	8.4	4.0
07	ZnFeZr 18:5:1	$18Zn^{2+}$: $5Fe^{3+}$: $1Zr^{4+}$	45.7	6.7	3.7
08	ZnFeZr 4:1:1	$4Zn^{2+}$: $1Fe^{3+}$: $1Zr^{4+}$	12.2	6.9	9.9
09	ZnFeZr 3.6:0.2:1	$3.6Zn^{2+}$: $0.2Fe^{3+}$: $1Zr^{4+}$	9.9	6.6	8.1
10	ZnFeZr 10:1:1	$10Zn^{2+}$: $1Fe^{3+}$: $1Zr^{4+}$	23.7	6.5	5.5
11	ZnFeZr 6:1:1 @ Magnetic Particles	$6Zn^{2+}$: $1Fe^{3+}$: $1Zr^{4+}$	56	8.0	25.0

The chemicals used for the synthesis were: zinc chloride ($ZnCl_2$), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), aluminium chloride hexahydrate ($AlCl_3 \cdot 6H_2O$), calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$), zirconium (IV) oxychloride octahydrate ($ZrOCl_2 \cdot 8H_2O$), hydrochloric acid (HCl, 36%), iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), sodium hydroxide (NaOH) [8]. The synthesis of all metal oxyhydroxide nanocomposites was carried out by Dr. Asya Drenkova-Tuhtan in the first phase of the "NanoPhosTox" project [1], and the nanocomposites were provided as ready water suspensions for the further toxicity tests performed in this thesis.

Because of its high solubility in water, $ZnCl_2$ is capable of leaching potentially harmful free Zn^{2+} ions and forming ZnO nanoparticles in solutions. The presence of elevated concentrations of Zn can have a significant adverse impact on ecosystems, as Zn is highly toxic and its toxicity is influenced by pH [58]. Measurements were taken to make sure that pH level was acceptable and this could not be affecting the results.

According to European Chemicals Agency Substance Infocard, zinc chloride and zinc oxide are very toxic to aquatic life, with long lasting effects [59], [60].

Figure 2.1 Photograph of the 11 metal oxyhydroxide nanocomposite materials (stock suspensions in deionized water) tested in this work for acute toxicity to crustacean *Daphnia magna*.

2.2 Toxicity tests with *Daphnia magna*

The acute toxicity test was chosen in the current study to evaluate the toxicity of the tested composites on *Daphnia magna*. This test is relatively easy to perform, quick, sensitive and cost effective.

The testing utilized Daphtoxkit F [61]. One of the main benefits of Toxkit microbiotests, as opposed to traditional bioassays, is that the test organisms are present in the kits in a "dormant" or "immobilized" state and can be activated as needed prior to conducting the toxicity test. Daphtoxkit tests follow testing protocols established by various national and international organizations, such as The Organization for Economic Cooperation and Development (OECD), International Organization for Standardization (ISO), European Economic Community (EEC), United States Environmental Protection Agency (USEPA), and American Society for Testing and Materials (ASTM). Each Daphtoxkit includes all the necessary materials to conduct 6 complete bioassays, the only equipment required is an incubator or a temperature-controlled room set to 20-25°C, a small light table or a dissection microscope, and standard laboratory glassware (Daphtoxkit procedure).

2.2.1 Acute immobilisation test

An acute immobilization test was conducted with *Daphnia magna* to evaluate nanocomposite materials toxicity, with immobilization recorded at both 24 and 48 hours. The test was conducted in accordance with OECD Guideline for testing chemicals "Test No. 202: *Daphnia sp*. Acute Immobilization Test" [51]. The dormant eggs of *Daphnia magna*, obtained from Microbiotest, Inc., Belgium (batch number DM121219), were used. Hatching, pre-feeding and testing (tested nanocomposite concentrations and control group) was carried out in artificial freshwater (prepared in ultrapure water (MilliQ

water) with 294 mg/L $CaCl_2 \cdot 2H_2O$, 123.25 mg/L $MgSO_4 \cdot 7H_2O$, 64.75 mg/L $NaHCO_3$, 5.75 mg/L KCl, and a pH of 7.8 ± 0.2). The artificial freshwater was aerated for at least 30 minutes before testing. The dormant eggs were thoroughly rinsed and transferred to a petri dish containing 15 ml of pre-aerated artificial freshwater, which was covered with another petri dish and incubated for up to 3 days at 6000 lux and temperature 20-22°C (Figure 2.2).

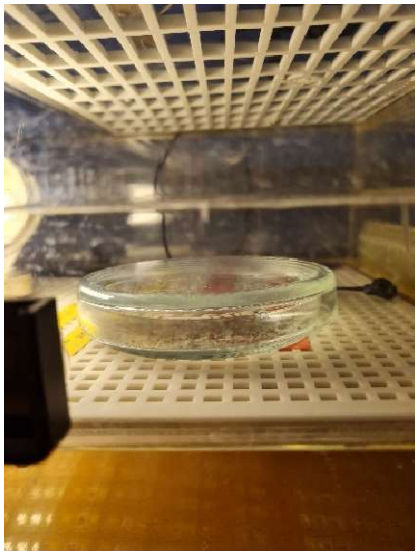

Figure 2.2 Hatching process. Dormant ephippia in Petri dish.

Daphnids less than 24 hours old were chosen for the test and were fed with microalgae *Raphidocelis subcapitata* (Figure 2.3) during two hours before testing commenced. The *Raphidocelis subcapitata* algae suspension used for pre-feeding was cultivated in the laboratory following OECD 201 guidelines [62], and a concentrated algae suspension was used to minimize the volume of algal culture medium. The algae were grown in artificial media (artificial freshwater) and subsequently centrifuged before being resuspended in the test medium in order to reduce the amount of algal culture medium required.

Figure 2.3 Pre-feeding with algae and dormant ephippia in Petri dish.

A range-finding test was conducted to perform initial estimation of the chemical toxicity. This test involved three concentrations, namely 1 mg/L, 10 mg/L, and 100 mg/L. The selection of 100 mg/L as the highest concentration was based on the OECD guideline [51]. In the definitive tests, six exposure concentrations (3.125 mg/L, 6.25 mg/L, 12.50 mg/L, 25 mg/L, 50 mg/L, and 100 mg/L) were selected based on the range-finding test results. Chemicals that showed low toxicity at the highest concentration in the range-finding test were tested in a definitive test with two concentrations (50 mg/L and 100 mg/L). The definitive test was performed three times (three repetitions, n=3) for chemicals that displayed toxicity and two times (n=2) for those that did not.

A test plate consisting of one rinsing well and four test wells was used (Figure 2.4). For each concentration or control group, 20 animals were divided into four groups of five animals each (5 per 10 ml), with 2 ml of test solution being provided for each animal. According to Guidelines, in the control group, mortality or immobility should not exceed 10%, otherwise the test would not be considered valid. Neonates which are unable to swim after gentle agitation of the liquid for 15 seconds are marked as immobilized, even if they are still capable of moving their antennae.

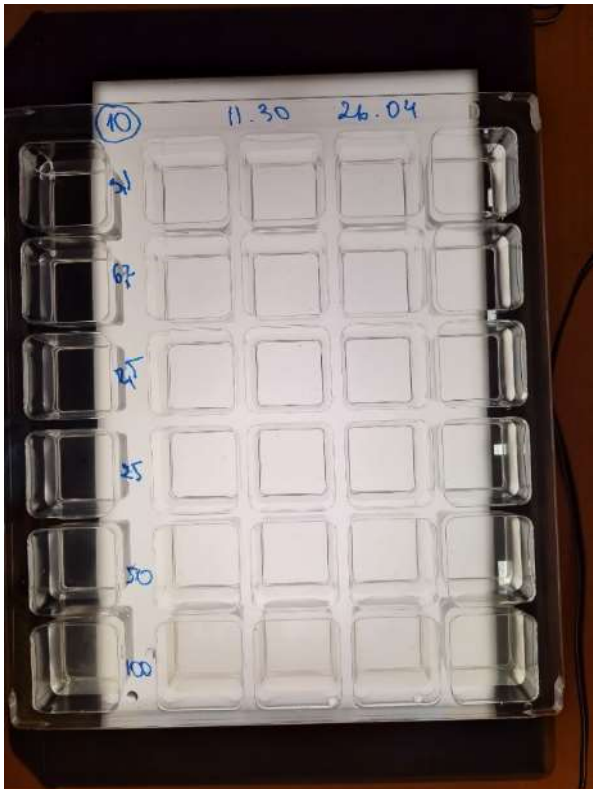

Figure 2.4 Test plate with *Daphnia magna* in composite 10 ZnFeZr 10:1:1 on light board.

Following the acute test, *Daphnia magna* specimens were examined under a Nikon SMZ1270 microscope (using a digital camera DS-Fi3 and software NIS-BR) to obtain further insights into how the chemicals interacted with the test organisms.

2.3 Elemental analysis of metals concentrations

The elemental analysis, concentrations of metals in the test media and eluates were analyzed with reflection X-ray fluorescence spectroscopy. The total metal concentrations in the test medium were determined using the Picofox S2 total reflection X-ray fluorescence spectrometer (TRXF) from Bruker AXSMicroanalysis GmbH, Germany. This instrument has detection limits ranging from ppb to ppm and is suitable for trace element analysis [63].

The total metal concentrations in the suspensions were measured in the beginning and at the end of the acute test. To control exposure concentrations, at the start of the test, test media samples were collected from the lowest concentration (3.125 mg/L if chemical is toxic or 50 mg/L if non-toxic) and from the highest concentration (100 mg/L) after shaking. At the end of the test, samples of all exposure concentrations were collected from the upper layer of the water column avoiding re-suspension (Figure 2.5).

Non-toxic chemicals were tested twice (n=2), while toxic chemicals were tested three times (n=3). The Picofox S2 was used for analysis of the samples due to the advantages that it requires low sample amounts and that device cooling is not necessary. Concentrated HNO_3, including 1 ppm Ga as an internal standard, was added to the samples before analysis. The elements analyzed were Zn, Fe, Zr, Ca, Mg. The limits of detection (LOD) for the individual metals for this device are: Zr, Mg 10-20 mg/L (ppm), Ca 50-100 µg/L (ppb), Zn, Fe 5-10 µg/L (ppb).

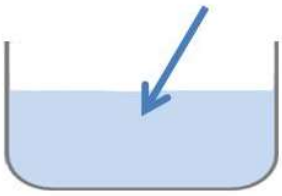

Figure 2.5 After testing, samples from the upper layer were collected avoiding re-suspension.

3. RESULTS

3.1 Concentration of the metals in the test solutions

Before the test, the test solutions were characterized by analyzing the lowest (3.125 mg/L for toxic and 50 mg/L for non-toxic nanocomposite material) and highest concentrations (100 mg/L) after shaking to find out the total metal concentrations in the solutions. The elements analyzed were Zn, Fe, Zr, Ca and Mg.

The nanocomposite material with the highest Zn total concentration before the test was material 10 (ZnFeZr 10:1:1) with a concentration of 46.56 mg/L Zn in 100 mg/L solutions and 1.73 mg/L Zn in 3.125 mg/L solutions. Following that was composite 07 with concentrations of 45.39 mg/L Zn in 100 mg/L solutions and 1.48 mg/L Zn in 3.125 mg/L solutions (Appendix 1).

In order to assess Zn concentrations in the water column after 48 hours of exposure, samples were taken from the upper level of exposure cells avoiding re-suspension (Figure 2.5) as non-dissolved composite particles have settled down on the bottom (Figure 3.1 as an example). The nanocomposites are heavy (average particle size >1 µm) and they settle very quickly to the bottom (already within the first 30 minutes). Figure 3.1 shows how the nanocomposite material 06 CaFe 2:1 looked like in the testing wells in concentrations 50 mg/L and 100 mg/L at the beginning of the test. Situation after 48 hours, by the end of the test, is depicted on the right-side picture, where it is clearly visible how the particles have settled to the bottom. The metal concentrations measured in the water column by the end of test allow to evaluate most bioavailable fractions of the tested substances (ionic form and remained suspended unsettled nanoparticles) in the exposure solution. We assume that Zn ions are accounted for the largest share of the Zn concentration measured in the water column and other Zn compounds (e.g., suspended particles of nanocomposite) constitute a smaller part.

Figure 3.1 Nanocomposite 06 CaFe 2:1 during the test (concentrations 100 mg/L).

Chemical analysis of test solutions showed different solubility of tested nanocomposites. For example, at the beginning, total amount of Zn in the 09 at concentration 3.125 mg/L was 1.03 mg/L, which dissolved completely in the solution after 48 hours. However, in all the other nanocomposite materials tested at 3.125 mg/L Zn concentrations measured after 48 h were lower than initial ones (Table 3.1).

In Table 3.1 displayed only Zn and Fe concentrations are displayed because all the Ca dissolved and Zr and Mg were undetectable at nominal composite concentration 3.125 mg/L.

Table 3.1 Measured metal concentrations (mg Me/L) in the exposure solutions of Zn containing composites at the beginning (0 min) after shaking and at the end (48 h) of the tests.

Composites		**Zn (mg/L)**	**Fe (mg/L)**	**Number of measurements**
01 ZnFeZr 6:1:1	in the beginning	1.3 ± 0.03	0.36 ± 0.21	2
(3.125 mg/L)	at the end	1.1 ± 0.02	0.14 ± 0.08	3
03 CaZnFeZr :3:1:1	in the beginning	0.86 ± 0.1	2.24 ± 2.7	2
(3.125 mg/L)	at the end	0.5 ± 0.04	0.10 ± 0.09	3
05 MgZnFe 1:1:1	in the beginning	0.74 ± 0.15	0.66 ± 0.1	2
(3.125 mg/L)	at the end	0.53 ± 0.18	0.24 ± 0.05	3
07 ZnFeZr 18:5:1	in the beginning	1.48 ± 0.12	0.5 ± 0.01	2
(3.125 mg/L)	at the end	1.0 ± 0.12	0.14 ± 0.17	3
08 ZnFeZr 4:1:1	in the beginning	1.38 ± 0.3	0.7 ± 0.45	2
(3.125 mg/L)	at the end	0.7 ± 0.09	0.09 ± 0.10	3
09 ZnFeZr 3.6:0.2:1	in the beginning	1.03 ± 0.06	0.17 ± 0.05	2
(3.125 mg/L)	at the end	1.0 ± 0.01	0.11 ± 0.09	3
10 ZnFeZr 10:1:1	in the beginning	1.73 ± 0.34	0.27 ± 0.1	2
(3.125 mg/L)	at the end	1.4 ± 0.03	0.15 ± 0.08	3

We have not found a strong link between Zn concentrations in the water column and the nominal concentrations in the test solutions (Figures 3.2, 3.3; Table 3.1, 3.2). Results revealed that higher nominal concentrations do not necessarily result in greater Zn concentration in the water column, with the exception of 07 where solubility appears to increase with concentration. Nanocomposite materials 01, 03, 08, 09, and 10 demonstrated that Zn solubility is higher at intermediate concentrations. In the case of 01 and 08, Zn concentration was higher at a concentration of 25 mg/L. For 03, Zn dissolved more at a concentration of 50 mg/L, while for chemical 09, Zn dissolved more at a concentration of 12.5 mg/L (twice as much as in a concentration of 100 mg/L).

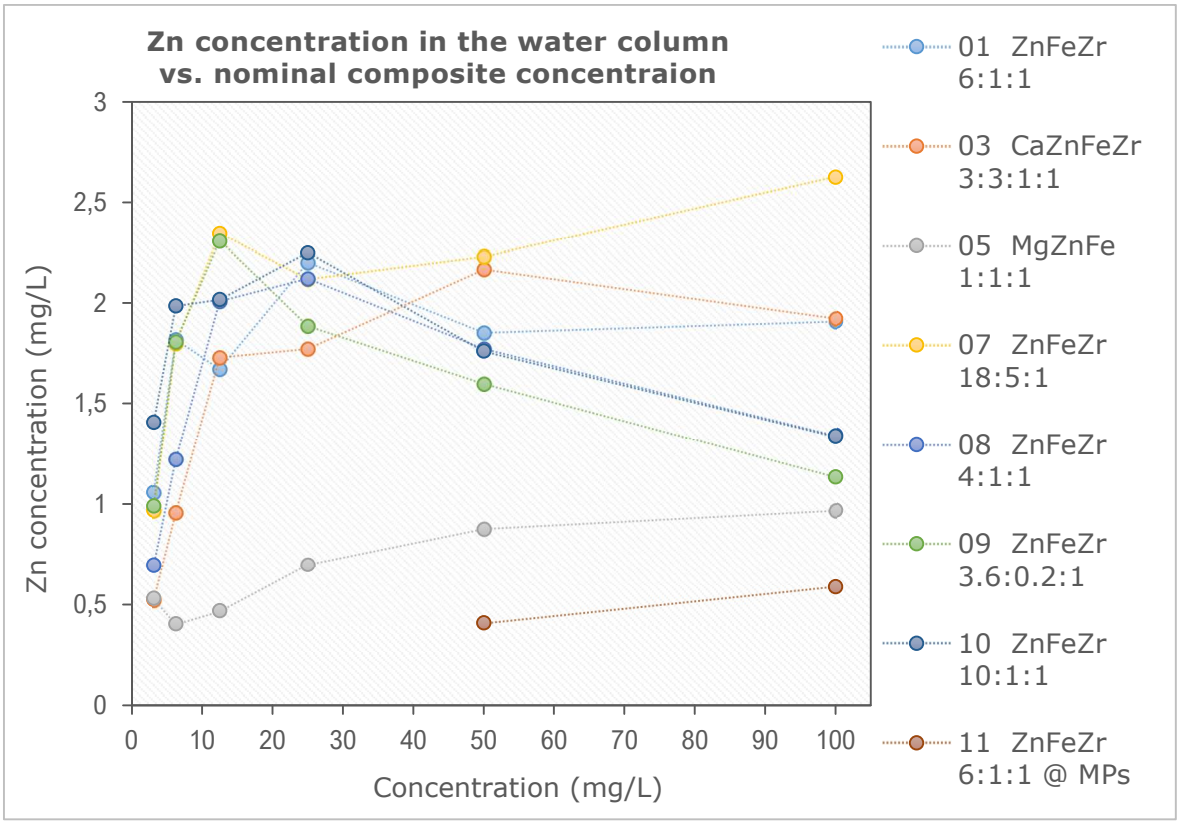

Figure 3.2 Zn concentration in the water column vs. nominal nanocomposite concentration.

After the 48-hour test, samples were collected from the upper layer of the test media to prevent re-suspension to determine metals in the test solutions. Table 3.2 provides information on the amount of metal ions and nanoparticles have leached into the solutions. Chemical analysis revealed the presence of metals in the water column, with Zn being most prevalent in material 07, despite 10 having the highest initial concentration of Zn. In sample 10, Zn did not dissolve to the same extent as in 07 (Table 3.2). Material 10 exhibited the highest Zn solubility at concentrations of 6.25 and 12.5 mg/L (again, twice as much as in a concentration of 100 mg/L).

Magnesium (Mg) was below the detection limit (< LOD). Also, Zr was hard to detect. Zr could be detected only in original 100 mg/L concentration in the beginning of the test and after shaking (Table 3.2). At the end of the test Zr could not be detected at all (< LOD for all samples). This shows that Zr particles are too heavy or Zr does not dissolve in the solutions, or that the reliability of the Zr-results measured with Picofox is questionable and must be verified with another method.

This could also mean that Mg and Zr are properly immobilized within the structure of the nanocomposite material containing these metals. The same was concluded by Kevin Uke in his MSc thesis work (2022) [38].

The lower than expected values of Ca concentrations indicate that Ca may have originated solely from the used in the experiment artificial freshwater made from four salts ($CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, $NaHCO_3$, and KCl). The artificial freshwater had Ca concentration of 80 mg/L, but Picofox measurements showed lower concentrations, possibly due to (i) the absorbent nature of the composites or (ii) underestimation of elements such as Zr, Ca, and Mg by used analytical method (Picofox). Similar observations were made by Towett et al. (2013) [64], who found that TXRF consistently underestimated element concentrations compared to ICP-MS (inductively coupled plasma mass spectrometry), requiring spectrometer recalibration. He showed that after single-element recalibration, accurate determination of total element concentrations for Al, K, Ti, V, Cr, Mn, Fe, Ni, Cu, Zn, and Ga was achieved. However, other elements such as P, Ca, As, Rb, Sr, Y, Pr, Ta, and Pb were generally somewhat under- or overestimated ($R2 > 0.60$). Even after recalibration, underestimations for Na, Mg, Ba, Ce, Hf, La, Nd, W, and Sm and overestimations for Bi, Tl, and Zr still occurred compared to ICP-MS. Thus, it could be concluded that analytical method applied in the current study (X-ray fluorescence spectroscopy using Picofox S2 device) has certain limitations, especially regarding the measuring of Zr. However, this does not concern Zn, which is the element of main focus and highest interest for the current work, and all Picofox data on Zn measurements can be considered reliable and accurate.

Table 3.2 Concentrations of the metals in the water column after 48 h exposure.

Composite	Nominal exposure concentration (mg/L)	Zn (mg/L)	Fe (mg/L)	Zr (mg/L)	Ca (mg/L)	Mg (mg/L)	Number of measurements (n)
01 ZnFeZr 6:1:1	3.125	1.06 ± 0.03	0.14 ± 0.08	<LOD	57.31 ± 5.49	<LOD	3
	6.25	1.82 ± 0.08	0.12 ± 0.12	<LOD	52.90 ± 9.92	<LOD	3
	12.5	1.67± 0.56	0.14 ± 0.10	<LOD	55.18 ± 8.46	<LOD	3
	25	2.21 ± 0.54	0.14 ± 0.06	<LOD	52.04 ± 6.75	<LOD	3
	50	1.85 ± 0.70	0.15 ± 0.11	<LOD	51.53 ± 2.82	<LOD	3
	100	1.91 ± 0.38	0.13 ± 0.08	<LOD	51.22 ± 10.74	<LOD	3
02 CaFeZr 6:1:1	50	0.10± 0.11	0.07 ± 0.0	<LOD	43.26 ± 15.43	<LOD	2
	100	0.03 ± 0.01	0.25 ± 0.41	<LOD	42.43 ± 23.59	<LOD	2
03 CaZnFeZr 3:3:1:1	3.125	0.53 ± 0.04	0.10 ± 0.09	<LOD	55.56 ± 4.50	<LOD	3
	6.25	0.96 ± 0.11	0.11 ± 0.09	<LOD	56.15 ± 7.55	<LOD	3
	12.5	1.73 ± 0.19	0.13 ± 0.08	<LOD	54.91 v 13.64	<LOD	3
	25	1.77 ± 0.85	0.11 ± 0.07	<LOD	54.48 ± 3.44	<LOD	3
	50	2.17 ± 0.28	0.09 ± 0.09	<LOD	52.23 ± 10.03	<LOD	3
	100	1.92 ± 0.82	0.10 ± 0.10	<LOD	47.27 ± 9.97	<LOD	3
04 MgFeZr 6:1:1	50	0.07 ± 0.02	0.09 ± 0.03	<LOD	41.48 ± 15.48	<LOD	2
	100	0.07 ± 0.03	0.06 ± 0.0	<LOD	40.16 ± 17.20	<LOD	2
05 MgZnFe 1:1:1	3.125	0.53 ± 0.22	0.24 ± 0.05	<LOD	56.28 ± 4.66	<LOD	3
	6.25	0.41 ± 0.12	0.11 ± 0.07	<LOD	52.69 ± 6.74	<LOD	3
	12.5	0.47 ± 0.14	0.17 ± 0.10	<LOD	52.89 ± 5.33	<LOD	3
	25	0.70 ± 0.02	0.20 ± 0.12	<LOD	56.80 ± 3.72	<LOD	3
	50	0.88 ± 0.05	0.18 ± 0.10	<LOD	51.90 ± 15.06	<LOD	3
	100	0.97 ± 0.15	0.16 ± 0.10	<LOD	55.40 ± 11.45	<LOD	3
06 CaFe 2:1	50	0.05 ± 0.02	0.08 ± 0.02	<LOD	39.59 ± 6.37	<LOD	2
	100	0.04 ± 0.01	0.08 ± 0.04	<LOD	37.51 ± 6.14	<LOD	2

07 ZnFeZr 18:5:1	3.125	0.97 ± 0.15	0.14 ± 0.17	<LOD	56.32 ± 11.00	<LOD	3
	6.25	1.80 ± 0.23	0.11 ± 0.12	<LOD	49.53 ± 10.98	<LOD	3
	12.5	2.35 ± 0.14	0.14 ± 0.05	<LOD	53.55 ± 19.37	<LOD	3
	25	2.12 ± 0.64	0.19 ± 0.10	<LOD	51.13 ± 14.61	<LOD	3
	50	2.23 ± 0.67	0.17 ± 0.13	<LOD	50.04 ± 8.59	<LOD	3
	100	2.63 ± 1.18	0.19 ± 0.12	<LOD	56.77 ± 5.25	<LOD	3
08 ZnFeZr 4:1:1	3.125	0.70 ± 0.11	0.09 ± 0.10	<LOD	47.66 ± 4.64	<LOD	3
	6.25	1.22 ± 0.11	0.12 ± 0.09	<LOD	49.03 ± 8.23	<LOD	3
	12.5	2.01 ± 0.10	0.28 ± 0.23	<LOD	54.90 ± 6.60	<LOD	3
	25	2.12 ± 0.26	0.11 ± 0.09	<LOD	48.24 ± 6.25	<LOD	3
	50	1.77 ± 0.30	0.12 ± 0.10	<LOD	50.21 ± 6.29	<LOD	3
	100	1.34 ± 0.24	0.14 ± 0.07	<LOD	47.86 ± 9.54	<LOD	3
09 ZnFeZr 3.6:0.2:1	3.125	1.0 ± 0.01	0.11 ± 0.09	<LOD	65.50 ± 5.44	<LOD	3
	6.25	1.81 ± 0.01	0.10 ± 0.07	<LOD	60.73 ± 6.10	<LOD	3
	12.5	2.31 ± 0.13	0.11 ± 0.10	<LOD	55.99 ± 11.91	<LOD	3
	25	1.89 ± 0.17	0.11 ± 0.11	<LOD	54.76 ± 12.52	<LOD	3
	50	1.60 ± 0.43	0.13 ± 0.08	<LOD	53.0± 13.13	<LOD	3
	100	1.14 ± 0.19	0.13 ± 0.08	<LOD	59.17 ± 10.59	<LOD	3
10 ZnFeZr 10:1:1	3.125	1.41 ± 0.04	0.15 ± 0.08	<LOD	58.26 ± 17.60	<LOD	3
	6.25	1.99 ± 0.44	0.11 ± 0.07	<LOD	53.96 ± 1.21	<LOD	3
	12.5	2.02 ± 0.56	0.13 ± 0.07	<LOD	63.79 ± 4.62	<LOD	3
	25	2.25 ± 0.39	0.15 ± 0.09	<LOD	69.47 ± 8.56	<LOD	4
	50	1.76 ± 0.07	0.15 ± 0.09	<LOD	74.97 ± 5.11	<LOD	4
	100	1.34 ± 0.11	0.18 ± 0.07	<LOD	72.46 ± 11.74	<LOD	4
11 ZnFeZr 6:1:1 @ MP	50	0.41 ± 0.09	0.08 ± 0.01	<LOD	62.78 ± 0.46	<LOD	2
	100	0.59 ± 0.20	0.23 ± 0.21	<LOD	63.01 ± 9.20	<LOD	2

Displayed in Table 3.3, are the total metal concentrations measured with Picofox before the test, along with nominal exposure metal concentrations based on inductively coupled plasma optical emission spectroscopy (ICP-OES) measurements conducted separately and independently within the "NanoPhosTox" project [1]. Within ICP-OES measurements, the nominal concentrations of Ca and Mg do not include the additional Ca and Mg in the system coming from the artificial freshwater, as it is in the case of the Picofox measurements. The results indicated that Picofox measurements for Zr are unreliable, as Zr was either not detected at all (Zr < LOD) or its concentration was significantly overestimated compared to the ICP-OES measurements. The average limit of detection for Mg was high (LOD=10-20 mg/L) and Mg was not detected with Picofox neither in the beginning, nor at the end of the test (Mg < LOD), despite the nominal concentration of Mg in the artificial freshwater being 12 mg/L.

The nominal metal concentrations measured with ICP-OES indicate that Zn and Fe measurements with Picofox are reliable due to good compliance between the Picofox with the ICP-OES values. However, collecting the samples was challenging due to the rapid settling of particles, which was visible to the naked eye during the test. The particles in the solution agglomerated and settled immediately when shaking stopped, a phenomenon also confirmed by Kevin Uke in his MSc thesis work (2022) [38]. Agglomeration was faster in 2% NaCl solution (within 30 minutes) than in deionized water, possibly due to the increased ionic strength in the salt media and a shift in the surface charge of the nanocomposites. Uke observed that all the samples, except for ZnFeZr composites and CaZnFeZr, were out of the nano-range, confirmed by the fast sedimentation already within 30 minutes in 2% NaCl solution and equally fast sedimentation for half of the samples also in deionized water. The exceptions were all five ZnFeZr-based composites and CaZnFeZr which indicates that these samples remained in the nano-range. [38]

According to the European Commission's definition, a nanomaterial is a natural, incidental, or manufactured material containing particles, in an unbound state or as an aggregate or agglomerate, and where, for 50% or more of the particles in the number size distribution, one or more external dimensions are in the size range 1 nm - 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%. [65]

Before analysis, test solutions were shaken (vortexed) and concentrated HNO_3 was added, which included 1 ppm Ga as an internal standard. The samples (test solution together with acid and internal standard) were shaken (vortexed) again before analysis.

Table 3.3 Measured metal concentrations (mg Me/L) in the homogenized (shaken) water samples collected before the tests.

Composite name	Nominal concentration	Zn (mg/L)	Fe (mg/L)	Zr (mg/L)	Ca (mg/L)	Mg (mg/L)
07 ZnFeZr 18:5:1 Nominal concentration measured with ICP-OES Concentration measured with Picofox n=3	(100 mg/L)	52.7 45.4 ± 7.9	13.0 10.2 ± 1.5	3.7 31.4	-	-
10 ZnFeZr 10:1:1) Nominal concentration measured with ICP-OES Concentration measured with Picofox n=4	(100 mg/L)	55.0 46.6 ± 8.9	5.1 3.2 ± 1.5	7.2 < LOD	-	-
01 ZnFeZr 6:1:1 Nominal concentration measured with ICP-OES Concentration measured with Picofox n=3	(100 mg/L)	43.7 37.1 ± 4.5	6.8 5.5 ± 0.6	9.5 26.8	-	-
08 ZnFeZr 4:1:1 Nominal concentration measured with ICP-OES Concentration measured with Picofox n=3	(100 mg/L)	38.9 32.3 ± 6.8	9.5 7.5 ± 1.8	13.6 25.5	-	-
09 ZnFeZr 3.6:0.2:1 Nominal concentration measured with ICP-OES Concentration measured with Picofox n=3	(100 mg/L)	41.6 36.4 ± 21.1	2.4 1.6 ± 1.2	16.9 51.8	-	-
06 CaFe 2:1 Nominal concentration measured with ICP-OES Concentration measured with Picofox n=2	(100 mg/L)	-	56.2 49.7 ± 2.3	-	1.9 60.9 ± 2.0	-
02 CaFeZr 6:1:1 Nominal concentration measured with ICP-OES Concentration measured with Picofox n=2	(100 mg/L)	-	21.7 14.7 ± 0.04	31.2 113.3 ± 30.8	1.7 40.6 ± 12.8	-
03 CaZnFeZr 3:3:1:1 Nominal concentration measured with ICP-OES Concentration measured with Picofox n=3	(100 mg/L)	31.3 27.8 ± 3.5	11.2 9.1 ± 0.8	16.0 90.1 ± 45.9	0.0 50.9 ± 2.6	-

04 MgFeZr 6:1:1 Nominal concentration measured with ICP-OES Concentration measured with Picofox n=2	(100 mg/L)	-	20.6 14.7 ± 0.08	29.3 117.0 ± 3.0	-	1.8 < LOD
05 MgZnFe 1:1:1 Nominal concentration measured with ICP-OES Concentration measured with Picofox n=3	(100 mg/L)	28.8 26.2 ± 0.04	25.9 21.5 ± 1.2	-	-	3.5 < LOD
11 ZnFeZr 6:1:1 @ MPs Nominal concentration measured with ICP-OES Concentration measured with Picofox n=2	(100 mg/L)	8.7 12.6± 0.1	>50 due to Fe_3O_4 MPs 22 ± 0.7	1.9 17.4 ± 2.4	-	-

Remark: The sum of the individual metals within one compound is < 100 mg/L due to the presence of other ions in the composition of the tested nanocomposite materials.

3.2 Toxicity evaluation of nanocomposite materials

3.2.1 Range finding test

Toxicity assessment began with a range-finding test to determine range of toxic concentration of tested compounds to select exposure concentration in the definitive test (Appendix 1). Results of range finding test identified the most likely toxic nanocomposite materials (starting from the most toxic chemical) in the following order: 01 (ZnFeZr 6:1:1) > 03 (CaZnFeZr 3:3:1:1) > 07 (ZnFeZr 18:5:1) > 09 (ZnFeZr 3.6:0.2:1) > 08 (ZnFeZr 4:1:1). However, the results of the tests for materials 05 (MgZnFe 1:1:1) and 10 (ZnFeZr 10:1:1) were rather unfeasible, as both contain Zn and were therefore anticipated to be toxic. Further testing was conducted with six concentrations for 05 and three concentrations for 10 to confirm these results.

Nanocomposite materials 04 (MgFeZr 6:1:1) > 06 (CaFe 2:1) > 11 (ZnFeZr 6:1:1 @ MPs) > 02 (CaFeZr 6:1:1) were found to be non-toxic at maximal concentration (100 mg/L).

3.2.2 Definitive test

The test was conducted three times with each nanocomposite material exhibited toxicity at concentrations less than 100 mg/L in the range finding test, except for composite 10 (ZnFeZr 10:1:1), whose toxicity was initially unclear and later tested once with concentrations 25, 50, and 100 mg/L, followed by three tests with six concentrations. The definitive test revealed toxicity in six materials, namely 07 (ZnFeZr 18:5:1) > 08 (ZnFeZr 4:1:1) > 09 (ZnFeZr 3.6:0.2:1) > 10 (ZnFeZr 10:1:1) > 03 (CaZnFeZr 3:3:1:1) > 01 (ZnFeZr 6:1:1).

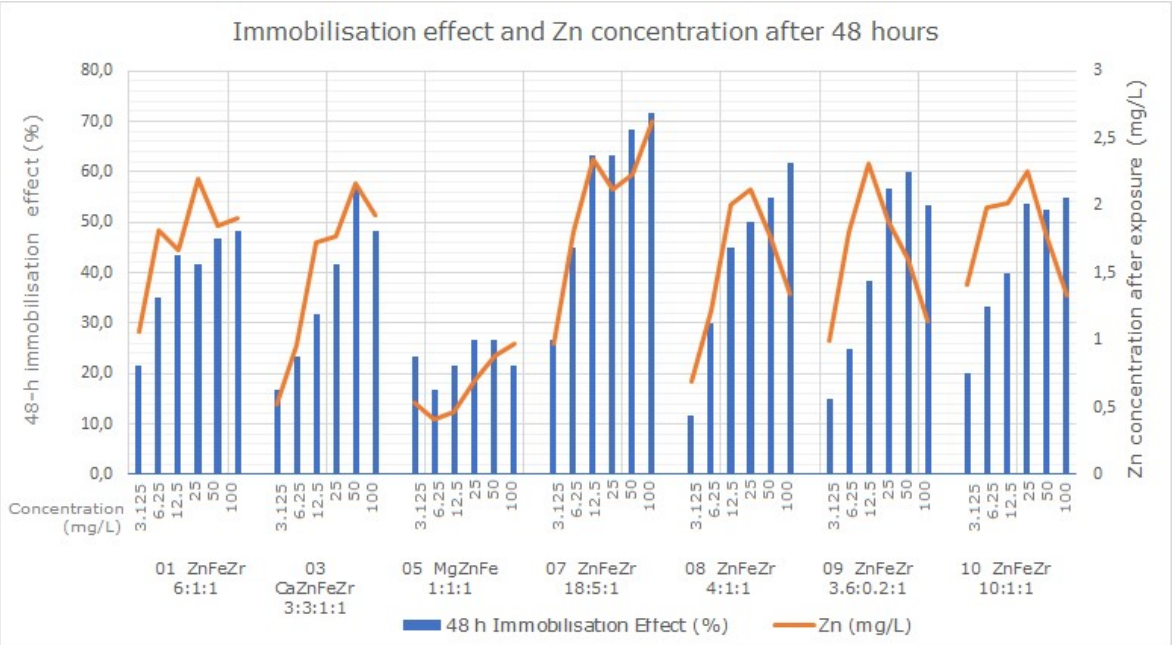

Figure 3.3 Immobilization effect on *D. magna* and associated Zn concentrations in the water column after 48 hours.

In Figure 3.3 toxicity of materials 01, 03, 05, 07, 08, 09 and 10 to *D. magna* at six concentrations 3.125, 6.25, 12.5, 25, 50, 100 mg/L is presented. The test results show that toxicity of composites in general depend on Zn concentrations in the water column. The results also can be attributed to the fact that ZnFeZr sorbents are composed of nanoparticles made of crystalline zinc oxide (ZnO NPs) surrounded by amorphous zinc, iron, and zirconia (oxy)hydroxides, as stated in Drenkova-Tuhtan's (2017) study [7]. The concentration of zinc present in nanoZnO may have an impact on *Daphnia magna*. Several studies have shown that ZnO toxicity is associated with the dissolution of Zn^{2+} [11], [12], [66]. However, at the largest exposure concentrations in same cases this pattern does not work. For example, the Zn concentration is not at its highest peak at maximum 100 mg/L concentrations, in composites 01, 08 and 10 the effect is the highest. This could be explained by fact what at the high nominal concentrations adsorption of the composite particles on the exoskeleton may also adversely affect survival of the daphnides (Figure 3.4).

Zn is a crucial essential trace element for the growth of organisms and serves as a critical metal co-factor for various enzymes involved in the metabolism of proteins, nucleic acids, carbohydrates, and lipids. Although zinc in certain concentrations promotes fish growth, excessive accumulation can be detrimental to exposed fish. Zn is a prevalent contaminant in aquatic systems and can originate from urban runoff, soil erosion, industrial discharges, pharmaceuticals, pesticides, and various other sources. The persistence of Zn in the environment is concerning because it cannot be biologically

decomposed and can only transform from one oxidation state or organic complex to another. [67], [68]

As dissolution of nano ZnO depends on the particle size, the toxicity of ZnO nanoparticles with different size differ. In terms of the acute toxicity of ZnO NPs towards the freshwater microcrustacean *Daphnia magna*, the particle size affects the EC_{50}. Blinova et al. (2010) [66] reported a value of EC_{50} (48h) at 2.6 mg/L when testing NPs that were 70 nm in size. Santo et al. (2014) found the EC_{50} (48h) value to be 3.1 mg/L for NPs > 100 nm and 1.9 mg/L for NPs < 50 nm [69].

Zn toxicity is a complex issue, where several factors have a significant role to play. These factors include the particle size, pH levels and water hardness. Although the pH level and Ca concentration were constant in the tests and measurements carried out in this work, these are still important factors that must not be forgotten and could potentially significantly affect the outcome [58]. For instance, Berglind and Dave (1984) determined that the 24-hour EC_{50} value of zinc was 3.0 mg/L in hard water (300 mg $CaCO_3$/L) and 5.3 mg/L in soft water (50 mg $CaCO_3$/L). The study was carried out with chemical $ZnCl_2$ [70].

Nanocomposite materials 05 (MgZnFe 1:1:1) > 11 (ZnFeZr 6:1:1 @ MPs) > 06 (CaFe 2:1) > 04 (MgFeZr 6:1:1) > 02 (CaFeZr 6:1:1) were found to be non-toxic with EC_{50} values >100 mg/L. Notably, composite 11 (ZnFeZr 6:1:1 @ MPs) had a significantly lower zinc content compared to the other composites (approximately 6 wt%). This is because the ZnFeZr fraction only constituted 20 wt% of the composite mass, with the remaining 80 wt% being made up of the carrier magnetite-silica matrix.

05 (MgZnFe 1:1:1) did not exhibit toxicity, despite having a similar Zn total concentration at the beginning of test as 03, which was toxic, but its soluble Zn concentration in eluates was lower. Therefore, it can be concluded that Zn in 03 is more prone to dissolution than in 05, leading to lower toxicity in the latter. This proves the fact that Zn^{2+} plays a critical role in composite toxicity. Materials 05 and 10 have the same amount of dissolved Zn at 100 mg/L solutions but the effect is the opposite. Material 05 is non-toxic but 10 is toxic. The explanation for this could be related to particle size or how the particles interacted with *Daphnia magna*.

07 was found to be the most toxic due to the presence of soluble Zn, with a soluble Zn concentration of 1.0 mg/L even at the lowest concentration of 3.125 mg/L. The results show that the effect of chemical 07 increases with increasing Zn concentration. As the concentration of the solution increases, the solubility of Zn also increases. The solubility

of Zn and its effect have almost a linear relationship. For other nanocomposite materials, the solubility of Zn is not the highest at high solution concentrations.

Despite having the second highest initial Zn concentration before the test, 10 had a lower soluble Zn concentration than 07 after the test, which explains why the effect in 10 was also lower than in 07. Materials 01, 07, and 08 showed the highest effect at a concentration of 100 mg/L. The highest effect at 50 mg/L occurred in 03, while the highest effect in 09 occurred at 25 mg/L and 50 mg/L. In 05, the dissolved Zn was higher at 100 mg/L, but the highest effect was observed at 25 and 50 mg/L. This may be due to the interaction of particles with *Daphnia magna*.

Only composites containing Zn are listed in Table 3.3 since Zn^{2+} plays a critical role in composite toxicity. The percentage of Zn in the composite varies from 6% to 33%. Composites with less than 12% Zn are non-toxic, confirming that Zn is the primary cause of toxicity.

According to the literature, the Zn-based EC_{50} values for *Daphnia magna* vary in the range 1.4-2.6 mg/L, which is in compliance with the results for the nanocomposites in this study with nominal EC_{50} values of 1.3-2.2 mg/L (Table 3.4, Table 3.5).

Table 3.4 compares literature EC_{50} values for the five metals relevant for this study regarding their toxicity to two common test organisms for aquatic toxicity: crustacean *Daphnia magna* and bacteria *Vibrio fischeri.* According to the data given in the table, *Daphnia magna* is more sensitive than *Vibrio fischeri.* The acute toxicity data for *Vibrio fischeri* was available only for Zn.

Table 3.4 Acute toxicity of selected metals to *Daphnia magna* and *Vibrio fischeri* with EC_{50} values (mg/L) collected from literature.

Test organism/metal	Zn	Fe	Ca	Mg	Zr
Daphnia magna	1.4-2.6[a]-3.1[b]	12.9–17.3[c]	870[d]-2400[e]	290[d]	>400[d,f]
Vibrio fischeri	3.8-4.8[g]	-	-	-	-

[a] Blinova et al., 2010 (bulk ZnO or nano ZnO) [66]
[b] Gonçalves et al., 2018 (nano ZnO) [69]
[c] Blinova et al., 2018 [71]
[d] Okamoto et al., 2014 ($CaCl_2{\cdot}2H_2O$, $MgSO_4{\cdot}5H_2O$, $ZrCl_4$) [72]
[e] ECHA database $CaCl_2{\cdot}2H_2O$ [73]
[f] Załęska-Radziwiłł et al., 2016 (ZrO_2 EC_{50} > 400 mg/L) [74]
[g] Mortimer et al., 2008 (bulk ZnO or nano ZnO) [75]
*$MgCl_2{\cdot}6H_2O$ no EC_{50} data available [76]
*$FeCl_3{\cdot}6H_2O$ non-toxic all acute LC_{50} and EC_{50} > 100 mg/L & chronic NOEC > 1 mg/L [77]
*$ZrOCl_2{\cdot}8H_2O$ no EC_{50} data available [78]

It is interesting that the most toxic composite 07 ZnFeZr 18:5:1 EC_{50} value, based only on Zn ions, is 1.3 mg/L. This is the lowest value compared to the other composites. The reason lies in Zn^{2+}, which makes the composite more toxic. Additionally, Table 3.2 suggests that from this material Zn is more prone to leach out. This may also indicate that the toxicity to *Daphnia magna* is more complex and could not solely depend on Zn concentration in water but also how the particles interact with the environment (agglomerate, ingested by *Daphnia magna*, etc.). Composite 07 ZnFeZr 18:5:1 particle size is 3.7 µm, which was not the smallest (05 has 3.2 µm) but under the microscope, it could not be detected that particles of composite 05 have been ingested by *Daphnia magna*.

Table 3.5 Mass fraction of Zn (wt%) in the nanocomposites, their respective EC_{50} and Zn-normalized EC_{50} values.

No	Composite name	Mass of Zn^{2+} (g)	Molar mass of the NCs (g)	Zn fraction in mass percentage	EC_{50} value of the whole composite (mg/L)	EC_{50} value based only on Zn^{2+} (mg/L)
07	ZnFeZr 18:5:1	1177	4127	29%	4.4	1.3
09	ZnFeZr 3.6:0.2:1	235	867	27%	5.7	1.5
10	ZnFeZr 10:1:1	654	1955	33%	6.5	2.1
01	ZnFeZr 6:1:1	392	1410	28%	7.7	2.2
08	ZnFeZr 4:1:1	262	1138	23%	9	2.1
03	CaZnFeZr 3:3:1:1	196	1442	14%	12.5	1.8
05	MgZnFe 1:1:1	65	610	11%	>100	
11	ZnFeZr 6:1:1 @ MP	392	1410	6%	>>100	

The absence of Zr in the eluates leads to the conclusion that Zr particles are either too heavy to be present or are present in such small quantities that they cannot be detected by the Picofox. Additionally, according to the research by Okamoto et al. (2014) [72] and Załęska-Radziwiłł et al. (2016) [74], zirconium was found non-toxic with EC_{50} > 400 mg/L [72], [74].

In the case of Fe, Ca and Mg, while they can be toxic at higher concentrations, their exposure concentration in the current study were much lower than toxic level for these elements [74].

The findings suggest that the distress experienced by *Daphnia magna* cannot be attributed solely to the presence of soluble Zn. Materials 01 and 03, which have higher levels of soluble Zn in 100 mg/L solutions concentration after 48 hours compared to 08, 09, or 10, exhibit a lower effect on *Daphnia magna*. This shows that Zn in the water column was presented as ion and other less bioavailable Zn compounds. These results suggest that the interaction between particles and *Daphnia magna*, as well as their consumption of the particles, may also play a significant role in determining the observed effects. Similar findings have been presented in Nabi et al. (2021) [79].

After the acute test, *Daphnia magna* was inspected under the microscope to investigate particles interaction with the water flea. The total metal concentrations found in the test medium indicate that immobilization cannot solely be attributed to dissolved Zn. Under the microscope, we can see.

1. Aggregation of nanocomposites was the major effect observed under the microscope and was visible even at low concentrations in all of the materials tested (Figure 3.4). The small particle size, large surface area, and strong surface tension of nanoparticles have been reported to cause their aggregation, which is an important factor to consider when studying their toxicity. The mechanical adhesion of NPs on the organism's surface / on the body surface of aquatic animals can lead to various toxic effects, such as molting disturbances and changes in gene expressions related to molting, energy metabolism, and genetic material. [80]

Figure 3.4 Adhesion of NPs on the organism's surface and accumulation in the gut of nanocomposite material 07 ZnFeZr 18:5:1 (concentration 12 mg/L) and nanocomposite material 06 CaFe 2:1 (concentration 100 mg/L). Scale 0.5 mm.

2. *Daphnia* typically feed on small suspended particles in the water, with a particle size ranging from approximately 1 µm to 50 µm [81]. All the tested nanocomposite materials particle sizes were from 3.2 µm (composite 05) up to 25 µm (composite 11). This suggests that *Daphnia magna* would be able to eat all the tested materials in the matter of size. Ingestion of nanocomposite materials by *Daphnia magna* was observed for all tested chemicals except for material 05, which may explain why it was not toxic despite having a similar total Zn concentration as 03, which was toxic (Figure 3.5). However, composite 05 had the smallest particle size of 3.2 µm. Thus, the ingestion of particles by *Daphnia magna* needs to be further investigated.

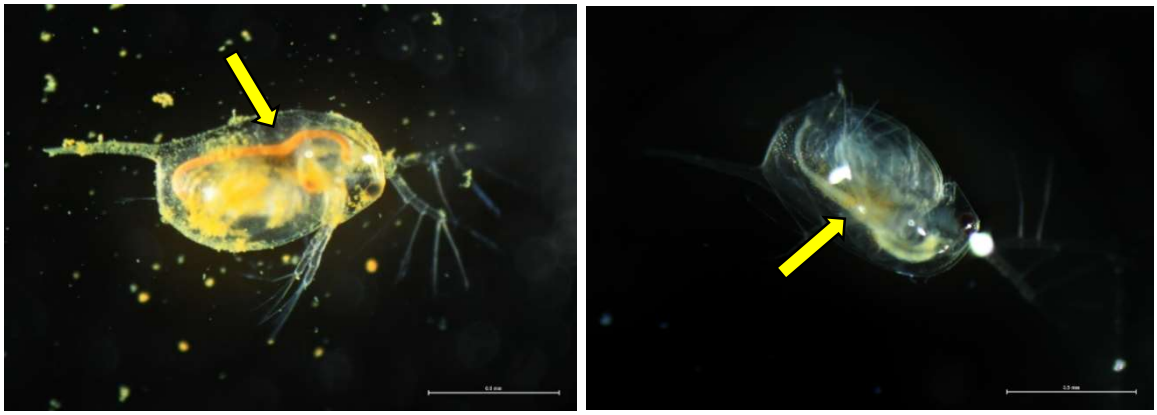

Figure 3.5 *Daphnia magna* has eaten the nanocomposite material 06 CaFe 2:1 (concentration 50 mg/L) and nanocomposite material 10 ZnFeZr 10:1:1, 50 mg/L). Scale 0.5 mm.

3. In some cases, chemicals appeared to prevent molting (Figure 3.6). This was observed in 08 ZnFeZr 4:1:1 at concentrations of 3.125 mg/L, 6.25 mg/L, and 12.5 mg/L, as well as in 05 at a concentration of 50 mg/L and in 01 at a concentration of 10 mg/L. This effect is difficult to confirm under the microscope, as the shell may remain in the well or move when lifting the *Daphnia magna* from the well.

   It was shown by Wang et al. (2021) [80] that Daphnia's molting was inhibited by dissociated ZnO NPs, which was caused by Zn^{2+} ion release. The gene expressions of eip, scot, and idh were also inhibited by dissociated ZnO NPs, showing a similar trend as bulk ZnO and $ZnSO_4 \cdot 7H_2O$ under low-dose exposure conditions. This indicates that the toxic effects of dissociated ZnO NPs were primarily caused by the release of Zn ions. The results provide direct evidence of the effect of nanoparticles on molting and reveal that the toxicity mechanisms of dissociated NPs differ from undissociated NPs. Some researchers suggest that molting is an important mechanism for eliminating nano-oxide particles for *Daphnia pulex* [80]. This could also be true for *Daphnia magna*, which is larger than *pulex*.

   Figure 3.6 shows a shell that has detached from the *Daphnia magna* in the following picture. The Daphnia in the image was immobilized in well but was able to move its antennae. After lifting it onto the microscope slide, the shell came off.

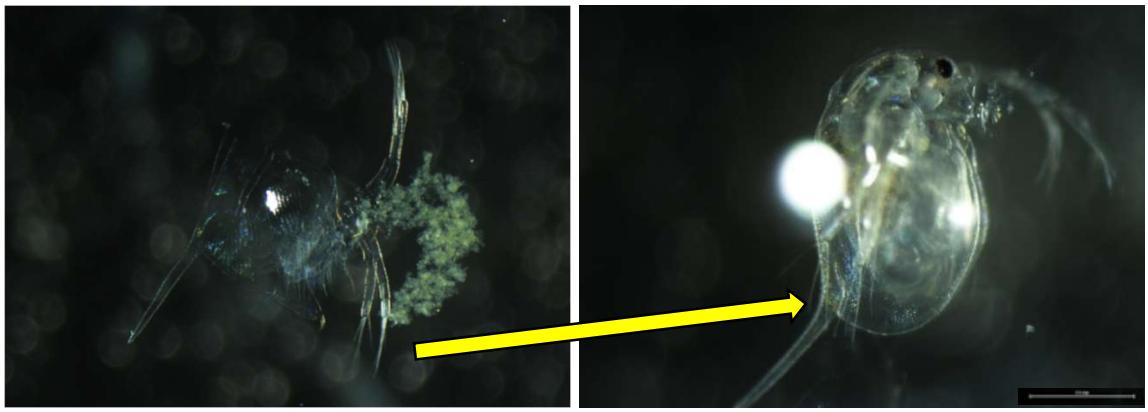

Figure 3.6 Light microscope image of *Dapnia magna* in nanocomposite material 08 ZnFeZr 4:1:1 (concentration 6.25 mg/L), showing that particles can prevent molting. Scale 0.5 mm.

3.3 Evaluation of the potential hazard of tested compounds

Aquatic toxicity is defined as the inherent property of a substance to cause harm to aquatic organisms upon exposure to the substance over short- or long-term periods. Although waterborne exposure to substances is the primary route of exposure, aquatic organisms may also be exposed through food, particularly to lipophilic substances. [82]

The hazardous of chemical compounds to the aquatic environment may be classified based on EC_{50} values. According to these criteria, a substance is considered extremely toxic if its EC_{50} value is ≤ 0.1 mg/L, very toxic if 0.1 < EC_{50} ≤ 1 mg/L, toxic if 1 < EC_{50} ≤ 10 mg/L, harmful if 10 < EC_{50} ≤ 100 mg/L, and not classified if EC_{50} > 100 mg/L (Table 3.6). The upper concentration limit of 100 mg/L was chosen based on the hazard ranking criteria for the aquatic environment, which specifies that substances with an L(E)C_{50} value greater than 100 mg/L are not considered harmful and are therefore classified as "not classified". [83]

Based on previous experiments with bacteria *Vibrio fischeri*, two nanocomposite materials were classified as harmful: 07 (ZnFeZr 18:5:1), 10 (ZnFeZr 10:1:1) (Uke 2022) [38]. The data from scientific literature (Table 3.4) indicates that bacteria *Vibrio fischeri* are less sensitive to metals test organism than crustacean *Daphnia magna.* Similar results were drawn in this study which are displayed in Table 3.6.

Based on the toxicity data collected in this study and from previous research by the supervisor Dr. Drenkova-Tuhtan and her former MSc student Kevin Uke [38], the chemicals were classified as shown in Table 3.6 The data for *Vibrio fischeri* was generated and provided by Dr. Drenkova-Tuhtan and included in Uke's master thesis 2022 [38], where 30-min EC_{50} values are statistically calculated based on three repetitive tests (n=3) with two parallels each. For *Daphnia magna*, the number of tests varied depending on the composite. Composites that were believed to be toxic (composites 07, 01, 08, 09, 03, 05) were tested three times, except for composite 10 which was tested four times. Non-toxic composites (06, 02, 04, and 11) were tested twice.

Table 3.6 Hazard classification of 11 composites based on the results of toxicity testing with *Daphnia magna* and *Vibrio fischeri*. The listed EC_{50} values are average with the respective 95% confidence intervals included in parenthesis. Representative for three repetitive tests (n=3) with two parallels each in *Vibrio fischeri* and four to two repetitive tests with four parallels each for *Dapnia magna*. All concentrations are nominal and refer to mg-compound/L.

		Daphnia magna	***Vibrio fischeri***
No	Composites	48-h EC_{50} (mg/L)	30-min EC_{50} (mg/L)
07	ZnFeZr 18:5:1	**4.38 (4.06-4.83)**	**36.86 (28.97-49.05)**
09	ZnFeZr 3.6:0.2:1	**5.74 (4.66-7.20)**	168.2 (141.0-177.0)
10	ZnFeZr 10:1:1	**6.48 (5.31-7.78)**	**53.91 (40.97-69.96)**
01	ZnFeZr 6:1:1	**7.69 (5.59-9.88)**	> 100
08	ZnFeZr 4:1:1	**9.01 (7.34-11.23)**	> 100
03	CaZnFeZr 3:3:1:1	**12.54 (9.94-14.98)**	> 100
05	MgZnFe 1:1:1	> 100	>> 100
11	ZnFeZr 6:1:1@MPs	>> 100	>> 100
06	CaFe 2:1	>> 100	>> 100
02	CaFeZr 6:1:1	>> 100	>> 100
04	MgZnFe 6:1:1	>> 100	>> 100

Not classified / not harmful	**Harmful**	**Toxic**	**Very toxic**	**Extremely toxic**
EC_{50} >100 mg/L	**10< EC_{50} ≤100 mg/L**	**1< EC_{50} ≤10 mg/L**	**0.1< EC_{50} ≤1 mg/L**	**EC_{50} ≤0.1 mg/L**

According to Table 3.6, one composite 03 CaZnFeZr 3:3:1:1 was classified as harmful and five composites 07 ZnFeZr 18:5:1, 10 ZnFeZr 10:1:1, 01 ZnFeZr 6:1:1, 08 ZnFeZr 4:1:1, 09 ZnFeZr 3.6:0.2:1 as toxic. This table shows clearly that *Daphnia magna* is more sensitive than *Vibrio fischeri*. Five materials out of eleven were classified as not harmful. These were 05 MgZnFe 1:1:1, 11 ZnFeZr 6:1:1@MPs, 06 CaFe 2:1, 02 CaFeZr 6:1:1, 04 MgZnFe 6:1:1. According to all the information gathered in this study the recommended nanocomposite engineered particles for application in phosphorus

recovery are those, which do not contain Zn 06 CaFe 2:1, 02 CaFeZr 6:1:1, 04 MgZnFe 6:1:1 (EC_{50} >>100 mg/L) and two which contain Zn 05 MgZnFe 1:1:1 (EC_{50} >100 mg/L), 11 ZnFeZr 6:1:1@MPs (EC_{50} >>100 mg/L). The best candidate is 11 ZnFeZr 6:1:1@MPs (EC_{50} >>100 mg/L) because it is already coated with magnetic particles.

While classification is determined based on available information, a comprehensive comparison with the criteria would necessitate data on the acute aquatic toxicity of the substance to fish, Daphnia, and algae [82]. Therefore additional research on the composites is necessary to further assess the risks.

SUMMARY

The objective of the current study was to assess potential hazard of eleven nanocomposite sorbent materials designed for phosphorus recovery from wastewater to aquatic ecosystems. The toxicity of nanocomposite materials' to crustacean *Daphnia magna* was evaluated in 48 h acute immobilization test (OECD202). The main hypothesis of the thesis was that due to the release of toxic Zn ions, Zn-containing nanocomposites are more toxic to aquatic organisms than the ones not containing Zn. This hypothesis was confirmed. Concentrations of the metals - main components of the tested composites - in the exposure media were measured using reflection X-ray fluorescence spectroscopy (Bruker Picofox).

The main outcomes of the study are:

1. Five nanocomposite materials out of eleven materials analysed were found to be toxic, with ZnFeZr 18:5:1 (EC_{50} 4.4 mg/L) being the most toxic, followed by ZnFeZr 10:1:1 (EC_{50} 6.5 mg/L), ZnFeZr 6:1:1 (EC_{50} 7.7 mg/L), ZnFeZr 4:1:1 (EC_{50} 9.0 mg/L) and ZnFeZr 3.6:0.2:1 (EC_{50} 5.7 mg/L). Composite CaZnFeZr 3:3:1:1 was harmful (EC_{50} 12,5 mg/L). The non-toxic nanocomposite materials included MgZnFe 1:1:1 (EC_{50} >100 mg/L), ZnFeZr 6:1:1 @ MPs (EC_{50} >>100 mg/L), CaFe 2:1 (EC_{50} >>100 mg/L), MgFeZr 6:1:1 (EC_{50} Z>100 mg/L), and CaFeZr 6:1:1 (EC_{50} >>100 mg/L).
2. The toxicity of nanocomposites depended on their chemical composition and especially on the release of toxic Zn into exposure media. All the nanocomposites which did not include Zn were not toxic to *D. magna*.
3. Zn concentrations in the water column did not depend on the nominal concentrations in the test solutions.
4. Investigation of test organisms under the microscope revealed that at the high nominal concentrations adsorption of the composite particles on the Daphnia's exoskeleton and ingestion of the nanocomposites by daphnids may also adversely effect daphnids.
5. The elemental analysis using reflection X-ray fluorescence spectroscopy (Bruker Picofox) revealed that it is not the most suitable method to measure concentrations of Zr, Mg and Ca and that other analytical methods could be applied to quantify these metals in the aqueous solutions.
6. It was confirmed that the crustacean *Daphnia magna* is more sensitive to the tested nanocomposites compared to bacteria *Vibrio fischeri*.

KOKKUVÕTE

Käesoleva lõputöö eesmärgiks oli hinnata 11 nanokomposiitmaterjali võimalikku kahjulikku mõju veeökosüsteemidele, valides testorganismiks vesikirbu Daphnia magna ja kasutades standardset OECD202 testi. Püstitati hüpotees, et tingituna mürgiste tsingiioonide vabanemisest on tsinki (Zn) sisaldavad nanokomposiidid veeorganismidele mürgisemad kui need komposiidid, mis Zn ei sisalda. Hüpotees leidis katsetes kinnitust. Lisaks määrati materjalide põhikomponentide kontsentratsioone, kasutades peegeldus-röntgen-kiirguse fluorestsents-spektroskoopia meetodit ja aparaati Picofox (Bruker).

Uuringu peamised tulemused on järgmised:

1. Leiti, et viis nanokomposiitmaterjali üheteistkümnest analüüsitust klassifitseerisid kui *'mürgised veeorganismidele'*, kusjuures kõige mürgisem oli ZnFeZr 18:5:1 (EC50 4,4 mg/L), millele järgnes ZnFeZr 10:1:1 (EC50 6,5 mg/L), ZnFeZr. 6:1:1 (EC50 7,7 mg/L), ZnFeZr 4:1:1 (EC50 9,0 mg/L) ja ZnFeZr 3,6:0,2:1 (EC50 5,7 mg/L). Komposiit CaZnFeZr 3:3:1:1 klassifitseeriti kui *'kahjulik veeorganismidele'* (EC50 12,5 mg/L). *'Mittetoksiliseks veeorganismidele'* klassifitseerisid järgmised kuus nanokomposiitmaterjali: MgZnFe 1:1:1 (EC50 >100 mg/L), ZnFeZr 6:1: 1 @ MPs (EC50 >>100 mg/L), CaFe 2:1 (EC50 >>100 mg/L), MgFeZr 6:1:1 (EC50 Z>100 mg/L) ja CaFeZr 6: 1:1 (EC50 >>100 mg/L).
2. Nanokomposiitide toksilisus sõltus nende keemilisest koostisest ja eelkõige Zn ioonide eraldumisest materjalist testkeskkonda. Lahustunud Zn kontsentratsioonid lahuses samas ei sõltunud materjalide nominaalkontsentratsioonidest testkeskkonnas. Kõik nanokomposiitmaterjalid, mis ei sisaldanud Zn, klassifitseerisid *Daphnia magna* testi alusel '*mittetoksilisteks*'.
3. Uurides katseorganisme mikroskoobi all, tuvastati komposiitosakeste kuhjumist vesikirbu seedeekstraktis – ka see võib organismidele kahjulikult mõjuda.
4. Nanokomposiitmaterjalide koostise analüüs näitas, et kasutatud meetod ja aparatuur (Bruker Picofox) ei osutunud kõige sobivamaks Zr, Mg ja Ca kontsentratsioonide määramiseks vees ja nende metallide mõõtmiseks tuleks kasutada muid analüütilisi meetodeid.
5. Kinnitust leidis, et vesikirpudel (*Daphnia magna*) on võrreldes bakteritega (*Vibrio fischeri*) suurem tundlikkus testitud nanokomposiitmaterjalide suhtes.

LIST OF REFERENCES

[1] A. Drenkova-Tuhtan, "Nanocomposite Engineered Particles for Phosphorus Recovery and Toxicological Risk Assessment for the Aquatic Environment," 2020. [Online]. Available: https://cordis.europa.eu/project/id/867457.

[2] D. Cordell and S. White, "Life's Bottleneck: Sustaining the World's Phosphorus for a Food Secure Future," *Annual Review of Environment and Resources,* vol. 39, pp. 161-188, 2014.

[3] W. J. Brownlie, M. A. Sutton, D. Cordell, D. S. Reay, K. V. Heal, P. J. A. Withers, I. Vanderbeck and B. M. Spears, "Phosphorus price spikes: A wake-up call for phosphorus resilience," *Frontiers in Sustainable Food Systems,* vol. 7, 2023.

[4] P. J. A. Withers, K. G. Forber, C. Lyon, S. Rothwell, D. G. Doody, H. P. Jarvie, J. Martin-Ortega, B. Jacobs, D. Cordell, M. Patton, M. A. Camargo-Valero and R. Cassidy, "Towards resolving the phosphorus chaos created by food systems," *Ambio,* vol. 49, pp. 1076-1089, 2020.

[5] "Urban Waste Water Treatment Directive (91/271/EEC)," European Commission, [Online]. Available: https://www.era-comm.eu/EU_water_law/stand_alone/part_4/index.html#:~:text=Directive%2091%2F271%2FEEC%20concerning,industrial%20sectors%20(Article%201)%20by. [Accessed 20 05 2023].

[6] M. Schneider, A. Drenkova-Tuhtan, W. Szczerba, C. Gellermann, C. Meyer, H. Steinmetz, K. Mandel and G. Sextl, "Nanostructured ZnFeZr oxyhydroxide precipitate as efficient phosphate adsorber in waste water: understanding the role of different material-building-blocks," *Environmental Science: Nano,* no. 1, 2017.

[7] A. Drenkova-Tuhtan, M. S. M. Franzreb, C. Meyer, C. Gellermann, G. Sextl, K. Mandel and H. Steinmetz, "Pilot-scale removal and recovery of dissolved phosphate from secondary wastewater effluents with reusable ZnFeZr adsorbent @ Fe3O4/SiO2 particles with magnetic harvesting," *Water Research,* vol. 109, pp. 77-87, 2017.

[8] A. Drenkova-Tuhtan, M. Schneider, K. Mandel, C. Meyer, C. Gellermann, G. Sextl and H. Steinmetz, "Influence of cation building blocks of metal hydroxide precipitates on their adsorption and desorption capacity for phosphate in

wastewater - A screening study," *Colloids and Surfaces A: Physicochemical and Engineering Aspects,* vol. 488, pp. 145-153, 2016.

[9] A. Drenkova-Tuhtan, K. Mandel, A. Paulus, C. Meyer, F. Hutter, C. Gellermann, G. Sextl, M. Franzreb and H. Steinmetz, "Phosphate recovery from wastewater using engineered superparamagnetic particles modified with layered double hydroxide ion exchangers," vol. 47, no. 15, pp. 5670-5677, 2013.

[10] A. Drenkova-Tuhtan, E. K. Sheeleigh, E. Rott, C. Meyer and D. L. Sedlak, "Sorption of recalcitrant phosphonates in reverse osmosis concentrates and wastewater effluents – influence of metal ions," *Water Science & Technology,* vol. 83, no. 4, p. 934–947, 2021.

[11] M. Heinlaan, A. Ivask, I. Blinova, H.-C. Dubourguier and A. Kahru, "Toxicity of nanosized and bulk ZnO, CuO and TiO2 to bacteria Vibrio fischeri and crustaceans Daphnia magna and Thamnocephalus platyurus," *Chemosphere,* vol. 71, no. 7, pp. 1308-1316, 2008.

[12] F. Chen, L. Wu, X. Xiao, L. Rong, M. Li and X. Zou, "Mixture toxicity of zinc oxide nanoparticle and chemicals with different mode of action upon Vibrio fischeri," *Environmental Sciences Europe,* vol. 23, 2020.

[13] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts and P. Walter, in *Molecular biology of the cell*, New York, Garland Science, pp. 401-401.

[14] H. Lambers, "Phosphorus Acquisition and Utilization in Plants," *Annual Review of Plant Biology,* vol. 73, pp. 17-42, 2022.

[15] J. Tian, F. Ge, D. Zhang, S. Deng and X. Liu, "Roles of Phosphate Solubilizing Microorganisms from Managing Soil Phosphorus Deficiency to Mediating Biogeochemical P Cycle," *Biology,* vol. 10, no. 2, 2021.

[16] L. Bordenave, G. Tcherkez, C. LeRoy and G. Farquhar, "Phosphorus metabolism in plants: an overview," *Plant Physiology,* vol. 179, no. 2, pp. 731-746, 2019.

[17] M. Oliveira and A. Machado, "The role of phosphorus on eutrophication: a historical review and future perspectives," *Environmental Technology Reviews,* vol. 2, no. 1, pp. 117-127, 2013.

[18] P. G. A. Njock, A. Zhou, Z. Yin and S.-L. Shen, "Integrated risk assessment approach for eutrophication in coastal waters: Case of Baltic Sea," *Journal of Cleaner Production,* vol. 387, 2023.

[19] H. Yu, X. Lu, T. Miki, K. Matsubae, Y. Sasaki and T. Nagasaka, "Sustainable phosphorus supply by phosphorus recovery from steelmaking slag: a critical review," *Resources, Conservation and Recycling,* vol. 180, 2022.

[20] H. Steinfeld, P. J. Gerber, T. Wassenaar and V. Castel, Livestock's Long Shadow: Environmental Issues and Options, United Nations Food and Agriculture Organization, 2006.

[21] A. Witek-Krowiak, K. Gorazda, D. Szopa, K. Trzaska, K. Moustakas and K. Chojnacka, "Phosphorus recovery from wastewater and bio-based waste: an overview," *Bioengineered,* vol. 13, no. 5, pp. 13474-13506, 2022.

[22] S. R. Carpenter, N. F. Caraco, D. L. Correll, R. W. Howarth, A. N. Sharpley and V. H. Smith, "Nonpoint Pollution of Surface Waters with Phosphorus and Nitrogen," *Ecological Applications,* vol. 8, no. 3, pp. 559-568, 1998.

[23] D. Cordell, J.-O. Drangert and S. White, "The story of phosphorus: Global food security and food for thought," *Global Environmental Change,* vol. 19, no. 2, pp. 292-305, 2009.

[24] S. V. Vassilev, C. G. Vassileva and J. B. , "Content, modes of occurrence, and significance of phosphorous in biomass and biomass ash," *Journal of the Energy Institute,* vol. 108, 2023.

[25] V. Smil, "Phosphorus in the Environment: Natural Flows and Human Interferences," *Annual Review of Energy and the Environment ,* pp. 53-88, 2002.

[26] X. Liu, Y. Zhang, M. Cheng, S. Jiang and Z. Y. , "Recycling phosphorus from waste in China: Recycling methods and their environmental and resource consequences," *Resources, Conservation and Recycling,* vol. 188, 2023.

[27] A. R. Jupp, S. Beijer, G. C. Narain, W. Schipperc ja J. C. Slootweg, „Phosphorus recovery and recycling – closing the loop," *Chemical Society Reviews,* nr 1, 2021.

[28] M. Sena, M. Seib, D. R. Noguera and A. Hicks, "Environmental impacts of phosphorus recovery through struvite precipitation in wastewater treatment," *Journal of Cleaner Production,* vol. 280, no. 1, 2021.

[29] P. Achilleos, K. Roberts and I. Williams, "Struvite precipitation within wastewater treatment: A problem or a circular economy opportunity?," *Heliyon,* vol. 8, no. 7, 2022.

[30] G. J. Kortstee, K. J. Appeldoorn, C. F. Bonting, E. W. v. Niel and H. W. v. Veen, "Biology of polyphosphate-accumulating bacteria involved in enhanced biological phosphorus removal," *FEMS Microbiology Reviews,* vol. 15, no. 2-3, p. 137–153, 1994.

[31] G. Tchobanoglus, F. Burton and H. D. Stensel, "Tchobanoglus, George; Burton, Franklin; Stensel, H David.," *American Water Works Association,* vol. 95, no. 1, 2003.

[32] C. Chan, A. Guisasola and J. A. Baeza, "Living on the edge: Prospects for enhanced biological phosphorus removal at low sludge retention time under different temperature scenarios," *Chemosphere,* vol. 258, 2020.

[33] M. C. Chrispim, M. Scholz and M. A. Nolasco, "Phosphorus recovery from municipal wastewater treatment: Critical review of challenges and opportunities for developing countries," *Journal of Environmental Management,* vol. 248, 2019.

[34] B. W. Liping Fang and I. M. Lo, "Fabrication of silica-free superparamagnetic ZrO2@Fe3O4 with enhanced phosphate recovery from sewage: Performance and adsorption mechanism," *Chemical Engineering Journal,* vol. 319, pp. 258-267, 2017.

[35] Y. Chen, R. X. Y. Li, Y. Liu, Y. Wu, Y. Chen, J. Z. S. Chen, H. Yin, Z. Zeng, S. Wang and Z. Peng, "La(OH)3-modified magnetic CoFe2O4 nanocomposites: A novel adsorbent with highly efficient activity and reusability for phosphate removal," *Colloids and Surfaces A: Physicochemical and Engineering Aspects,* vol. 599, 2020.

[36] M. Sürmeli, H. Yazıcı, M. Kılıç and M. Karaboyacı, "Screening of optimum composition of superparamagnetic nanocomposite microparticles modified with various layered double hydroxides for phosphorus removal," *Journal of Water Process Engineering,* vol. 49, 2022.

[37] A. Drenkova-Tuhtan, "Phosphorus Elimination and Recovery from Wastewater with Reusable Nanocomposite Magnetic Particles," doctoral thesis, 2018.

[38] K. Uke, "Evaluating the environmental safety of nanocomposite sorbent materials for phosphorus recovery from wastewater to bacteria Vibrio fischeri," master thesis, Tallinn University of Technology, 2022.

[39] G. E. Santos-Medrano and R. Rico-Martínez, "Acute sensitivity comparison among Daphnia magna Straus, 1820 Daphnia pulex Leydig, 1860 and Simocephalus vetulus Müller, 1776, exposed to nine toxicants.," *Turkish Journal of Fisheries and Aquatic Sciences,* vol. 19, no. 7, pp. 615-623, 2018.

[40] C. Gupta S., Biomarkers in Toxicology, 2019.

[41] C. M. Piovezan, E. A. da Silva, L. B. Silva, T. C. Pereira, R. L. Azevedo and E. C. de Oliveira, "Evaluation of water quality and toxicity of two neotropical rivers using freshwater shrimp," *Environmental Monitoring and Assessment,* vol. 194, no. 2, p. 236, 2020.

[42] L. Wang, W. Wang, P. He and X. Li, "Acute and chronic toxicity of copper to the freshwater shrimp Macrobrachium nipponense," *Ecotoxicology and Environmental Safet,* vol. 140, pp. 327-334, 2017.

[43] X. Ren, Y. Z. Yao Xu, X. W. P. Liu and J. L. , "Comparative accumulation and transcriptomic analysis of juvenile Marsupenaeus japonicus under cadmium or copper exposure," *Chemosphere,* vol. 249, 2020.

[44] S. M. Iliff, R. J. Harris and E. W. Stoner, "Effects of chronic pesticide exposure on an epibenthic oyster reef community," *Marine Pollution Bulletin,* vol. 146, 2019.

[45] "European Commission, Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation,Authorisation and Restriction of Chemicals (REACH)," [Online]. Available: http://data.europa.eu/eli/reg/2006/1907/2022-12-17. [Accessed 25 05 2023].

[46] G. Persoone, R. Baudo, M. Cotman, C. Blaise, K. C. Thompson, M. Moreira-Santos, B. Vollat, A. Törökne ja T. Han, „Review on the acute Daphnia magna toxicity test – Evaluation of the sensitivity and the precision of assays performed with organisms from laboratory cultures or hatched from dormant eggs," *Knowledge and Management of Aquatic Ecosystems,* kd. 393, nr 1, 2009.

[47] N. Tatarazako and S. Oda, "The water flea Daphnia magna (Crustacea, Cladocera) as a test species for screening and evaluation of chemicals with endocrine

disrupting effects on crustaceans," *Ecotoxicology volume,* vol. 16, pp. 197-203, 2007.

[48] I. Altshuler, B. Demiri, S. Xu, A. Constantin, N. D. Yan and M. E. Cristescu, "An Integrated Multi-Disciplinary Approach for Studying Multiple Stressors in Freshwater Ecosystems: Daphnia as a Model Organism," *Integrative and Comparative Biology,* vol. 51, no. 4, pp. 623-633, 2011.

[49] K. Reilly, L.-J. A. Ellis, H. H. Davoudi, S. Supian, M. T. Maia, G. H. Silva, Z. Guo, D. S. T. Martinez and I. Lynch, "Daphnia as a model organism to probe biological responses to nanomaterials—from individual to population effects via adverse outcome pathways," *Frontiers in Toxicology,* vol. 5, 2023.

[50] D. Ebert, "Daphnia as a versatile model system in ecology and evolution," *EvoDevo,* vol. 13, 2022.

[51] OECD, "Daphnia sp., Acute Immobilisation Test. OECD Guidelines for the Testing of Chemicals, Section 2, Test No. 202.," 2004.

[52] ASTM, "Standard Guide for Conducting Daphnia magna Life-Cycle Toxicity Tests, ASTM E1193-20," 2020.

[53] ISO, "Water quality — Determination of the inhibition of the mobility of Daphnia magna Straus (Cladocera, Crustacea) — Acute toxicity test," 1996.

[54] OECD, "Test No. 211: Daphnia magna Reproduction Test," 2008.

[55] EPA, "Guidelines for Ensuring and Maximizing the Quality, Objectivity, Utility, and Integrity of Information Disseminated by the Environmental Protection Agency," 2002.

[56] A. Beasley, S. E. Belanger, J. L. Brill and R. R. Otter, "Evaluation and comparison of the relationship between NOEC and EC10 or EC20 values in chronic Daphnia toxicity testing," *Environmental Toxicology,* vol. 34, no. 10, 2015.

[57] K. Mandel, A. Drenkova-Tuhtan, F. Hutter, C. Gellermann, H. Steinmetz and G. Sextl, "Layered double hydroxide ion exchangers on superparamagnetic microparticles for recovery of phosphate from waste water," *Journal of Materials Chemistry A,* no. 5, 2013.

[58] A. Fettweis, K. D. S. and E. Smolders, "Zinc toxicity to Daphnia magna in a two-species microcosm can be predicted from single-species test data: The effects of

phosphorus supply and pH," *Environmental Toxicology,* vol. 37, no. 8, pp. 2153-2164, 2018.

[59] "European Chemicals Agency database, Zinc oxide Substance Infocard," [Online]. Available: https://echa.europa.eu/substance-information/-/substanceinfo/100.013.839. [Accessed 20 05 2023].

[60] "European Chemicals Agency database, Zinc chloride Substance Infocard," [Online]. Available: https://echa.europa.eu/substance-information/-/substanceinfo/100.028.720. [Accessed 20 05 2023].

[61] "Daphtoxkit F, Crustacean Toxicity Screening Test, Standard Operating Produre," [Online]. Available: https://www.microbiotests.com/wp-content/uploads/2019/07/daphnia-toxicity-test_daphtoxkit-f_standard-operating-procedure.pdf. [Accessed 20 05 2023].

[62] OECD, "Freshwater Alga and Cyanobacteria, Growth Inhibition Test No. 201," 2006.

[63] M. K. Tiwari, B. Gowrishankar, V. K. Raghuvanshi, R. V. Nandedkar and K. J. S. Sawhney, "Development of a total reflection X-ray fluorescence spectrometer for ultra-trace element analysise," *Bulletin of Materials Scienc,* vol. 25, pp. 435-441, 2002.

[64] E. K. Towett, K. D. Shepherd ja G. Cadisch, „Quantification of total element concentrations in soils using total X-ray fluorescence spectroscopy (TXRF)," *Science of The Total Environment,* Kd-d. %1/%2463-464, pp. 374-388, 2013.

[65] "European Commission, Chemicals," [Online]. Available: https://environment.ec.europa.eu/topics/chemicals_en. [Accessed 20 05 2023].

[66] I. Blinova, A. Ivask, M. Heinlaan, M. Mortimer and A. Kahru, "Ecotoxicity of nanoparticles of CuO and ZnO in natural water," *Environmental Pollution,* vol. 158, no. 1, pp. 41-47, 2010.

[67] S. Hussain, M. Khan, T. M. M. Sheikh, M. Z. Mumtaz, T. A. Chohan, S. Shamim and Y. Liu, "Zinc essentiality, toxicity, and its bacterial bioremediation: A comprehensive insight," *Frontiers in Microbiology,* vol. 13, 2023.

[68] M. Abdel-Tawwab, G. O. El-Sayed and S. H. Shady, "Growth, biochemical variables, and zinc bioaccumulation in Nile tilapia, Oreochromis niloticus (L.) as

affected by water-born zinc toxicity and exposure period," *International Aquatic Research,* vol. 8, pp. 197-206, 2016.

[69] R. A. Gonçalves, A. L. d. O. F. Rossetto, D. J. Nogueira, D. S. Vicentini and W. G. Matias, "Comparative assessment of toxicity of ZnO and amine-functionalized ZnO nanorods toward Daphnia magna in acute and chronic multigenerational tests," *Aquatic Toxicology,* vol. 197, pp. 32-40, 2018.

[70] M. B. Ergönül, S. Atasağun and T. Beşer, "The acute toxicity of zinc chloride on Daphnia magna straus," *Gazi University Journal of Science,* vol. 25, no. 2, pp. 313-316, 2012.

[71] I. Blinova, H. Vija, A. Lukjanova, M. Muna, G. Syvertsen-Wiig and A. Kahru, "Assessment of the hazard of nine (doped) lanthanides-based ceramic oxides to four aquatic species," *Science of The Total Environment,* vol. 618, no. 6, pp. 1171-1176, 2018.

[72] A. Okamoto, M. Yamamuro and N. Tatarazako, "Acute toxicity of 50 metals to Daphnia magna," *Journal of Applied Toxicology,* vol. 35, no. 7, 2014.

[73] "European Chemicals Agency database, Calcium chloride Substance infocard," [Online]. Available: https://echa.europa.eu/et/registration-dossier/-/registered-dossier/15461/6/2/4. [Accessed 21 05 2023].

[74] M. Załęska-Radziwiłł and N. Doskocz, "Ecotoxicity of zirconium oxide nanoparticles in relation to aquatic invertebrates," *Desalination and Water Treatment ,* vol. 57, no. 3, 2016.

[75] M. Mortimer, K. Kasemets, H. M, K. I and K. A, "High throughput kinetic Vibrio fischeri bioluminescence inhibition assay for study of toxic effects of nanoparticles," *Toxicology in Vitro,* vol. 22, no. 5, 2008.

[76] "European Chemicals Agency database, Magnesium chlorid Substance Infocard," [Online]. Available: https://echa.europa.eu/et/registration-dossier/-/registered-dossier/15140/7/9/1. [Accessed 21 05 2023].

[77] "European Chemicals Agency database, Iron sulphate Substance Infocard," [Online]. Available: https://echa.europa.eu/et/registration-dossier/-/registered-dossier/15513/4/1. [Accessed 21 05 2023].

[78] "European Chemicals Agency database, Zirconium tetrachloride Substance Infocard," [Online]. Available: https://echa.europa.eu/et/registration-dossier/-/registered-dossier/12108/5/1. [Accessed 21 05 2023].

[79] G. Nabi and S. W. Jeong, "Toxicity of zinc oxide nanoparticles on Daphnia magna: a comparison of the role of dissolved and nanoparticulate zinc," *Environmental Science and Pollution Research,* vol. 28, no. 5, pp. 5315-5324, 2021.

[80] W. Wang, Y. Yang, L. Yang, T. Luan and L. Lin, "Effects of undissociated SiO2 and TiO2 nano-particles on molting of Daphnia pulex: Comparing with dissociated ZnO nano particles," *Ecotoxicology and Environmental Safety,* vol. 222, 2021.

[81] D. Ebert, Ecology, Epidemiology, and Evolution of Parasitism in Daphnia, National Center for Biotechnology Information, 2005.

[82] European Chemicals Agency, "Guidance on information requirements and chemical safety assessment," [Online]. Available: https://echa.europa.eu/documents/10162/17235/information_requirements_part_b_en.pdf/7e6bf845-e1a3-4518-8705-c64b17cecae8?t=1323782779823. [Accessed 20 05 2023].

[83] V. Aruoja, S. Pokhrel, M. Sihtmäe, M. Mortimer, L. Mädler and A. Kahru, "Toxicity of 12 metal-based nanoparticles to algae, bacteria and protozoa," *Environmental Science: Nano,* no. 6, 2015.

APPENDIX 1

Appendix 1 Range finding and definitive test effect after 48 hours and Zn total and ion concentration in the samples

Toxicant / Chemical	Toxicant Nominal Conc. (mg/L)	Range finding test Effect % after 48h	Definitive test Zn total concentration mg/L before the test	Definitive test Effect % after 48h	Definitive test Zn ion concentration mg/L after the test	Range finding and definitive test together Effect % after 48 h	Definitive test EC50 48h
Chem 01 ZnFeZr 6:1:1	1.0	10					7.7 mg/L
	3.1	-	1.3 ± 0.03	21.7 ± 8.5	1.1 ± 0.02		
	6.3	-		35 ± 12.2	1.8 ± 0.06		
	10.0	35					
	12.5	-		43.3 ± 14.3	1.7 ± 0.46		
	25.0	-		41.7 ± 10.3	2.2 ± 0.44		
	50.0	-		46.7 ± 13.1	1.9 ± 0.57		
	100.0	75	37.1 ± 3.66	48.3 ± 15.5	1.9 ± 0.31	55 ± 17.9	
Chem 02 CaFeZr 6:1:1	1.0	15					>>100 mg/L
	10.0	0					
	50.0	-	0.10	5 ± 5	0.1 ± 0.07		
	100.0	0	0.04	5 ± 0	0.0 ± 0.01	3.3 ± 2.4	
Chem 03 CaZnFeZr 3:3:1:1	1.0	15					12.5 mg/L
	3.1		0.86 ± 0.08	16.7 ± 9.4	0.5 ± 0.04		
	6.3	-		23.3 ± 10.3	1.0 ± 0.09		
	10.0	25					
	12.5	-		31.7 ± 11.8	1.7 ± 0.16		
	25.0	-		41.7 ± 4.7	1.8 ± 0.70		
	50.0	-		56.7 ± 6.2	2.2 ± 0.23		
	100.0	65	27.78 ± 2.83	48.3 ± 15.5	1.9 ± 0.67	52.5 ± 15.2	

Chem 04 MgFeZr 6:1:1	1.0	0					>>100 mg/L
	10.0	0					
	50.0	-	0.14	10 ± 5	0.07 ± 0.01		
	100.0	15	0.05	7.5 ± 2.5	0.07 ± 0.02	10 ± 4.1	
Chem 05 MgZnFe 1:1:1	1.0	5					>100 mg/L
	3.1		0.74 ± 0.10	23.3 ± 6.2	0.53 ± 0.18		
	6.3	-		16.7 ± 2.4	0.4 ± 0.09		
	10.0	25					
	12.5	-		21.7 ± 6.2	0.5 ± 0.11		
	25.0	-		26.7 ± 10.3	0.7 ± 0.02		
	50.0	-		26.7 ± 13.1	0.9 ± 0.04		
	100.0	5	26.17 ± 0.30	21.7 ± 12.5	1.0 ± 0.12	17.5 ± 13	
Chem 06 CaFe 2:1	1.0	10					>>100 mg/L
	10.0	0					
	50.0	-	0.06	17.5 ± 7.5	0.0 ± 0,00		
	100.0	10	0.05	12.5 ± 2.5	0.0 ± 0,00	11.67 ± 2.4	
Chem 07 ZnFeZr 18:5:1	1.0	0					4.4 mg/L
	3.1	-	1.48 ± 0.09	26.7 ± 17	1.0 ± 0.12		
	6.3	-		45 ± 14.7	1.8 ± 0.19		
	10.0	52.3					
	12.5	-		63.3 ± 15.5	2.3 ± 0.11		
	25.0	-		63.3 ± 18.9	2.1 ± 0.52		
	50.0	-		68.3 ± 20.9	2.2 ± 0.54		
	100.0	60	45.39 ± 6.51	71.7 ± 13.1	2.6 ± 0.97	68.75 ±12.4	
Chem 08 ZnFeZr 4:1:1	1.0	5					9.0 mg/L
	3.1	-	1.38 ± 0.21	11.7 ± 2.4	0.7 ± 0.09		
	6.3	-		30 ± 16.3	1.2 ± 0.09		
	10.0	30					
	12.5	-		45 ± 22.7	2.0 ± 0.08		

	25.0	-		50 ± 16.3	2.1 ± 0.21		
	50.0	-		55 ± 16.3	1.8 ± 0.24		
	100.0	50	32.26 ± 5.59	61.7 ± 14.3	1.3 ± 0.20	58.75 ±13.4	
Chem 09 ZnFeZr 3.6:0.2:1	1.0	0					5.7 mg/L
	3.1	-	1.03 ± 0.05	15 ± 10.8	1.0 ± 0.01		
	6.3	-		25 ± 4.1	1.8 ± 0.01		
	10.0	40					
	12.5	-		38.3 ± 4.7	2.3 ± 0.11		
	25.0	-		56.7 ± 18.9	1.9 ± 0.14		
	50.0	-		60 ± 14.7	1.6 ± 0.35		
	100.0	55	36.4 ± 14.94	53.3 ± 6.2	1.1 ± 0.16	53.75 ± 4.3	
Chem 10 ZnFeZr 10:1:1	1.0	15					6.5 mg/L
	3.1	-	1.73 ± 0.25	20 ± 10.8	1.4 ± 0.03		
	6.3	-		33.3 ± 6.2	2.0 ± 0.36		
	10.0	25					
	12.5	-		40 ± 5	2.0 ± 0.45		
	25.0	-		53.75 ± 6.3	1.7 ± 0.34		
	50.0	-		52.5 ± 7.5	1.3 ± 0.06		
	100.0	35	46.56 ± 7.34	55 ± 18.7	1.0 ± 0.09	51 ± 18.5	
Chem 11 ZnFeZr 6:1:1 @ MPs	1.0	0					>>100 mg/L
	10.0	10					
	50.0	-	6.14	12.5 ± 2.5	0.4 ± 0.06		
	100.0	0	12.64 ± 0.10	15 ± 0	0.6 ± 0.14	10 ± 7.1	