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GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL

**TOXICITY OF ZnFeAl-LAYERED DOUBLE HYDROXIDES
(LDHs) ON MARINE AND FRESHWATER ALGAE**

ZEHRA AKDAĞ

A THESIS OF MASTER OF SCIENCE
DEPARTMENT OF BIOTECHNOLOGY

ADVISOR: PROF. DR. MELEK ÖZKAN

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LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

**ZnFeAl KATMANLI ÇİFT HİDROKSİTLERİN DENİZ
VE TATLI SU ALGLERİ ÜZERİNDEKİ TOKSİSİTESİ**

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*To my fiancé,
family and friends*

ABSTRACT

The layered double hydroxides (LDHs) are a two-dimensional structured class of biocompatible inorganic nanomaterials with a wide range of applications for solving today's environmental and industrial problems. The layered structure, tunable physicochemical properties composition, high anion exchange capacity, biocompatibility and controlled release capabilities of LDHs make them have a wide range of potential applications, such as drug delivery, engineering fields, water treatment, catalysis, environmental remediation, agricultural product development and various industrial processes. However, their increasing release into aquatic systems raises concerns about their potential ecotoxicological effects.

This study investigates and compares the acute toxicity of newly synthesized LDH, ZnFeAl, on two microalgal species from distinct aquatic environments. Freshwater microalgae *Chlorella vulgaris* and marine diatom *Cyclotella cryptica* were cultivated with different concentrations of ZnFeAl LDHs (0, 10, 25, 50, 100, 250 and 500 mg.L⁻¹) for 15 days. At the end of fifteen days, morphological changes of algal cells, changes in chlorophyll and carotenoid content, changes in anthocyanin and phenolic content and antioxidant enzyme activities were examined.

The results show that the effects of nanoparticles on algae growth can vary depending on various factors and can generally inhibit algae growth. Exposure to high concentrations of LDHs also caused reduced photosynthetic pigment content and cellular damage in microalgae. At lower concentrations, the effects may be less severe or may trigger different biochemical responses. Direct interaction of LDHs with the cell surface, solubilization of toxic elements, or production of reactive oxygen species (ROS) may contribute to toxicity. The toxicity of LDHs varies depending on the type of organism exposed. The results revealed a concentration-dependent toxic response, with *C. cryptica* showing greater sensitivity to LDH exposure compared to *C. vulgaris*. A concentration of 10 mg/L ZnFeAl was found to show toxic effect to *C. cryptica*. SOD antioxidant enzyme activity findings indicate that the freshwater alga *C. vulgaris* is more affected and activates its antioxidant defenses more strongly than the marine diatom *C. cryptica*. CAT activity was observed to increase as LDH concentrations increased in both algae. These species-specific responses highlight the importance of biochemical biomarkers in assessing sublethal stress caused by nanomaterials in aquatic organisms. These findings suggest that marine ecosystems may be more susceptible to LDH pollution. These results reflect the importance comprehensive risk assessments of nanomaterials that can contaminate aquatic environments.

Keywords: *Cyclotella cryptica*, *Chlorella vulgaris*, Ecotoxicity, ZnFeAl nanoparticles.

ÖZET

Katmanlı çift hidroksitler (LDH'ler), günümüzün çevresel ve endüstriyel sorunlarını çözmek için geniş bir uygulama yelpazesine sahip iki boyutlu yapılandırılmış bir biyoyumlu inorganik nanomalzeme sınıfıdır. Katmanlı yapı, ayarlanabilir fizikokimyasal özellikler bileşimi, yüksek anyon değişim kapasitesi, biyoyumluluk ve LDH'lerin kontrollü salınım kabiliyetleri, ilaç dağıtımı, mühendislik alanları, su arıtma, kataliz, çevresel iyileştirme, tarımsal ürün geliştirme ve çeşitli endüstriyel süreçler gibi geniş bir potansiyel uygulama yelpazesine sahip olmalarını sağlar. Ancak, su sistemlerine artan salınımları, potansiyel ekotoksikolojik etkileri konusunda endişelere yol açmaktadır.

Bu çalışmada, yeni sentezlenmiş bir LDH olan ZnFeAl nin farklı sucul ortamlardan iki mikroalg türü üzerindeki akut toksisitesi araştırılmıştır. Tatlı su mikroalgi *Chlorella vulgaris* ve deniz diatomu *Cyclotella cryptica*, 15 gün boyunca farklı ZnFeAl LDH konsantrasyonları (0, 10, 25, 50, 100, 250 ve 500 mg.L⁻¹) varlığında inkübe edilmişlerdir. On beş günün sonunda, alg hücrelerinin morfolojik değişimleri, klorofil ve karotenoid içeriğindeki değişimler, antosiyanin ve fenolik içeriğindeki değişimler ve antioksidan enzim aktiviteleri incelendi.

Sonuçlar, söz konusu nanopartiküllerin alg büyümesi üzerindeki etkilerinin çeşitli faktörlere bağlı olarak değişebileceğini ve genel olarak alg büyümesini engelleyebileceğini göstermektedir. Yüksek ZnFeAl konsantrasyonlarına maruz kalma, mikroalglerde fotosentetik pigment içeriğinin azalmasına ve hücresel hasara da neden olmuştur. Daha düşük konsantrasyonlarda, etkiler daha az şiddetli olmakla birlikte farklı biyokimyasal tepkileri tetikleyebilir. LDH'lerin hücre yüzeyiyle doğrudan etkileşimi, toksik elementlerin çözünmesi veya reaktif oksijen türlerinin ROS üretimi toksisiteye katkıda bulunabilmektedir. Sonuçlar ZnFeAl'nin toksisitesinin maruz kalan organizmanın türüne bağlı olarak değiştiğini göstermektedir. *C. cryptica*, *C. vulgaris* ile karşılaştırıldığında LDH maruziyetine karşı daha fazla duyarlılık göstermiştir. 10 mg/L ZnFeAl konsantrasyonunun *C. cryptica* için toksik etki gösterdiği görülmüştür. SOD antioksidan enzim aktivitesi bulguları, *C. vulgaris* tatlı su alginin daha fazla etkilendiğini ve antioksidan savunmalarını *C. cryptica* deniz diatomundan daha güçlü bir şekilde harekete geçirdiğini göstermektedir. Her iki algde de LDH konsantrasyonu arttıkça CAT aktivitesinin arttığı görülmüştür. Türler özgü tepkiler, suda yaşayan organizmalarda nanomalzemelerin neden olduğu subletal stresi değerlendirmede biyokimyasal biyobelirteçlerin önemini vurgulamaktadır. Bulgulara göre, deniz ekosistemleri LDH kirliliğine karşı daha duyarlı olabilir. Sonuçlar sucul ortamları kirletebilecek nanomalzemelerin kapsamlı risk değerlendirmelerinin gerekliliğini vurgulamaktadır.

Anahtar Kelimeler: *Cyclotella cryptica*, *Chlorella vulgaris*, Ekotoksisite, ZnFeAl nanopartikülleri

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LIST OF SYMBOLS AND ABBREVIATIONS

%	: Percentage
°C	: Celcius
g	: Gram
kg	: Kilogram
L	: Liter
v	: Volume
mL	: Mililiter
mM	: Milimolar
μL	: Microliter
μM	: Micromolar
p	: p-value
5FU	: 5-fluorouracil
Abs	: Absorbance
Al	: Aluminum
ANOVA	: Analysis of Variance
As	: Arsenic
BSA	: Bovine Serum Albumin
CAT	: Catalase
Cd	: Cadmium
Co	: Cobalt
Cu	: Copper
dH ₂ O	: Distilled water
DNA	: Deoxyribonucleic acid
DOX	: Doxorubicin
Eq	: Equation
Fe	: Iron
Fe ₂ O ₃	: Iron (III) oxide
FW	: Fresh weight
H ₂ O	: Water
H ₂ O ₂	: Hydrogen peroxide
HSD	: Tukey's Honest Significant Difference
kDa	: Kilodalton
LDH	: Layered double hydroxides
Mg	: Magnesium
min	: Minute
MRI	: Magnetic resonance imaging
NBT	: Nitroblue Tetrazolium
nm	: Nanometre

NM	: Nanomaterial
O ₂	: Oxygen
OD	: Optical density
O3D-LDH	: Organic three-dimensional layered double hydroxide
PBS	: Phosphate buffered saline
PCDE	: Polychlorinated diphenyl ethers
PEG	: Polyethylene glycol
Pb	: Lead
RNA	: Ribonucleic acid
ROS	: Reactive oxygen species
s	: Second
SD	: Standard deviation
Se	: Selenium
SEM	: Scanning electron microscopy
SEM EDS	: Energy Dispersive X-ray Spectroscopy
siRNA	: Small Interfering RNA
SOD	: Superoxide dismutase
TEM	: Transmission electron microscopy
TiO ₂	: Titanium dioxide
W	: Watt
Zn	: Zinc
ZnO	: Zinc oxide

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1. INTRODUCTION

The two-dimensional organized class of biocompatible inorganic nanomaterials known as layered double hydroxides (LDHs) has several uses in engineering, water treatment, catalysis, and drug delivery, among other areas, to address contemporary environmental and industrial issues [Arrabito et al., 2019]. They belong to the family of anionic clays, which are two-dimensional layered nanomaterials that can exfoliate in polymer matrices, intercalate with functional materials, and form hybrids with other superior nanomaterials to create a variety of applications [Arrabito et al., 2019; Chaillot et al., 2021]. Numerous disciplines, such as biology, electrochemistry, pharmacology, and environmental pollution treatment, use these compounds. LDHs can include nearly every stable element in the periodic table. Their interlayer space contains complexes of metal ions or non-metallic elements in their anionic state, while their framework is composed of smaller metal cations. Their physical-chemical properties are hence highly variable and have been summarized from a variety of angles, including energy-related applications, sensing, and catalysis. The low cost, chemical stability, high catalytic activity, and absorbent nature of LDHs have made them suitable materials for environmental remediation applications. Studies have been conducted on the detrimental effects and associated processes of various nanomaterials in a range of animals, despite their varied applications [Mishra et al., 2018]. The direct contact of nanomaterials with the cell surface, the solubility of dangerous substances, and the production of reactive oxygen species (ROS) are the three main mechanisms that have been found to cause oxidative stress in biological systems [Torbati et al., 2024]. In the introduction parts, LDHs and their toxic effects on different organisms, and, toxic responses and antioxidant enzymes as defence mechanisms of the cells are covered to give general knowledge about the thesis subject.

1.1. Ecotoxicological Effects of Pollutants

Environmental pollutants have various harmful effects on the environment and living organisms. Contaminants, particularly trace elements, have demonstrated significant dangers to human, plant, and ecology. Exposure to environmental pollutants is

associated with various adverse health conditions such as cancer, immune and reproductive dysfunction, and imbalance in the gastrointestinal microbiota [Demir & Kacew, 2024; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2012; Salk & Kennedy, 2020]. Some environmental chemicals (endocrine disrupting chemicals and metabolic disrupting chemicals) can disrupt the hormonal system, contribute to reproductive problems, developmental abnormalities, and cancer risk [Michelangeli, 2024]. They can also exacerbate metabolic syndrome diseases such as type 2 diabetes, cardiovascular disease, and fatty liver disease [Erradhouani et al., 2024].

Accumulation and ingestion of pollutants in edible plant tissues is an important route of exposure for humans. Contaminants such as mycotoxins found in foods and agricultural products pose a risk to human and animal health [M. Chen, 2024]. Sources have indicated the accumulation of lead (Pb) and arsenic (As) in the leaves of plants such as spinach and their potential carcinogenic and non-carcinogenic risks for children. Irrigation with wastewater may pose health risks by causing heavy metal accumulation and oxidative stress in plants [Amen et al., 2020; Natasha, Shahid, Farooq, et al., 2020; Shah et al., 2020; Shahid et al., 2012, 2017].

Industrial emissions from mining, metallurgical, and chemical industries and urban air pollution pose risks, including fine particles (<100 nm) that are hazardous to workers and the general population [Sutunkova, 2022]. Exposure to mineral fibers requires understanding the mechanisms involved in the toxicity and carcinogenicity of inhaled mineral fibers [Mirata et al., 2022]. For example, electroplating industry wastewater contains toxic substances such as heavy metals and requires treatment [Rajoria et al., 2022].

Environmental pollutants, in addition to causing direct toxic effects on biological organisms, cause cellular damage, physiological dysfunctions and deterioration of ecosystem health. These effects can be transmitted through the food chain and pose serious risks to human health, especially through substances such as heavy metals, organic chemicals, microplastics and nanoplastics [Demir & Kacew, 2024; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2012; Salk & Kennedy, 2020].

Various pollutants have direct harmful effects on living organisms at different trophic levels. For example, polychlorinated diphenyl ethers (PCDEs) are highly toxic to three

different aquatic organisms [W. Yang et al., 2022]. Chemicals leached from tire particles have caused growth inhibition in plants, mortality in water fleas, and abnormalities in zebrafish [Kim et al., 2023]. Nanoparticles and microplastics such as Nano-TiO₂ can have toxic effects on aquatic organisms such as algae and crustaceans, and these effects may increase or decrease depending on the surface properties of the microplastics [Thiagarajan et al., 2021]. Not only aquatic organisms but also organisms living in soil are affected by heavy exposure to pollutants such as fertilizers or pesticides. Soil microbiota play a central role in achieving sustainability goals such as maintaining soil health, recycling organic matter and reducing pollution. Soil pollution threatens soil sustainability, loss of diversity of soil microbes can impact ecosystem and organism health [Timmis & Ramos, 2021; Wassermann et al., 2019]. Toxicological effects such as growth inhibition, mortality, and abnormalities have been observed in exposed organisms. Acute or chronic exposure to a variety of environmental pollutants has been shown to have deleterious effects on mammalian and non-target model organisms at the genetic, cellular, and molecular levels [Kim et al., 2023; W. Yang et al., 2022].

Zinc and benzothiazole leached from tire particles were identified as the primary chemicals causing biotoxicity to tested organisms. [Kim et al., 2023]. Microplastics and nanoplastics can act as “Trojan Horses” carrying other environmental pollutants, causing damage to the enteric barrier [Thiagarajan et al., 2021].

Some synthetic organic chemicals (alkylphenols and brominated flame retardants) can cause cell death by disrupting intracellular calcium balance and triggering endoplasmic reticulum stress [Michelangeli, 2024; Michelangeli et al., 2024]. Industrial chemicals, such as mineral fibers, have different mechanisms of toxicity that cause DNA damage by producing ROS and cytokines in macrophages [Mirata et al., 2022; Sutunkova, 2022].

1.2. Environmental Toxicology

The study of toxicology looks at the potential causes of environmental contaminants' negative effects on human and other living organisms in different environments [Antoniadis et al., 2019; Shahid et al., 2021]. Toxicology is a large scientific discipline that studies the detrimental effects of chemicals and other environmental

elements on organisms and the environment, as well as the mechanisms, hazards, and evaluation techniques associated with these effects [Michelangeli, 2024; Shahid, Nadeem, et al., 2020]. Environmental toxicology examines industrial and technical advancements cause contaminants to circulate, and accumulate in various settings, hence polluting ecosystems [Shahid, Nadeem, et al., 2020]. One example of environmental toxicology is the use of agent-based monitoring techniques to evaluate how contaminants affect fish health in urbanized watersheds [Mehinto et al., 2021]. Determining the presence and effects of synthetic and naturally occurring pollutants in the environment (air, water, soil) is one of the main goals of environmental toxicology. Industrial and technological developments around the world, along with the unsustainable use of non-renewable resources, have led to pollutants that affect ecosystem health and integrity.

Toxicology research seeks to elucidate critical chemicals and their toxic metabolic signatures that result in detrimental effects by incorporating innovative concepts and methodologies, such as chemical exposomes (comprehensive environmental exposures throughout an individual's life) and metabolomics (identification of small molecule byproducts of cellular metabolism), to thoroughly examine the correlation between environmental exposures and human health [Gonzalez-Covarrubias et al., 2022; Vermeulen et al., 2020]. Beside, pollutants create potential hazards to aquatic ecosystems and affect organisms at different trophic levels. Model organisms representing different levels in the simple aquatic food chain, such as algae, water fleas, and fish, are used to study these effects [Domingo, 2006; W. Yang et al., 2022].

The importance of environmental toxicology for the environment and organisms can be explained by the detection and assessment of environmental pollution. Halogenated aromatic compounds, such as polychlorinated diphenyl ethers (PCDEs), are widely used in a variety of products, including hydraulic oils, electrical insulators, lubricants, flame retardants, and plasticizers [W. Yang et al., 2022]. Such contaminants have been frequently detected in a wide range of environmental and biological samples, including water, sediment, soil, atmosphere, fly ash, smoke, food, and wildlife [Demir & Kacew, 2024].

To avoid health risks from pollutants, it's critical to evaluate the risk of exposure. Risk assessment indices help with timely decision-making by estimating the possible

pathways and degrees of human exposure to contaminants [Demir & Kacew, 2024; Natasha et al., 2020].

The potential harmful effects of emerging pollutants such as nanoparticles (NPs) and microplastics on aquatic ecosystems are also being investigated. For example, zinc oxide (ZnO) and iron oxide (Fe₂O₃) nanoparticles have been observed to induce stressful physiological conditions in freshwater microalgae such as dose-dependent growth retardation, decreased chlorophyll content, and increased superoxide dismutase (SOD) and catalase enzyme activities [Saxena et al., 2021].

1.3. Toxicity at Three Trophic Levels

The study of the detrimental effects of different substances on living things at different levels in an ecosystem is known as toxicity at three trophic levels. Protecting natural habitats, requires an understanding of how harmful substances affect the environment. Three-trophic level toxicology, one of the methods created for this goal, is an extensive procedure that examines a substance's harmful effects on organisms at several trophic levels. This method seeks to comprehend the possible risks that contaminants pose to aquatic environments [Kim et al., 2023; W. Yang et al., 2022].

A trophic level refers to an organism's position on the food chain within an ecosystem. Model organisms that represent various food chain levels are frequently utilized in these kinds of investigations [Capolupo et al., 2020]. Primary producers (1st trophic level), photosynthetic organisms, usually algae and phytoplankton. Primary consumers (2nd trophic level), herbivorous aquatic invertebrates, such as planktonic species. Secondary/secondary consumers (3rd trophic level), carnivorous aquatic organisms, especially small fish species.

Green algae, or *Scenedesmus obliquus*, are the primary producers at the base trophic level. Plants (*Vigna radiata*) were also employed in another investigation [Kim et al., 2023; W. Yang et al., 2022].

Primary consumers (herbivores), water fleas (*Daphnia magna*) [Kim et al., 2023; W. Yang et al., 2022]. In a two-trophic level study, brine shrimp (*Artemia salina*) was also used as a primary consumer [Thiagarajan et al., 2021].

Secondary consumers (carnivores/fish), zebrafish (*Danio rerio*) [Kim et al., 2023; W. Yang et al., 2022]. These model organisms are often chosen as biological models in ecotoxicology studies. The algae-flea-fish combination represents a simple aquatic food chain.

Toxicity studies at three trophic levels help to assess how toxicity may differ both among species. This makes it possible to compare the sensitivity of different species to contaminants and the effect of their position in the food chain on toxicity.

1.4. Layered Double Hydroxides

In the literature, "layered nanostructures and anionic clays with a hydrotalcite-like crystal structure" are the definition of LDHs [Kiani et al., 2022]. Their distinctive layered structure is often composed of layers of water molecules, hydroxyl groups, exchangeable interlayer anions ($[An-]_{x/n} mH_2O$), and divalent (M^{2+}) and trivalent (M^{3+}) metal ions such as Mg^{2+} , Al^{3+} , Fe^{3+} , Cu^{2+} , Zn^{2+} , Mn^{2+} . $[M^{2+}_2- xM^{3+}_x(OH)_2]_x+[An-]_{x/n} mH_2O$ is their general formula, where "x" typically ranges from 0.16 to 0.33 [Gu et al., 2019; B. Liu et al., 2023; Maity et al., 2024; Xu et al., 2006]. Through hydrogen bonding and electrostatic interactions, the materials in between these layers create a stable structure.

Applications in engineering and biomedicine have shown a great deal of interest in LDHs. Their special qualities, being affordable and easy to synthesize, are the source of this interest [Koba-Ucun et al., 2021]. They can be quickly digested in the body and are thought to have strong biocompatibility, which reduces long-term biosafety hazards.

1.4.1. Key Features and Advantages of LDHs

They are easy to synthesize and low-cost materials [H. Wu et al., 2022, 2023; J. Zhao et al., 2021]. Their biocompatibility is considered good and they can be rapidly metabolized in the body, minimizing long-term biosafety risks [H. Wu et al., 2022; Yousefi et al., 2020]. Their chemical composition, morphology, particle size and surface charge are adjustable [Jing et al., 2022]. They have a large surface area and high drug loading capacity. This allows drugs and biomolecules such as peptides, proteins, nucleic acids to be placed in their interlayers [Kiani et al., 2022; Yan et al.,

2017]. Anion exchange capacity is important for the transport of drugs and other anionic molecules [Kiani et al., 2022; B. Liu et al., 2023; Pavlovic et al., 2022]. They can dissolve in slightly acidic pH conditions, such as the tumor microenvironment, and release their charged molecules in a controlled manner. This provides advantages in tumor targeting and drug delivery [Jing et al., 2022; Kiani et al., 2022; Maity et al., 2024]. Surface modification with biopolymers such as albumin can increase their colloidal stability and prevent aggregation [Pavlovic et al., 2022; Yan et al., 2017; J. Zhang et al., 2024]. Some LDHs may have superparamagnetic properties that can become magnetized when a magnetic field is applied, which can be used for targeted drug delivery [Ebadi et al., 2020].

1.4.2. Application Areas of LDHs

Because of its special qualities, LDHs are employed in many different fields.

Drug carriers and targeted drug delivery, in the treatment of cancer, they serve as nanocarriers that deliver anticancer drugs like doxorubicin (DOX), sorafenib, 5-fluorouracil (5FU) in a targeted and regulated manner [Aziz et al., 2022; Kiani et al., 2022]. Additionally, they have demonstrated the ability to enhance the therapeutic benefits and bioavailability of medications like glibenclamide for type II diabetes and phenytoin for epilepsy [Damasceno Leão et al., 2023; Kiani et al., 2022; J. Zhang et al., 2024].

Gene and biomolecule carriers, LDHs used to supply cells or tissues with genetic resources including deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and siRNA (small interfering RNA) [Bian et al., 2024; W. Yang et al., 2022].

Bone regeneration and reconstruction, LDHs might include ions like iron (Fe^{3+}) and magnesium (Mg^{2+}), which are crucial for bone metabolism and regeneration and can encourage osteogenesis and bone repair. They have been created as multipurpose platforms to aid in bone repair and stop tumor recurrence, particularly when treating osteosarcoma [Bian et al., 2024].

Anti-bacterial applications, both gram-positive and gram-negative bacteria have been shown to be susceptible to their antibacterial qualities. LDHs have the potential to prevent periprosthetic infections and treat bacterial infections that are resistant to treatment [Koba-Ucun et al., 2021][Bian et al., 2024].

Bioimaging and biosensing, LDHs can be utilized for biosensors and as contrast agents for magnetic resonance imaging (MRI) [J. Liu et al., 2022].

Nano-fertilizers, they can be applied as nanofertilizers to increase photosynthesis, encourage plant development, and minimize the harmful effects of heavy metal ions [H. Wu et al., 2023].

Water purification and catalysis, LDHs are also employed as catalysts for a variety of chemical reactions and as adsorbents in water purification systems [Kiani et al., 2022; Koba-Ucun et al., 2021].

1.4.3. Biocompatibility and Toxicity Assessment

Even while LDHs are usually biocompatible, there are some circumstances in which they could be toxic [Pavlovic et al., 2022; J. Zhang et al., 2024].

Aggregation and sedimentation, in the blood or physiological fluids (such as erythrocytes or saline), they frequently congregate. Significant systemic toxicity and even mortality may result from this aggregation, which can alter the distribution of nanoparticles and, particularly with repeated injections, cause buildup in the lungs and pulmonary embolism [Choi et al., 2013; Dong et al., 2014; Yan et al., 2017]. Biological mediums have high ion concentrations and protein content, LDH nanoparticles might decrease electrostatic repulsive forces to form aggregates and precipitate. They can cause erythrocyte aggregation by engaging directly with negative-charged erythrocytes (red blood cells) [Yan et al., 2017].

Surface charge and size, the stability of nanoparticles in the biological environment and their ability to interact with cells are influenced by both their positive surface charge and size. The stability and biodistribution of LDHs can be enhanced by negatively charged lipid membrane coating, which lowers toxicity. Precipitation can also result from large particle sizes [Yan et al., 2014, 2017; J. Zhang et al., 2024].

Release of metal ions and induction of oxidative stress, metal ions (zinc, iron, copper) liberated from LDHs can induce toxicity and provoke oxidative stress via the generation of ROS [Bundschuh et al., 2018; Kiani et al., 2022; Koba-Ucun et al., 2021].

Toxicological effects may result from LDH nanoparticles' ability to increase ROS generation and induce oxidative stress. Disruption to lipids, membranes, carbohydrates, and DNA can result from oxidative stress [Kiani et al., 2022; Koba-Ucun et al., 2021].

In the tumor microenvironment, which has a pH of 5.0 to 6.8, LDHs are able to dissolve and release their substance in a regulated way. The structural stability of LDH nanoparticles may alter at lower acidic levels, such as pH 4.5, which could have an impact on drug release. Low rates of dissolution occur at neutral pH [Kiani et al., 2022; N et al., 2023; Yan et al., 2017].

When nanoparticles come into direct contact with the cell surface, harmful reactions may result. One possible cause of toxicity, for instance, has been proposed to be the attachment or adsorption of LDHs to the outside surface of test organisms, such as daphnids (biological surface coating) [Bundschuh et al., 2018; Callaghan & MacCormack, 2017; Dabrunz et al., 2011; Koba-Ucun et al., 2021].

Even at low doses (30 mg/kg), repeated intravenous injections of naked LDH nanoparticles killed mice. Pulmonary embolism and lung deposition were linked to this [Yan et al., 2014, 2017]. It was discovered that naked LDHs seriously harmed the kidneys (increased creatinine levels) and liver cells. LDHs coated with lipid membranes have less of a negative impact on the kidney and liver [Yan et al., 2017].

Low cytotoxicity in vitro is not equal to excellent biocompatibility in vivo, despite the fact that LDHs are typically reported to be highly biocompatible [Kura et al., 2014; Kwak et al., 2004; Yan et al., 2014, 2017].

Because of their customizable structure, high loading capacity, pH sensitivity, and general biocompatibility, LDHs are inorganic nanomaterials with significant potential in a variety of biomedical applications, including drug delivery, cancer therapy, and bone regeneration. However, for them to move into clinical applications, it is essential to control the risks of systemic toxicity that can result from things like aggregation using techniques like surface alterations [Clemons et al., 2019; Jing et al., 2022; Kura et al., 2014].

1.4.4. Impact of LDHs on Aquatic Organisms

The main mechanisms of nanomaterials, and therefore LDHs, can cause potential toxicity include direct interaction with the cell surface, dissolution of toxic elements from nanoparticles, and inducing oxidative stress through the production of ROS. Aquatic living things at various trophic levels may be toxically affected by LDH nanoparticles [Koba-Ucun et al., 2021].

On microalgae (*Scenedesmus quadricauda*, *Pseudokirchneriella subcapitata*), LDHs have the potential to severely impede growth. This could be explained by the breakdown of microalgal barriers, oxidative stress, or the release of metal ions [Aruoja et al., 2009; Déniel et al., 2019; Ding et al., 2018; Franklin et al., 2007; Koba-Ucun et al., 2021].

LDHs have the potential to block bioluminescence of *Vibrio fischeri* bacteria. Metal ion release and preparation time affect toxicity [F. Chen et al., 2020; Koba-Ucun et al., 2021; Kurvet et al., 2017; Rossetto et al., 2014]. Freshwater crustaceans (*Daphnia magna*), depending on the dosage and duration of exposure of LDH, freshwater crustaceans (*D. magna*) can either kill or immobilize a person indirectly. Toxicological effects may also be caused by physical factors, such as particles adhering to daphnid antennae [Koba-Ucun et al., 2021]. Aquatic plants, such as duckweed (*Spirodela polyrhiza*), can block growth and alter their morphology, giving their leaves a pale yellow colour [Koba-Ucun et al., 2021; Mkandawire et al., 2014].

1.4.5. About *Chlorella vulgaris*

C. vulgaris is a freshwater microalgae that is considered an ideal model organism for assessing the effects of nanoparticles in aquatic ecosystems. It has a wide distribution and is widely used in toxicological assessments, making it an important biological indicator of water quality. Its low nutrient requirements, easy processing and the ability to perform a full life cycle assessment in a short time increase the suitability of this species [Saxena et al., 2021].

1.4.6. About *Cyclotella cryptica*

About half of the oceans' primary production is produced by the diatoms, a significant and varied group of phytoplankton, which includes the marine microalgae species *C. cryptica* [L. Wang et al., 2023]. The frustule, the silicified cell wall of the *C. cryptica* species, is one of its most distinctive physical traits. These characteristics are crucial for the species' ecological and biotechnological functions [L. Wang et al., 2023; X. Wang et al., 2023].

The structure of the *C. cryptica* frustule is cylindrical (**Figure 1.1**). This cylindrical frustule is made up of two circular valves, or shells: the hypotheca at the bottom and the epitheca at the top. Several overlapping girdle bands link these valves [L. Wang et al., 2023]. There is a center area and honeycomb striae (striations) on the valve's outer surface (distal surface). The striae are filled with pores the size of nanometers and are oriented radially. At the valve margin and, most of the time, in between the striae, there are numerous fultoportulae, which are tube-like structures. The middle region has one or two (rarely three) fultoportulae that are visible from both the inner and exterior surfaces [L. Wang et al., 2023; X. Wang et al., 2023]. Frustule is mainly composed of amorphous hydrated silica. It has a porous hierarchical structure. Morphological properties such as surface area, pore volume and pore diameter are critical factors affecting the biotechnological performance of the frustule (hemostatic activity) [L. Wang et al., 2023; X. Wang et al., 2023].

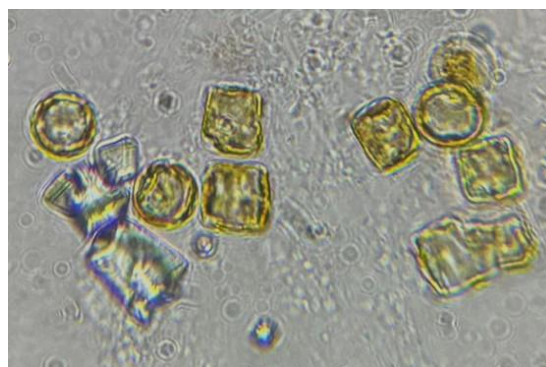


Figure 1.1: Light microscope image of *C. cryptica*, in 100x

No studies were found in the literature regarding the toxic effects of LDHs on marine algae.

1.5. Antioxidant Enzymes

One significant antioxidant enzyme that is essential to biological systems is catalase (CAT) [Samal et al., 2021; Sáringer et al., 2024]. In biological systems, superoxide dismutase (SOD), a crucial antioxidant enzyme, serves as the first line of defense for cells against oxidative damage brought on by ROS [Engelbrecht et al., 2023; H. Zhao et al., 2021].

1.5.1. Catalase Enzyme

One of the antioxidant enzymes that provides the first line of defense against damage brought on by ROS is CAT, along with SOD [Engelbrecht et al., 2023; H. Zhao et al., 2021]. Its primary job is to chemically convert the highly toxic hydrogen peroxide (H_2O_2) into molecular oxygen (O_2) and water (H_2O). It can convert millions of H_2O_2 molecules each second, demonstrating its immense conversion rate. Thus, it plays a vital role in preserving cellular homeostasis and shielding cells from oxidative stress [Engelbrecht et al., 2023; Jabłońska-Trypuć et al., 2017; Samal et al., 2021; Sáringer et al., 2024; H. Zhang et al., 2019]. The 232 kDa protein known as catalase has four heme groups that carry iron. Its interaction with its substrate, H_2O_2 , is facilitated by these heme groups. The detoxification of H_2O_2 also involves the heat-stable manganese catalase [Ballal et al., 2020; Chakravarty et al., 2019; Samal et al., 2021].

1.5.2. Clinical Significance of CAT

Numerous illnesses, such as cancer, Parkinson's disease, Alzheimer's disease, hypertension, and malaria, are linked to disruptions or decreases in catalase activity. [Escribano et al., 2015; Góth et al., 2004; Hepel & Andreescu, 2015; Samal et al., 2021]. Conversely, mice with mitochondrial catalase can have a 20% longer lifespan when CAT is overexpressed. The pathophysiology or advancement of several human diseases, including bronchial asthma, systemic sclerosis, and Alzheimer's disease, has been linked to the overproduction of ROS, which can heighten inflammatory responses [Bhatt et al., 2021; Doridot et al., 2019; Engelbrecht et al., 2023; Samal et al., 2021; Schriener et al., 2005; Vasconcelos et al., 2021].

CAT is an essential antioxidant with a variety of biological and industrial uses that shields cells from oxidative stress by detoxifying H_2O_2 . Numerous factors can impact its stability and activity, although tactics like immobilization with nanoparticles can enhance its performance [Engelbrecht et al., 2023; Galante & Formantici, 2005; Kaushal et al., 2018; Samal et al., 2021; Sáringer et al., 2024].

1.5.3. Function of CAT in Microalgae

In ROS detoxification, CAT and SOD are two antioxidant enzymes that work together as the body's first line of defense to remove ROS from cells [Chance, 1948; Engelbrecht et al., 2023; McCord & Fridovich, 1969]. The figure (Figure 1.2) shows a schematic that highlight the important steps in cellular ROS detoxification [Tripathi & Poluri, 2021]. Overproduction of ROS can cause oxidation of proteins, lipid peroxidation, and damage to nucleic acids, among other cellular problems [Bolduc et al., 2019; Hepel & Andreescu, 2015; Samal et al., 2021; H. Zhang et al., 2019]. By specifically turning H_2O_2 into water and molecular oxygen, catalase protects cells from its damaging effects. This is an essential role in preserving cellular homeostasis [Engelbrecht et al., 2023; Samal et al., 2021].

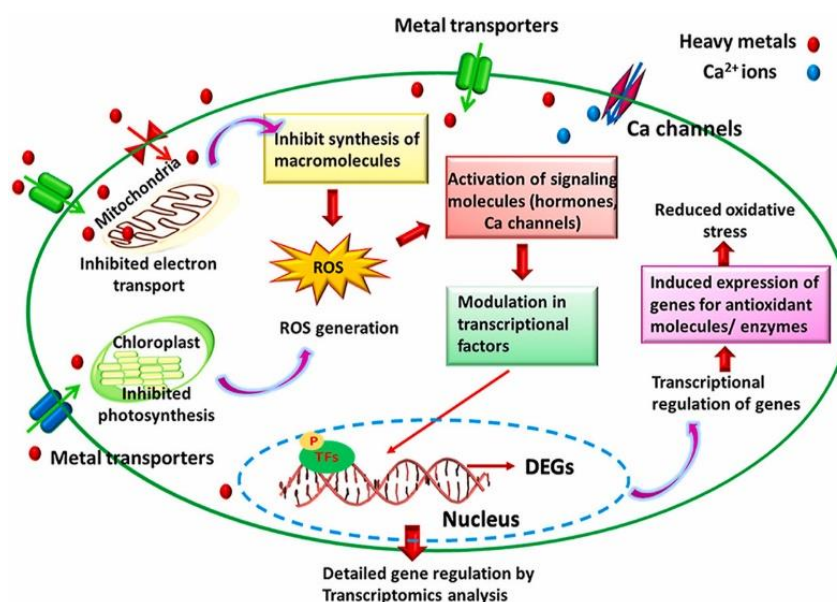


Figure 1.2: Diagram showing the several phases of cellular processes that detoxify ROS caused by heavy metals [Tripathi & Poluri, 2021].

Oxidative stress response, when microalgae are exposed to environmental contaminants including FeO₃ and ZnO nanoparticles, they undergo oxidative stress [Saxena et al., 2021]. Antioxidant enzymes like CAT and SOD become more active as a result, suggesting that the algae are under physiological stress. *C. vulgaris* treated to ZnO and FeO₃ nanoparticles exhibits dose-dependent increases in CAT activity, based on sources [Melegari et al., 2013; Saxena et al., 2021; Suman et al., 2015].

Despite the high CAT activity seen in algae, it was shown that this enzymatic defense system was insufficient to handle the oxidative stress that occurred when high dosages of nanoparticles (ZnO and FeO₃) were administered. Growth retardation, a reduction in chlorophyll content, and damage to the integrity of the cell membrane are among the harmful consequences of this deficiency [Melegari et al., 2013; Saxena et al., 2021; Ševců et al., 2011].

Variations in CAT activity can be used as a biomarker to evaluate the physiological state of stressed microalgae exposed to environmental pollutants such as nanoparticles [Saxena et al., 2021].

Briefly, the enzyme CAT plays a crucial role in *C. vulgaris* microalgae's natural defense mechanism against environmental stressors. By using ROS detoxification to safeguard cellular integrity, it may not be enough to protect the algae from harmful effects when pollutant concentrations are high [Saxena et al., 2021; Suman et al., 2015].

1.5.4. Superoxide Dismutase Enzyme

Primary job of SOD is to catalyze the creation of less toxic H₂O₂ and O₂ from the extremely dangerous superoxide radicals that are produced during aerobic metabolism or environmental interactions in cells. To avoid oxidative stress, this is an essential step. Together with CAT, this enzyme serves a crucial role in ROS detoxification; SOD transforms superoxide into H₂O₂, which CAT then breaks down into oxygen and water [Chance, 1948; Engelbrecht et al., 2023; McCord & Fridovich, 1969].

1.5.5. Significance of SOD

Overproduction of ROS can cause oxidative damage to proteins, lipids, and nucleic acids, among other cellular problems. SOD lowers these negative consequences,

assisting in the maintenance of cellular homeostasis [Engelbrecht et al., 2023; Jabłońska-Trypuć et al., 2017; H. Zhao et al., 2021].

FeO₃ and ZnO nanoparticles cause oxidative stress in microalgae like *C. vulgaris*. An increase in SOD activity is shown in this study, which is a defensive reaction that shows the algae are under physiological stress. Despite the high SOD activity seen in algal cells, it has been claimed that this enzyme defense mechanism is unable to deal with ROS at larger concentrations of nanoparticles [Bhaduri & Fulekar, 2012; Del Río et al., 2006; Saxena et al., 2021]. Environmental toxins such as lead, arsenic and mercury can significantly modulate the kinetic behavior of hepatic SOD by displacing essential metals [Kumar et al., 2020].

1.6. Significance of Pigments on Algal Cells

Pigments such as chlorophylls and carotenoids are important components of photosynthetic biomembranes in algae. Chlorophyll content is the basis of the photosynthetic mechanism of algae and is directly related to algal growth and development. Photosynthesis is an important process for energy production and organic matter synthesis in algae. A decrease in chlorophyll content indicates that microalgae are in a stressed physiological state and are experiencing growth inhibition. Algae are keystone organisms located at the lowest trophic level of aquatic ecosystems and are the main source of biomass production for organisms at higher trophic levels. Therefore, any changes in algae can negatively affect the structure and function of the entire marine ecosystem [Qiu et al., 2022; Saxena et al., 2021].

2. PURPOSE OF THE STUDY

The primary purpose of this study is to evaluate the potential toxic effects of newly synthesized Zinc-Iron-Aluminum (ZnFeAl) nanoparticles on freshwater and marine algae species. With the increasing application of nanoparticles in various industrial and environmental fields, understanding their ecological impact has become crucial. Algae, as primary producers in aquatic ecosystems, play a fundamental role in sustaining ecological balance; thus, any disruption to their growth or function can have cascading effects throughout the food chain. By comparing the responses of both freshwater and marine algae to ZnFeAl nanoparticles, this research aims to provide insight into nanoparticle-induced toxicity to environment and living things at the cell level, identify concentration-dependent effects, and contribute to the development of environmental risk assessments related to nanomaterial pollution.

3. EXPERIMENT

This study investigates and compares the acute toxicity of newly synthesized ZnFeAl LDH on two microalgal species from distinct aquatic environments. Freshwater microalgae *C. vulgaris* and marine diatom *C. cryptica* were cultivated with different concentrations of ZnFeAl (0, 10, 25, 50, 100, 250 and 500 mg.L⁻¹) for 15 days. At the end of fifteen days of incubation at room temperature at 350 rpm under Philips master TL-D 58 W light using orbital shaker of Jeiotech. Morphological changes of algal cells, changes in chlorophyll and carotenoid content, changes in anthocyanin and phenolic content and antioxidant enzyme activities which are SOD and CAT were examined.

3.1. Materials

Ethylenediaminetetraacetic acid (99.4%), Folin-Ciocalteu's phenol reagent (2 N), sodium carbonate (Na₂CO₃, ≥99.9 %), hydrochloric acid (37%), gallic acid monohydrate (C₇H₆O₅·H₂O, ≥98.0 %), methanol (gradient grade), acetone (100%), nitroblue tetrazolium (C₄₀H₃₀Cl₂N₁₀O₆, ≥90.0 %), riboflavin (C₁₇H₂₀N₄O₆, ≥98 %), and sodium hydroxide (NaOH, ≥97.0 %) were purchased from Sigma-Aldrich Chemie GmbH, Germany, L-methionine (C₅H₁₁NO₂S, 99.8 %) was purchased from Neofroxx GmbH, Germany.

3.2. Determination of Cell Growth

In 250 mL flasks, freshwater microalgae *C. vulgaris* SAG:211–12 and marine diatom *C. cryptica* CC 1070 were grown at room temperature in BG-11 and F/2 media, respectively. The Culture Collection of Algae (SAG), University of Göttingen, Germany, provided the *C. vulgaris* cells. The UTEX Culture Collection of Algae (UTEX #1269) is the original source of cultures of the marine centric diatom *C. cryptica*. They were separated by centrifugation once they reached the stationary phase of growth, and the resultant algae were used to inoculate media containing varying concentrations of ZnFeAl LDHs (0, 10, 25, 50, 100, 250, and 500 mg.L⁻¹). In all the mediums, about 10 × 10³ mL⁻¹ cells were inoculated. The growth of algae cells was

monitored by counting the cells in a hemocytometer on day 15 for each sample and measuring the optical density at 685 nm during the incubation period. The regression equation 1 and 2 are used for determination of cell number for the samples having only optic density values. Standard curves for cell count vs optical density was obtained for *C. cryptica*. The regression equation 3.1 was used for the *C. vulgaris* cell count in the hemocytometer [Erdoğan et al., 2024]. Regression equation 3.2 is obtained from the standard curve for *C. cryptica*.

The volume of each square on the hemocytometer is 10^{-4} cm^3 . 1 cm^3 equals 1 ml. Thus, the unit for cell count was 10^4 ml . The OD values measured over 15 days were substituted into the standard equation to calculate the cell count.

$$y = 1540.1 \times OD_{685} \quad (3.1)$$

$$y = 210.49 \times OD_{685} \quad (3.2)$$

y: cell number in 10^{-4} mL medium

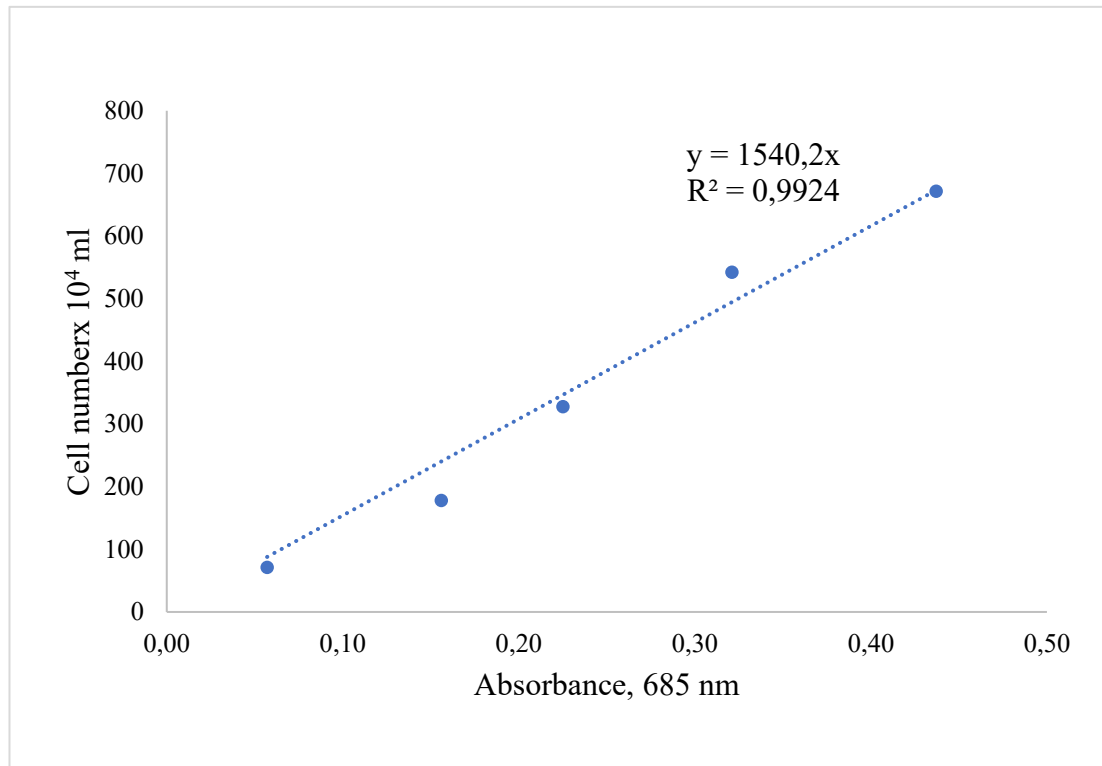


Figure 3.1: Standard curve of number of cells versus absorbance for *C. vulgaris*

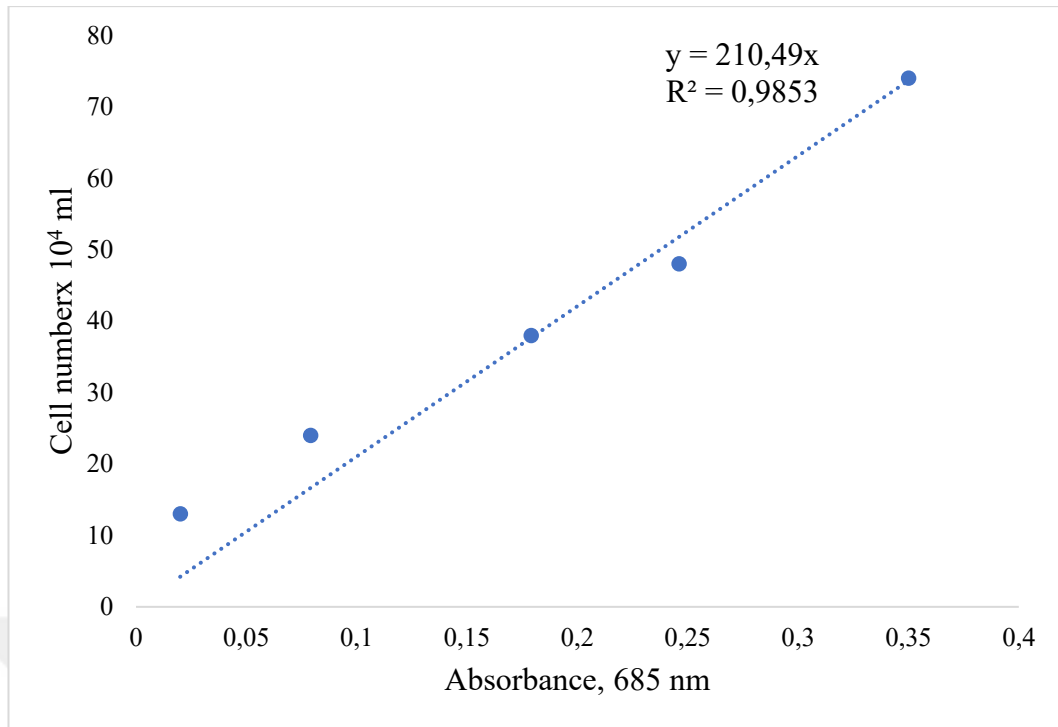


Figure 3.2: Standard curve of number of cells versus absorbance *C. cryptica*

To calculate the fresh weight, after 15 days, harvested cells were divided into four eppendorf tubes, each containing 500 μ l PBS. Because of the number of tests to be performed, the tubes were divided into four. These eppendorf tubes were left uncovered for one day at 36°C to allow the liquid to evaporate. The fresh weight was calculated by subtracting the previously measured empty eppendorf weight.

3.3. Morphological Analysis of Cells

Utilizing an optical microscope (Kern Optics, OBE 112, Kern&Sohn GmbH, Germany), the physiological impacts of LDHs on algae cells were observed. The algal cells were centrifuged and resuspended in a 4% glutaraldehyde solution for scanning electron microscopy (SEM) examination (Philips XL30 SFEG, Amsterdam) following a 15-day exposure to LDHs. Before the study, the cells were laid out on aluminum foil and allowed to dry overnight.

3.4. Determination of Chlorophyll and Carotenoid Content

The chlorophyll and carotenoid contents in *C. vulgaris* and *C. cryptica* cells were measured following the method [Lichtenthaler, 1987]. The cells were harvested and sonicated in 100% acetone for 20 minutes at 20°C in the dark and then they were centrifuged at 10,000 x g for 10 minutes at 4°C. The resulting supernatants were collected, and their optical densities were measured. Using equations 3.3–3.6, the amounts of carotenoids and chlorophyll were determined. The results obtained from the equations were divided into fresh weights.

$$C_a = 11.24 \times A_{661.6} - 2.04 \times A_{644.8} \quad (3.3)$$

$$C_b = 20.13 \times A_{644.8} - 4.19 \times A_{661.6} \quad (3.4)$$

$$C_{a+b} = 7.05 \times A_{661.6} - 18.09 \times A_{644.8} \quad (3.5)$$

$$C_{x+c} = \frac{1000 \times A_{470} - 1.90 \times C_a - 63.14 \times C_b}{214} \quad (3.6)$$

C_a = Chlorophyll-a, C_b = Chlorophyll-b, C_{a+b} =Total Chlorophyll, and C_{x+c} = Carotenoid concentrations.

3.5. Determination of Anthocyanin and Phenolic Content

Fresh algal samples were used to determine their anthocyanin content using spectrophotometry. After 14 days incubation, from the harvested cells, to extract the anthocyanin, the cells were combined with a 0.1% HCl solution and methanol that had been pre-cooled to -20°C. They were then maintained at 2°C for 24 hours. The initial optical density of 532 nm was found to have the highest anthocyanin absorption. By subtracting the maximal absorption value of chlorophylls ($0.24 \times \text{Abs}_{653}$), anthocyanin content was determined [Murray and Hackett, 1991]. The results obtained from the equation was divided into fresh weights.

To extract the phenolic content, algal pellets were treated with cold methanol and 0.1% HCl (-20°C) and kept at 2°C for 24 hours [van Alstyne, 1995]. 100 µL of Folin Ciocalteu reagent, 2.5 mL of dH₂O, and 100 µL of cell extract were combined. Following a 6-minute incubation period at room temperature, 150 µL of 20% w/v Na₂CO₃ was added, and the mixture was maintained at room temperature for 30 minutes. After measuring absorbance at 760 nm, the phenolic content per milligram of fresh algal mass was determined using a standard curve of gallic acid versus absorbance (**Figure 3.3**). The results obtained from the Figure 3.3 equation was divided into fresh weights.

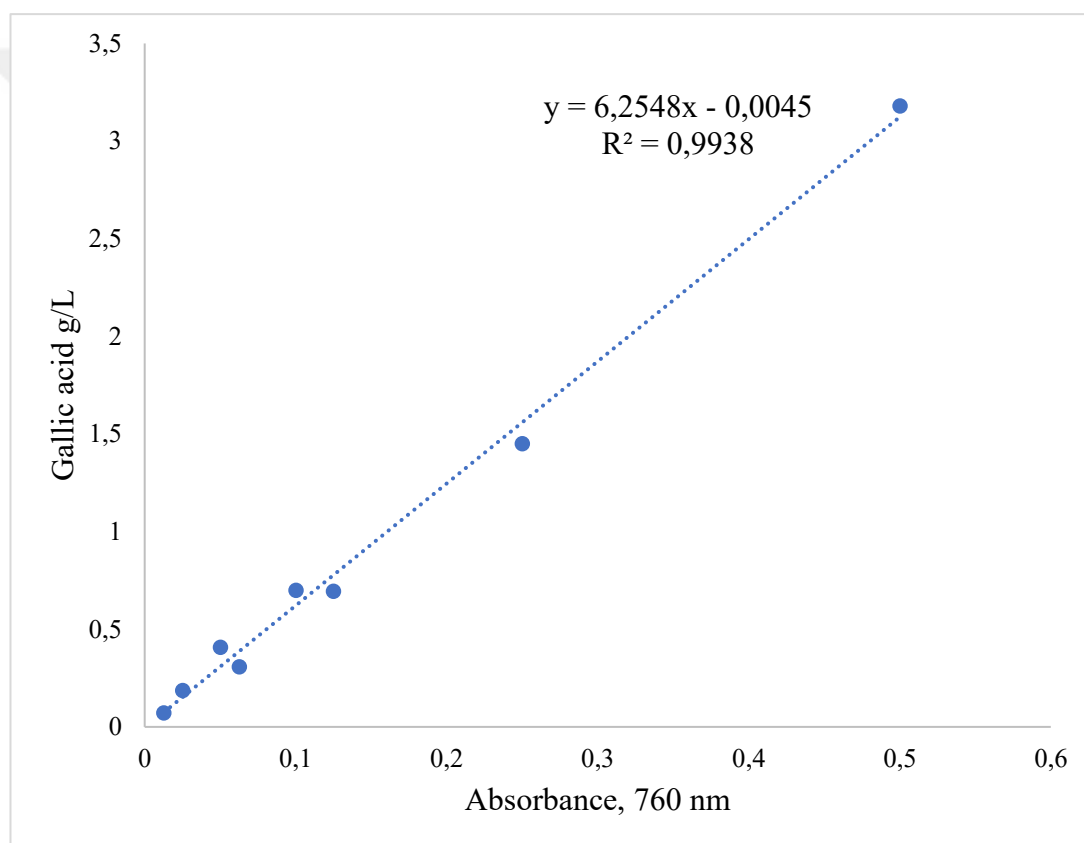


Figure 3.3: Standard curve of gallic acid versus absorbance

3.6. Determination of Protein Content

100 mL of algal cultures were centrifuged at 10,000 x g and 4°C for 10 minutes. Following centrifugation, the cell pellets were rinsed with phosphate-buffered saline (PBS). The pellets were resuspended in methanol: dichloromethane (2:1, v:v) and

sonicated for 60 minutes at 4°C in order to remove the proteins from the cells [Ding et al., 2018]. The concentration of total protein was determined using the Bradford test [Bradford, 1976]. As a standard, BSA solutions were prepared in different concentrations. After that, 200 µL of Bradford reagent was added to the standard BSA solutions or algal extracts until the total volume of the mixture reached 800 µL. After a dark incubation period of 10 minutes, the absorbance at 595 nm was measured. Protein concentrations in the samples were interpolated using the BSA standard curve (Figure 3.4).

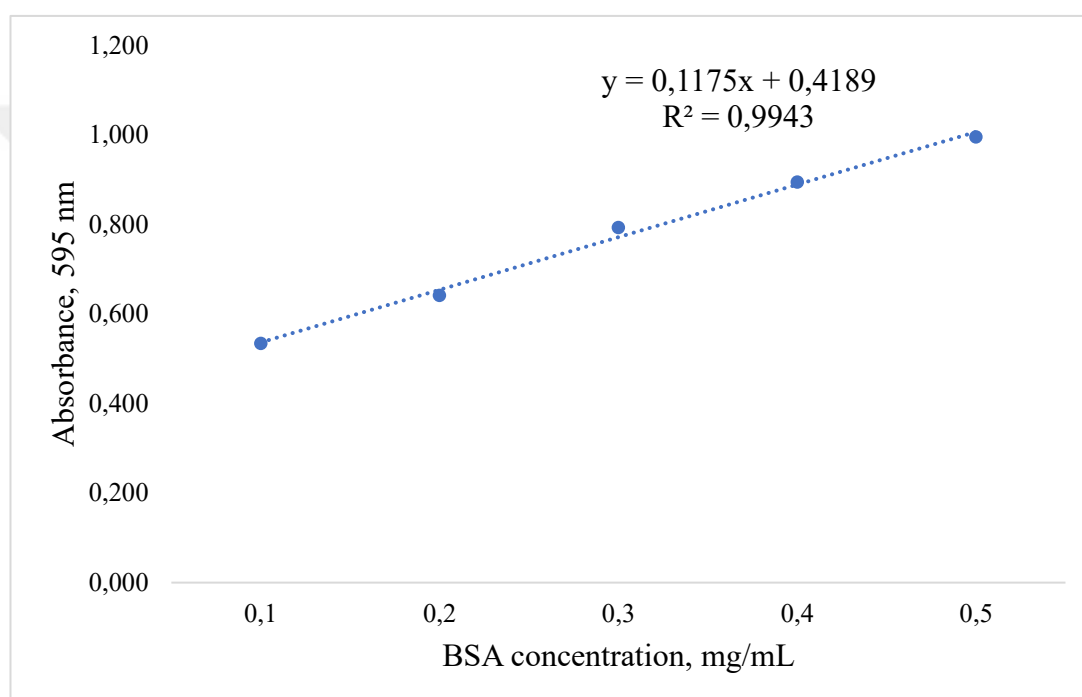


Figure 3.4: Standard curve of BSA

3.7. Determination of Antioxidant Enzyme Activities

SOD activity was measured using the method by [Beauchamp & Fridovich, 1971]. The suppression of nitroblue tetrazolium's (NBT) photochemical reduction is the fundamental idea behind this method. Basically, 20 µL of cell extract was combined with 1 mL of SOD reaction mixture (40 mM phosphate buffer; pH 7.8, 13 mM methionine, 75 µM NBT, 2 µM riboflavin, and 0.1 mM EDTA), and the combination was incubated for 15 minutes under a 20 W light source. At 560 nm, absorbance was

measured. NBT method for SOD assay is based on principle that NBT undergo photoreduction on exposure to light by superoxide radicals. NBT will produce less amount of coloured complex than control group. To find percent of inhibition of NBT, the treatment optical density value is subtracted from the control optical density value. Then divided to control optical density value and multiplies 100. 50% inhibition is equal to 1 unit of enzyme. Accordingly, the number of units of inhibition is found. This unit is from 20 μ L cell extract. Considering that the total enzyme extract volume was 1000 μ l, for total SOD activity the value is multiplies with 1000. SOD activity is found by dividing by fresh weight.

To determine the CAT activity description was followed [Aebi, 1984]. 0.5 mL of 30 mM H₂O₂ was combined with 1 mL of the cell extract. Enzyme activity was calculated using the recorded change in absorbance at 240 nm over 30 s. Equation 3.7 was used to determine the CAT activity.

$$\text{Catalase activity (mU/mg protein)} = 2.3 * \frac{-\log(\text{OD})}{\Delta t} * df * \frac{1}{[\text{protein}]} \quad (3.7)$$

Δt = the time (s), df = dilution factor of H₂O₂ in the mixture, $[\text{protein}]$ = protein concentration (mg.mL⁻¹)

3.8. Statistical Analysis

The data are presented as means $\pm$ the standard deviation (SD), and all decisions were made in triplicate. Using Microsoft Excel, the results of antioxidant enzyme activity were compared using an independent unpaired t-test. The p-value ($p < 0.05$) was used to assess the experiments' statistical significance. Tukey's Honestly Significant Difference (HSD) was applied to calculate significant ANOVA results ($p < 0.05$).

4. RESULTS

In this study, ZnFeAl LDHs effect on cell growth, chlorophyll and carotenoid content and antioxidant enzyme activity results were mentioned. This new LDH was synthesized by Prof. Dr. Ali Reza Khataee from Chemical Engineering Department of Istanbul Technical University. The co-precipitation method was employed to synthesize ZnFeAl LDH with a Zn/(Fe + Al). Since this LDH is new one, its toxic effect on aquatic environment needs to be analyzed. In the present study, two different algae representing freshwater and marine environments were used for toxicity study to see the effect of species difference in toxic responses.

4.1. Effect of LDHs on Algal Cell Growth

C. cryptica and *C. vulgaris* were grown in F/2 and BG-11 media representing marine and freshwater environments. The maximum cell density for both algae was attained on 15th day of the incubation. *C. cryptica* grew slowly, and their numbers were lower than those of *C. vulgaris* at same concentrations of ZnFeAl LDHs. The addition of even the lowest concentration of ZnFeAl (10 mg.L^{-1}) inhibited the growth of *C. cryptica* by more than 50%. Compared to *C. vulgaris*, *C. cryptica* is observed to be more responsive to lower dosages of nanoparticles. The number of cells in the *C. vulgaris* culture gradually dropped as the LDH dose increased. *C. cryptica* was affected at even lowest concentrations of LDH (10 mg.L^{-1}). In *C. vulgaris*, the LDH impact was particularly noticeable after day 7 (**Figure 4.1**). The findings imply that *C. vulgaris* is more tolerant to increased ZnFeAl nanoparticles concentrations. Optical density measurements of the 500 mg.L^{-1} concentration were made, but accurate measurements could not be made due to the particle density of the LDH.

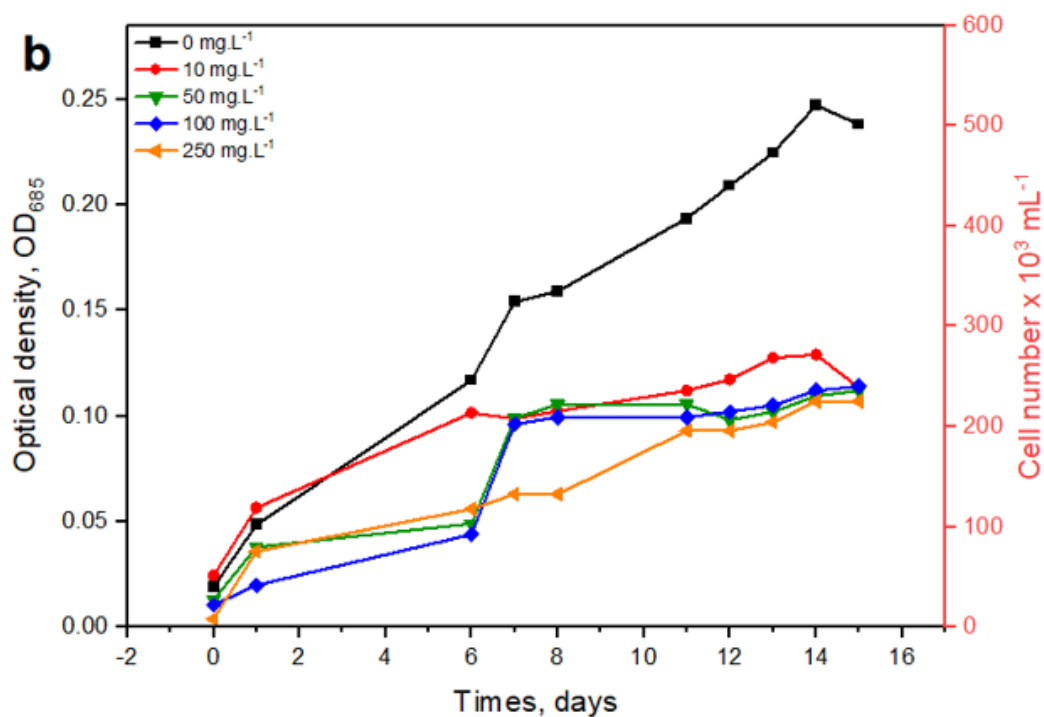
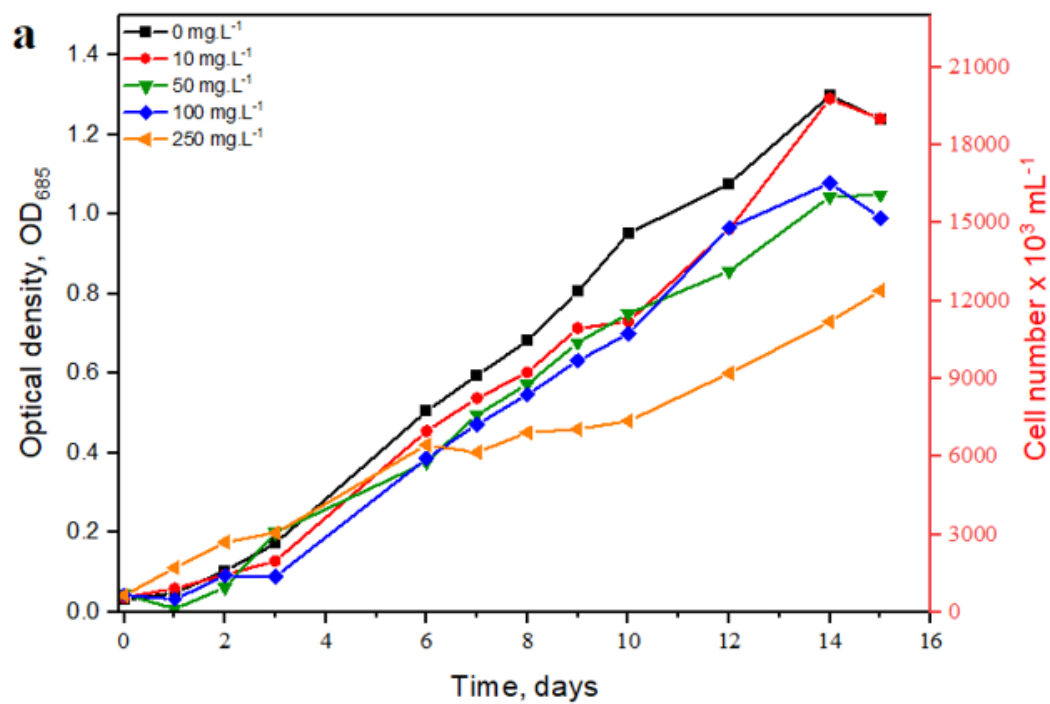


Figure 4.1: Effects of ZnFeAl NPs on *C. vulgaris* (a) and *C. cryptica* (b) growth exposed to 0-250 mg.L⁻¹ ZnFeAl LDH.

4.2. Effect of LDHs on Algal Cell Pigments and Antioxidants

In both algal species, the anthocyanin content decreased as the concentration of ZnFeAl LDHs increased. The high levels of anthocyanin at control (0 mg.L⁻¹ LDH) significantly dropped when ZnFeAl LDH was included in the culture media of both of the algae. The results clearly reveal the suppressive effect of LDH on algal cells (**Figure 4.2a, Figure 4.3a**). *C. cryptica* was affected more remarkably at the lowest ZnFeAl LDH concentration, supporting the findings on toxic effects on their growth.

The total chlorophyll (C_{total}) and carotenoid (C_{xc}) contents of algal cells in the presence of different dosages of LDHs for 15-day period are displayed in figures. (**Figure 4.2b, Figure 4.3b**). The pigment content of both algae gradually decreased as LDH concentration increased in the media. Higher LDH dosages caused a decrease in the amount of chlorophyll and carotenoid. The chlorophyll content of *C. vulgaris* cells is higher than *C. cryptica*. The total chlorophyll content of *C. vulgaris* showed sharp decrease when 10 mg.L⁻¹ LDH was added. This result shows that the presence of ZnFeAl LDH affects the chlorophyll content rather than cell growth for *C. vulgaris*.

Season, habitat, and other elements including light, salinity, nutrient availability, and UV irradiation all affect the amount of polyphenols [Cichoński & Chrzanowski, 2022]. The phenolic content of *C. vulgaris* changes in response to increased ZnFeAl LDH concentrations shown (**Figure 4.2c**). In the absence of ZnFeAl LDHs, the highest level of phenol was measured; at moderate amounts (25 and 50 mg.L⁻¹), it significantly decreased. However, phenolic content of *C. vulgaris* increased when the LDH dosage was raised to 250 mg.L⁻¹. Phenolic content of *C. cryptica* was found extremely low even in the absence of nanoparticles (0.199 µg.g⁻¹ FW) in the present study and could not be compared to that of *C. vulgaris*.

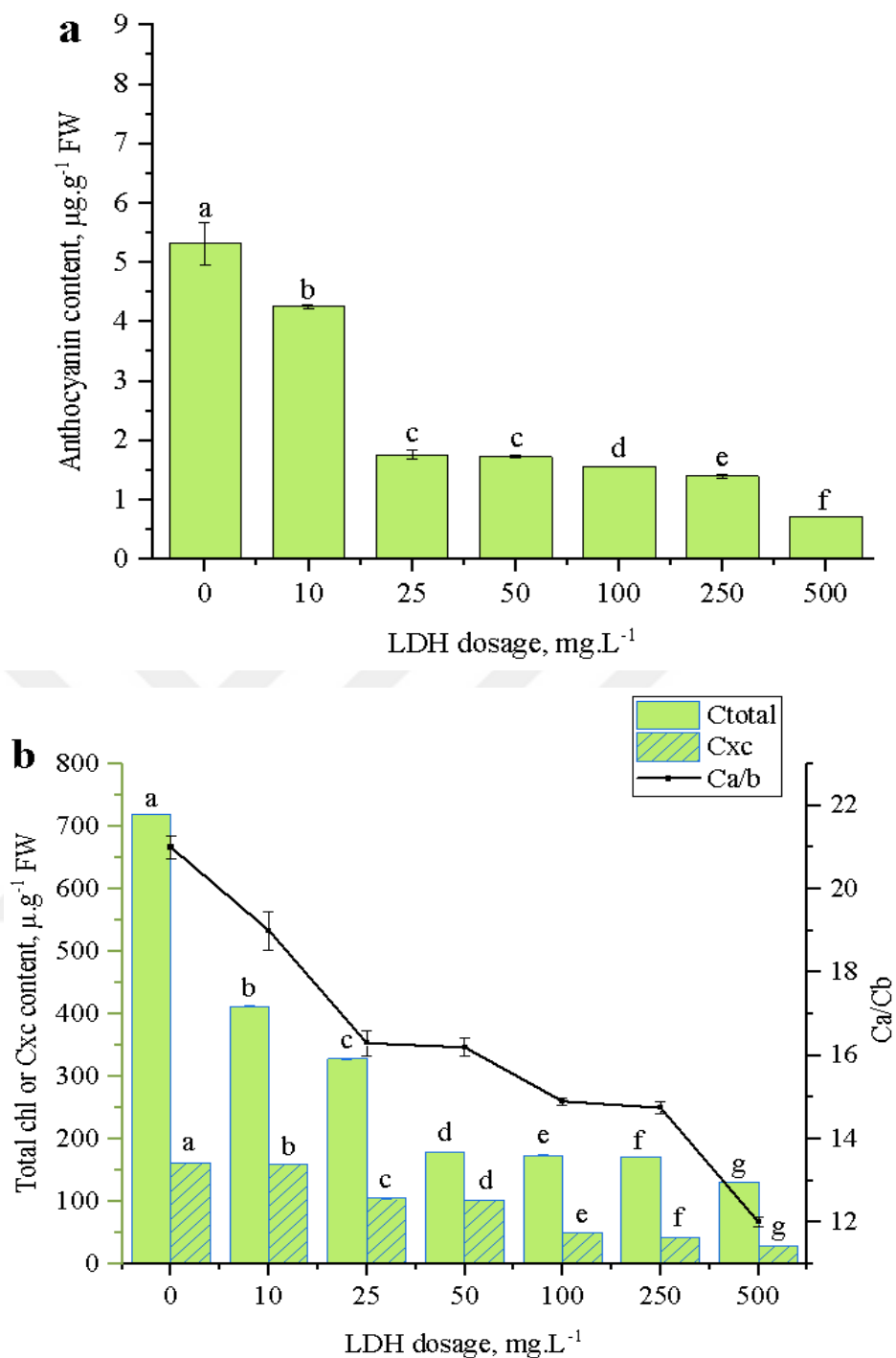


Figure 4.2: Anthocyanin content (a) of ZnFeAl LDHs exposed *C. vulgaris* cells. Effects of ZnFeAl LDHs on pigments (b) of *C. vulgaris*: chlorophyll (Ctotal), carotenoid (Cxc). Phenol content (c) of ZnFeAl LDHs exposed *C. vulgaris* cells. *ANOVA test was significant ($p < 0.05$) **Tuckey's HSD test is applied.

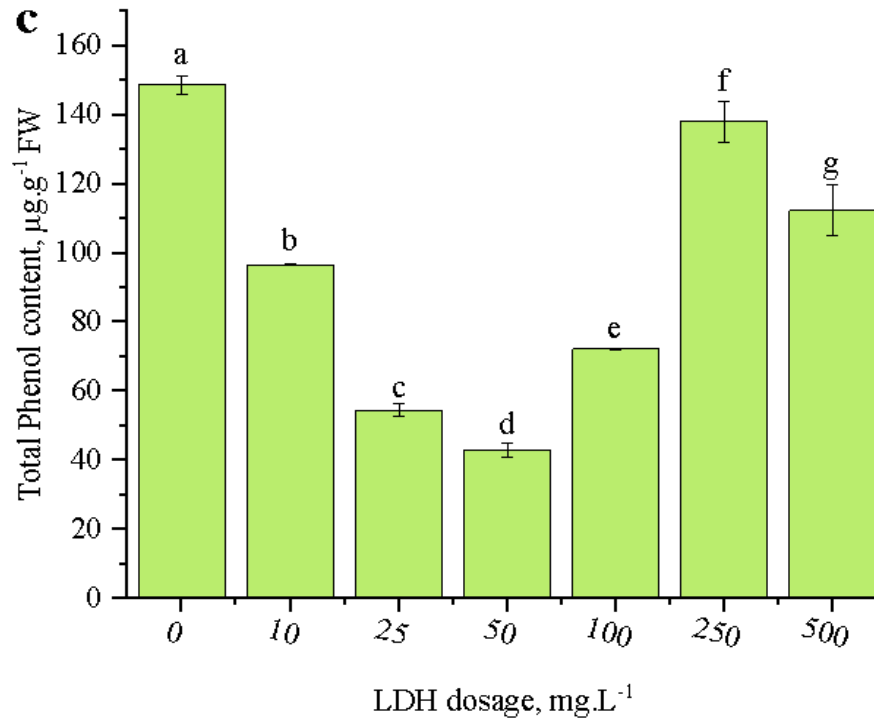


Figure 4.2 continued: Anthocyanin content (a) of ZnFeAl LDHs exposed *C. vulgaris* cells. Effects of ZnFeAl LDHs on pigments (b) of *C. vulgaris*: chlorophyll (C_{total}), carotenoid (C_{xc}). Phenol content (c) of ZnFeAl LDHs exposed *C. vulgaris* cells.*ANOVA test was significant ($p < 0.05$)**Tuckey's HSD test is applied.

The anthocyanin concentration of *C. vulgaris* was shown to be significantly affected by varying LDH dosages ($p < 0.05$). The anthocyanin content was highest in the control group (0 mg/L), which differed substantially from all other treatment groups. However, 100 mg/L, 250 mg/L and 500 mg/L dosages showed significant and meaningful decreases in anthocyanin content with increasing toxic effect. These results imply that algae lost color in response to increased ZnFeAl LDH dosages, which could be a sign of dose-dependent toxic stress (**Figure 4.2a**).

The C_{total} and C_{xc} value of *C. vulgaris* dropped significantly ($p < 0.05$). The chlorophyll (C_{total}) value is highest in the control LDH group. The C_{total} value of *C. vulgaris* statistically substantially drops when the LDH dose rises. For carotenoid content, the 0 mg.L⁻¹ LDH group had the highest (carotenoid) C_{xc} value. The C_{xc} value considerably drops as the LDH concentration rises. This shows that LDH has a suppressive effect on chlorophyll and carotenoid level depending on the dose (**Figure 4.2b**).

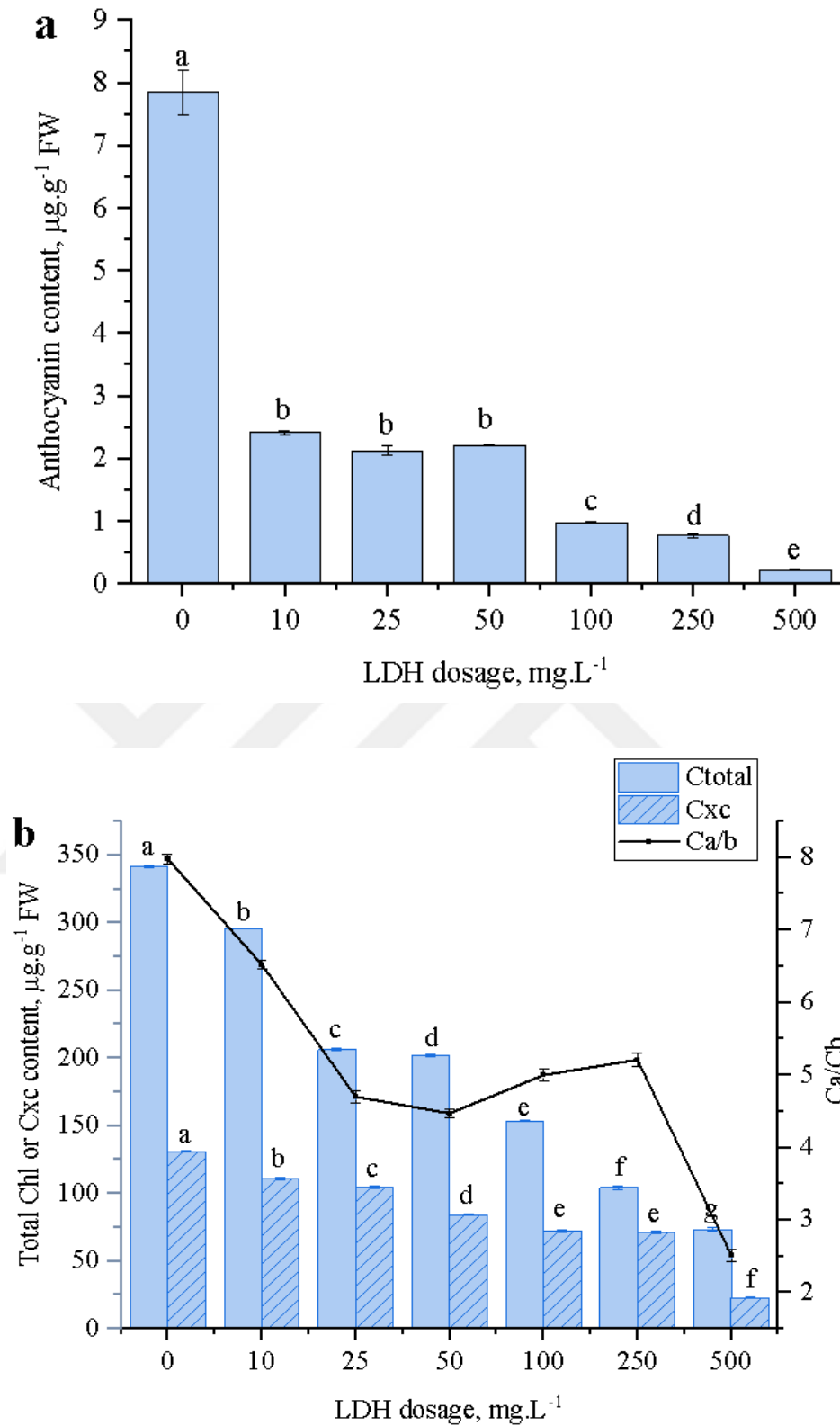


Figure 4.3: Anthocyanin content (a) of ZnFeAl LDHs exposed *C. cryptica* cells. Effects of ZnFeAl LDHs on pigments (b) of *C. cryptica*: chlorophyll (Ctotal), carotenoid (Cxc). *ANOVA test was significant ($p<0.05$) **Tuckey's HSD test is applied.

The figure (**Figure 4.3**) shows effect of LDH on anthocyanin and chlorophyll content of *C. cryptica*. The results are similar to that of *C. vulgaris*. It should be noted that phenol content of *C. cryptica* cells were negligible even in the absence of LDH. Anthocyanin content of all ZnFeAl LDH-treated groups and the control group of *C. cryptica* are significantly different ($p < 0.05$). Even very low levels of ZnFeAl LDH greatly inhibited the formation of anthocyanin. No statistically significant difference was observed between the 10, 25, and 50 mg/L dosages. These groups might be indicative of moderate suppression. Anthocyanin content decreased in groups of 100 mg/L and above. 250 and 500 mg/L groups show that the toxic effect of ZnFeAl LDH increased (**Figure 4.3a**).

The chlorophyll (C_{total}) and carotenoid (C_{xc}) of *C. cryptica* value dropped significantly ($p < 0.05$). This clearly points to an inhibitory impact is dose-dependent (**Figure 4.3b**). For C_{xc} , this figure provides statistical evidence that C_{xc} is successfully suppressed by rising ZnFeAl LDH concentrations (**Figure 4.3b**).

4.3. Morphological Effects of ZnFeAl on Algal Cells

Light microscopic image analyses showed the toxic effects of ZnFeAl nanoparticles on the algae species *C. vulgaris* and *C. cryptica*. Both species indicated dense and healthy cell populations in the 0 mg.L⁻¹, control group, but as nanoparticle concentrations increased, there was cell degradation and a loss of cell integrity (**Figure 4.4**). Algal cells showed extensive evidence of degradation, particularly at doses of 250 mg.L⁻¹ and 500 mg.L⁻¹ (**Figure 4.4**). *C. cryptica*, which was demonstrated to be more sensitive to ZnFeAl nanoparticles, experienced this effect more strongly than *C. vulgaris*, which had comparatively greater resistance up to higher doses. The results demonstrate that the harmful effect on algal cells rises with increasing nanoparticle concentration with species specific variations in this effect. Consequently, it was determined that ZnFeAl nanoparticles might be an ecotoxic risk factor in aquatic environments, and that *C. cryptica*, a species of diatom, might be a more sensitive biological indicator of these effects.

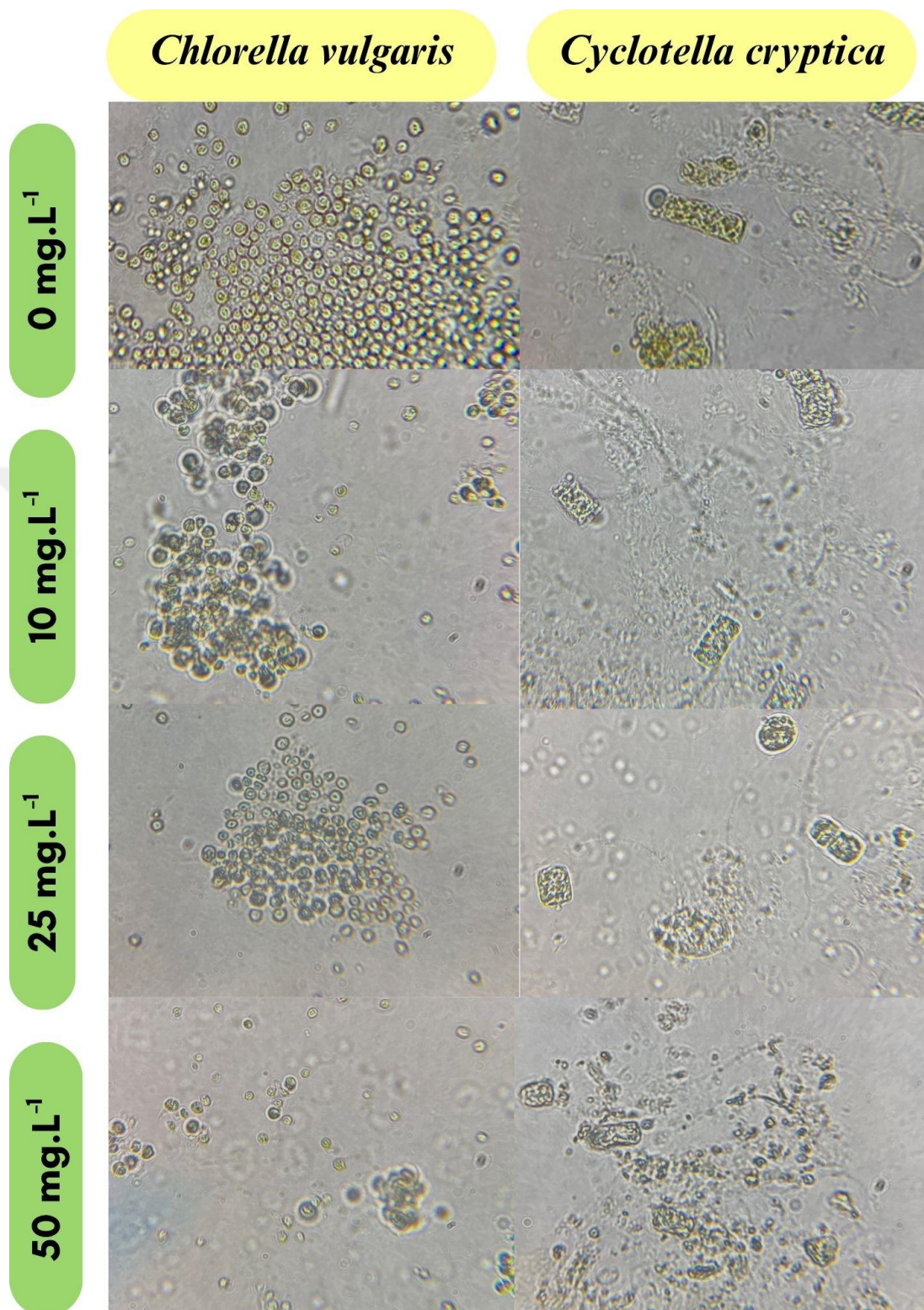


Figure 4.4: Light microscope images of ZnFeAl NPs exposed to *C. vulgaris* (left) and *C. cryptica* (right) in different dosage 0, 10, 25, 50, 100, 250, 500 mg.L⁻¹, in 100x.

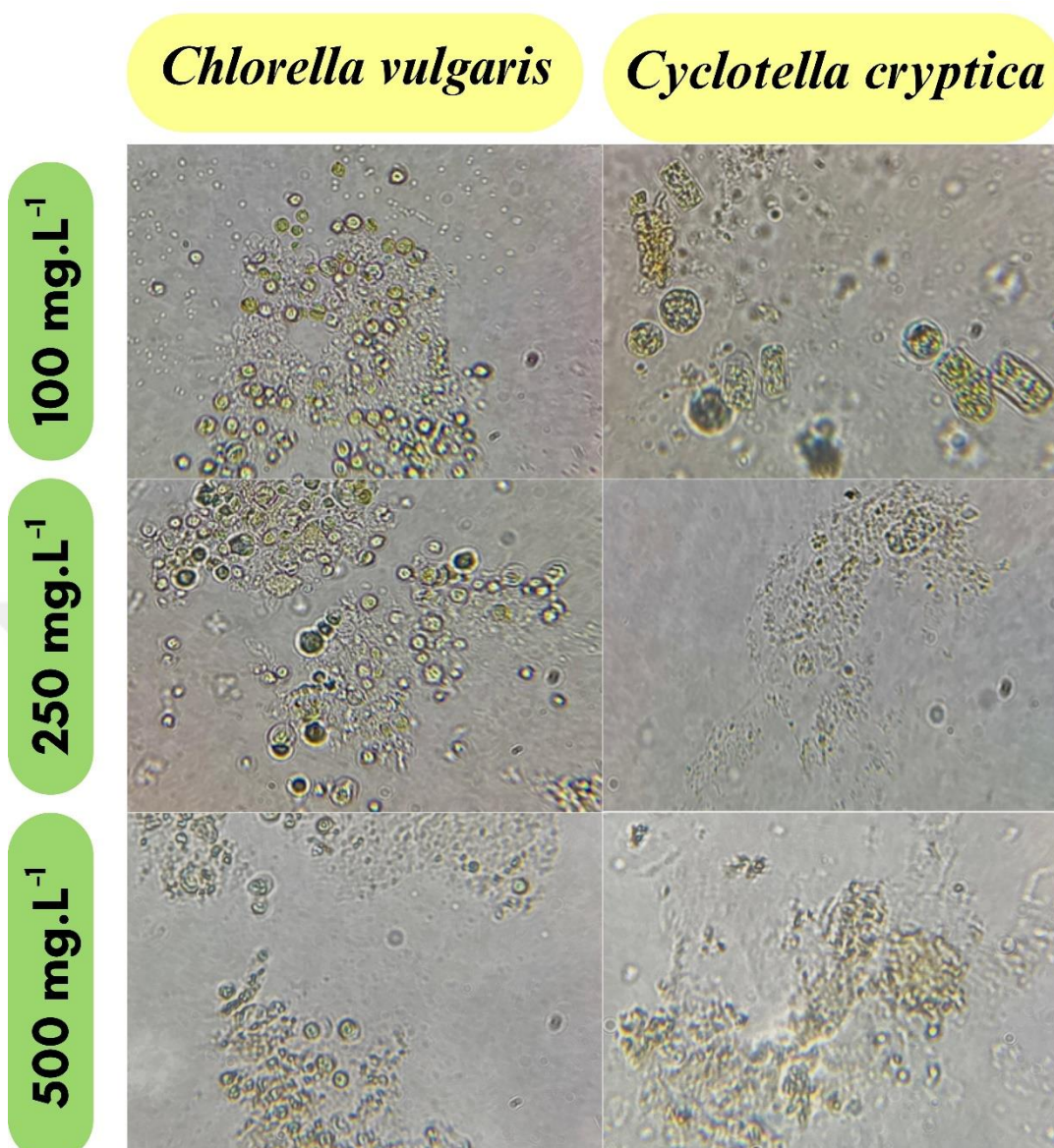


Figure 4.4 continued: Light microscope images of ZnFeAl NPs exposed to *C. vulgaris* (left) and *C. cryptica* (right) in different dosage 0, 10, 25, 50, 100, 250, 500 mg.L⁻¹, in 100x.

Following exposure to ZnFeAl LDH, the presence of NPs on freshwater and marine algae was confirmed by EDS analysis (Appendix A, B, C, D). The percentages of Zn, Fe, and Al elements increased in algae exposed to 250 mg.L⁻¹ LDH treatment, despite the fact that they were low in control tests. SEM pictures show that as LDH concentrations increased, *C. vulgaris* cells did not have structural damage as much as *C. cryptica*. Marine algae, *C. cryptica* exhibited notable structural deformation with exposure to a 250 mg.L⁻¹ concentration of ZnFeAl LDHs (**Figure 4.5**).

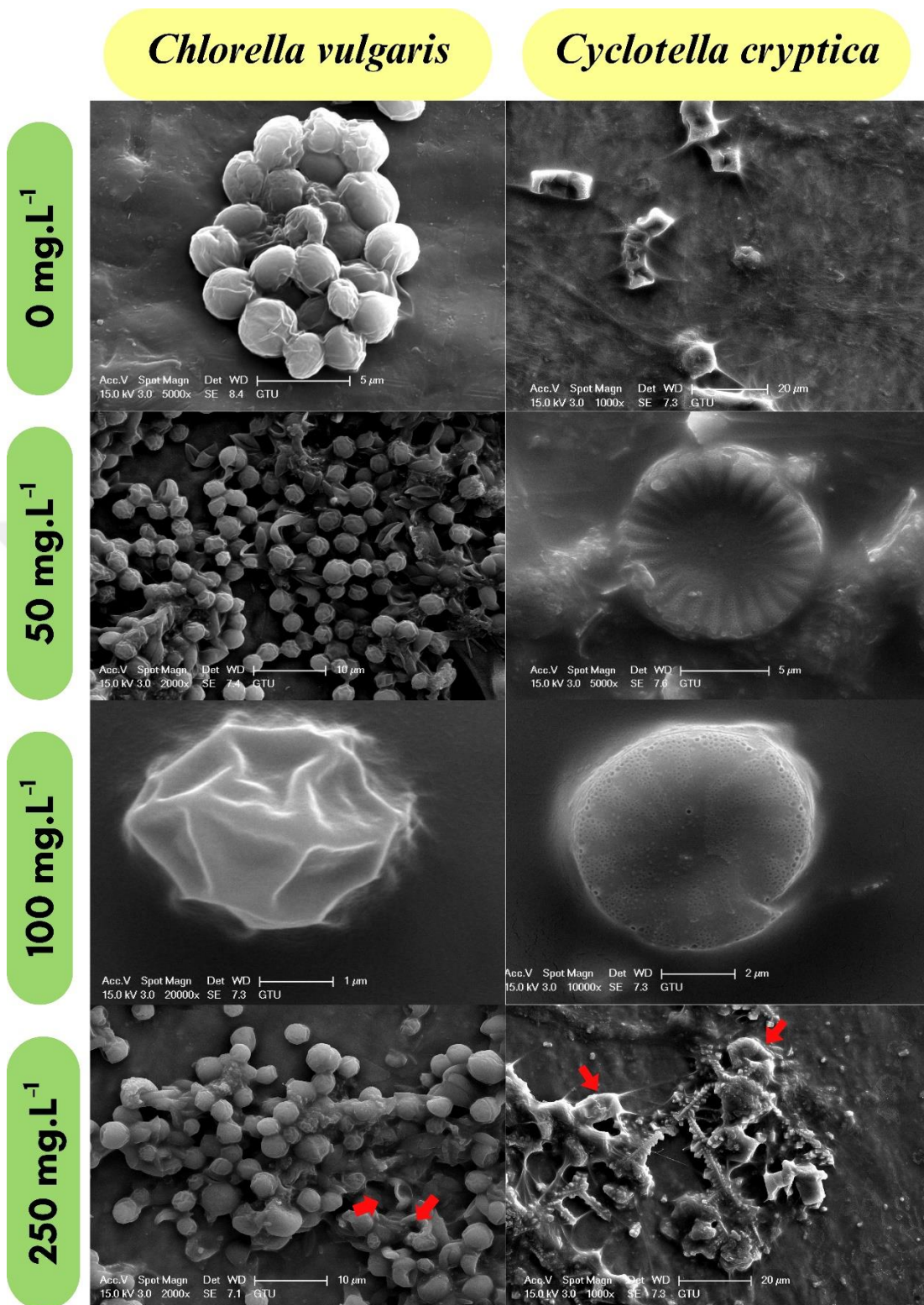


Figure 4.5: SEM images of ZnFeAl LDH treated *C. vulgaris* (left) and *C. cryptica* (right) cells. *concentration of ZnFeAl LDH increases from top to bottom.

4.4. Antioxidant Enzyme Activities

The figure (Figure 4.6) shows the SOD enzyme activity responses of *C. vulgaris* (a) and *C. cryptica* (b) exposure to varying concentrations of ZnFeAl LDHs. In *C. cryptica*, a sharp decrease in SOD activity was observed at lower concentrations (10 mg.L⁻¹) indicating possible enzyme inhibition or cellular damage at that concentration. However, SOD levels increased at 50 and 100 mg.L⁻¹ and then showed a small decline at higher concentrations (at 250 mg.L⁻¹). The results are parallel to the remarkable cell growth inhibition of *C. cryptica* in the presence of 10 mg.L⁻¹ ZnFeAl LDHs. The specific SOD activities of *C. vulgaris* are at least ten times higher than that of *C. cryptica*. *C. cryptica* may have very low SOD enzyme levels like their phenolic contents. For *C. vulgaris*, at 250 mg.L⁻¹, the maximum SOD activity was observed indicating toxic effect of that concentration to *C. vulgaris* (Figure 4.6a).

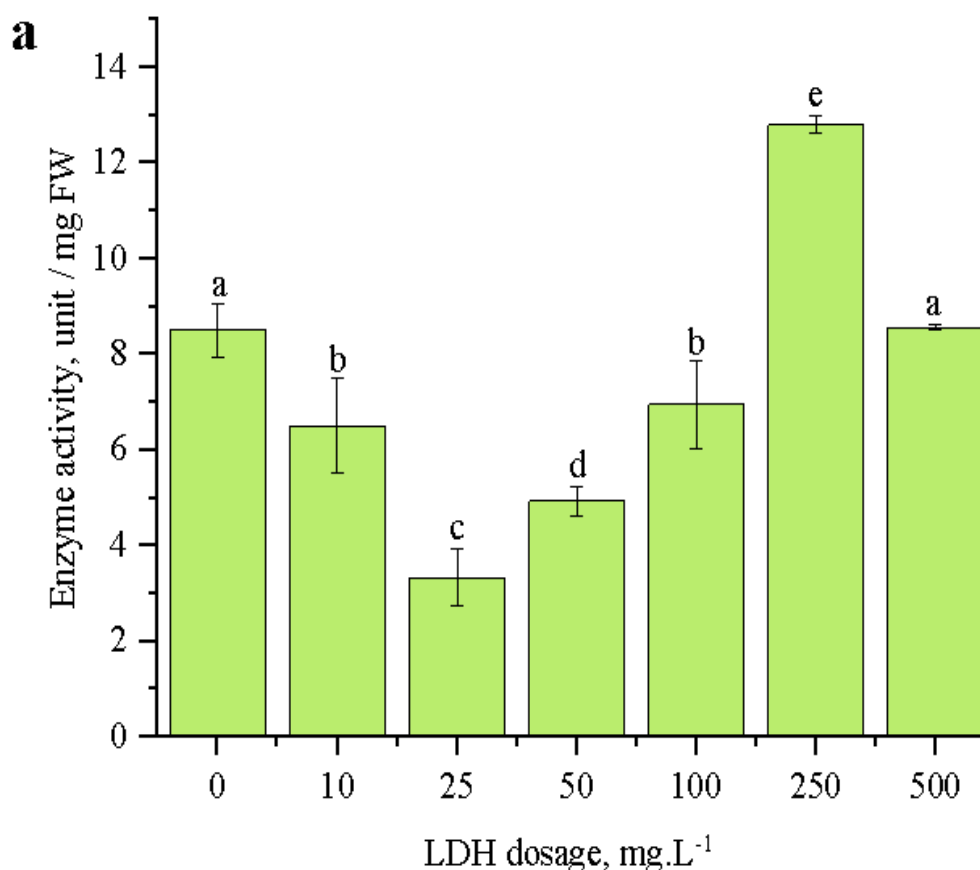


Figure 4.6: Antioxidative enzyme activities (SOD) of *C. vulgaris* (a) and *C. cryptica* (b) algal cells exposed to different dosages of ZnFeAl LDHs. *ANOVA test was significant ($p < 0.05$) **Tuckey's HSD test is applied.

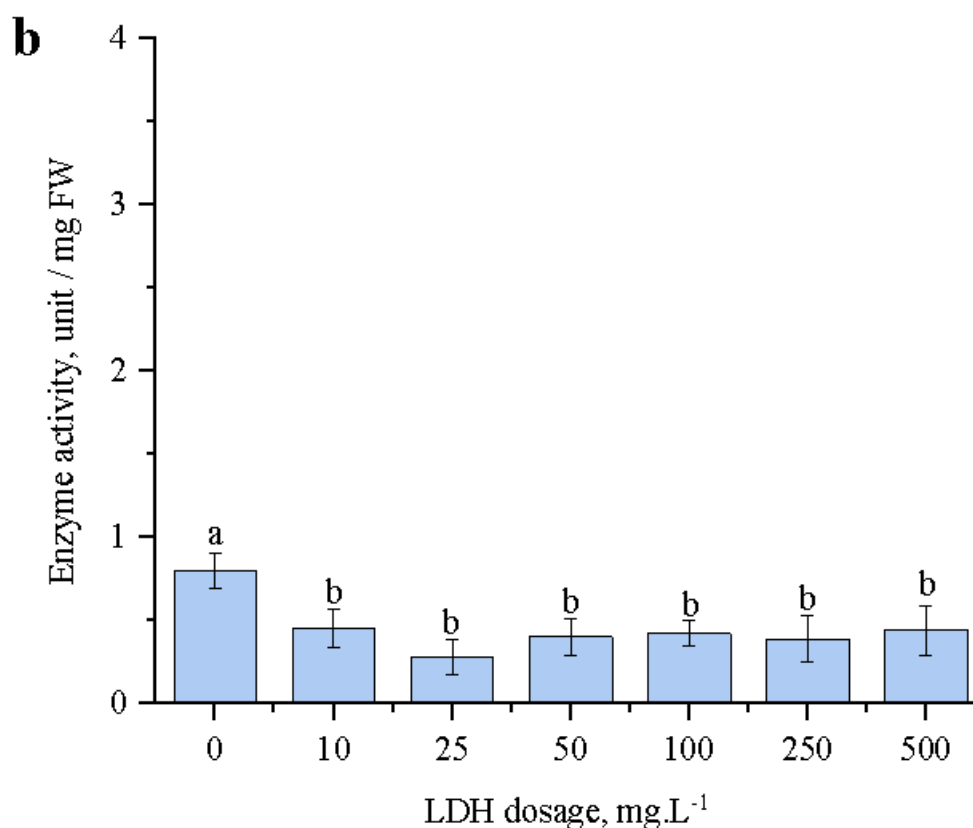


Figure 4.6 continued: Antioxidative enzyme activities (SOD) of *C. vulgaris* (a) and *C. cryptica* (b) exposed to different dosages of ZnFeAl LDHs. *ANOVA test was significant ($p < 0.05$) **Tuckey's HSD test is applied.

For *C. cryptica*, the SOD activity of control differs statistically from all other doses. There is no statistically significant difference between the other dosages of LDH (10–500 mg.L⁻¹). In this instance, LDH treatment decreased or maintained the SOD enzyme activity of *C. cryptica*, but the activity was not significantly affected by increasing the dose. The only group with noticeably higher enzyme activity than the others was the control group (**Figure 4.6b**).

The highest CAT activity was observed at 500 mg.L⁻¹ for *C. vulgaris* and 250 mg.L⁻¹ for *C. cryptica*. For *C. cryptica*, the difference in CAT activity is statistically insignificant (**Figure 4.7**). 500 mg.L⁻¹ LDH might affect the enzyme activity tests since high amount of particules adhere on the surface of the cells. Low cell density of *C. cryptica* affected its enzyme responses which did not change as remarkably as in *C. vulgaris*.

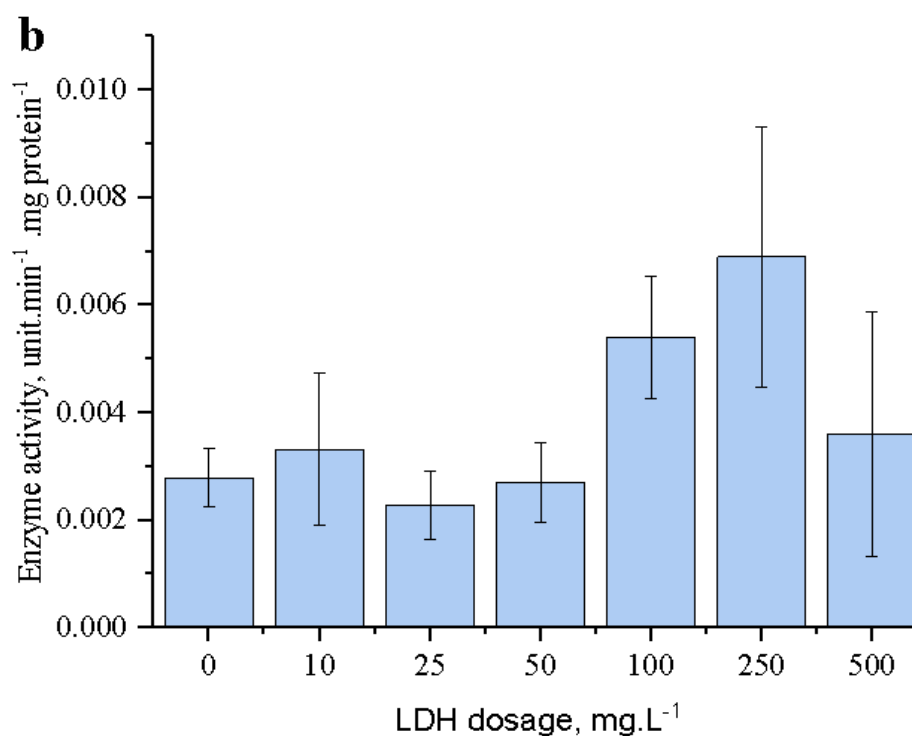
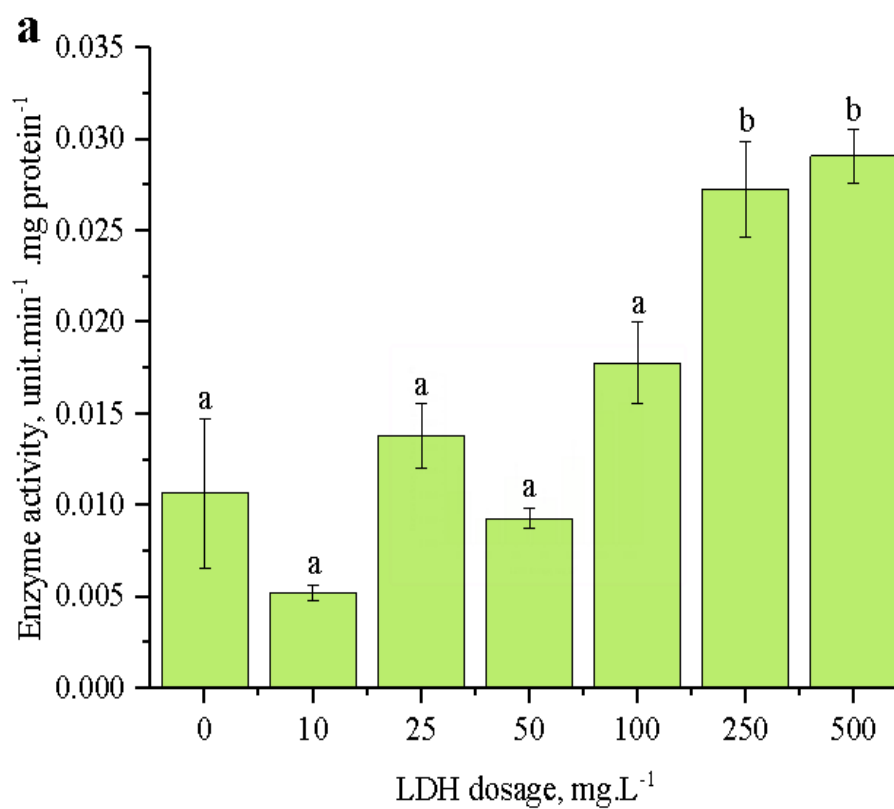


Figure 4.7: Antioxidative enzyme activities (CAT) of *C. vulgaris* (a) and *C. cryptica* (b) exposed to different dosages of ZnFeAl LDHs. * ANOVA test was significant ($p < 0.05$) (for a) **Tuckey's HSD test is applied to a

For *C. vulgaris*, according to Tuckey HSD test, there is no significant difference between 0, 10, 25, 50, and 100 mg.L⁻¹. It has been observed that CAT enzyme activity is increased by particularly high LDH dosages (**Figure 4.7a**) for both of the algae species. Since the *C. vulgaris* and *C. cryptica* were under stress at high concentrations of LDH, both SOD and CAT enzyme activity increased at higher doses.

Toxicity of ZnFeAl layered double hydroxide on different algae species was studied in the present study. It was shown that toxicity analysis on just one algae species might not be enough since other species may show different responses. Some aquatic species like *C. cryptica* are more vulnerable to toxic effect of nanoparticles. Results suggest that this novel ZnFeAl nanoparticle should not be released to aquatic environment more at higher concentrations to protect all the aquatic algal species.

5. DISCUSSION

In this study toxic effects of a newly synthesized ZnFeAl LDHs on *C. vulgaris* and *C. cryptica* representing freshwater and marine algae were studied. As toxic responses cell growth, pigment levels (chlorophyll, anthocyanin and phenols) and antioxidant enzyme activities (SOD, CAT) in response to increasing LDH concentrations were analyzed. For both of the algae, chlorophyll and anthocyanin content decreased, antioxidant enzyme activities increased at higher dosages of ZnFeAl LDHs. Microscopic examinations revealed that significant toxic effects such as decreased cell density, morphological deteriorations, and loss of cellular integrity occurred, especially at increasing concentrations. These results are consistent with the literature reporting that metal oxide nanoparticles, especially heavy metal-containing compounds such as zinc and iron, cause harmful effects on photosynthetic microorganisms by inducing oxidative stress, as outlined in detail in the following paragraphs.

The primary mechanisms of LDHs, may lead to potential toxicity involve direct interaction with the cell surface, release of toxic components from nanoparticles due to dissolution, and the induction of oxidative stress via ROS generation. LDH nanoparticles may have toxic effects on aquatic organisms across different trophic levels. Composite, SEM and transmission electron microscopy (TEM) analyses in the literature confirmed the accumulation of nanoparticles on the cell surface and the disintegration of the algal cell wall and organelles including chloroplasts [Long et al., 2012; Oliviero et al., 2017; Saxena et al., 2021; B. Xia et al., 2015].

Changes in the quantity of chlorophyll are one of the main signs of stress in photosynthetic organisms. The rate of photosynthesis and the growth of the organism are linked to the decrease in chlorophyll content [Songserm et al., 2024]. Several studies show that exposure to nanoparticles reduces the amount of chlorophyll in algae [Adochite & Andronic, 2021; X. Chen et al., 2018; Hazeem et al., 2019]. This drop can be attributed to mechanisms such as direct destruction of chlorophyll molecules by nanoparticles, suppression of chlorophyll synthesis, or oxidative stress in algal cells [Nguyen et al., 2020]. For example, as the concentration of Co NPs increased, the amount of chlorophyll in *Skeletonema costatum* decreased [X. Chen et al., 2018].

Similarly, another study reported that ZnO and TiO₂ nanoparticles gradually reduced the amount of chlorophyll-a in *Picochlorum sp.* [Hazeem et al., 2016].

For the *C. vulgaris*, organic three-dimensional layered double hydroxide (O3D-LDH) materials have demonstrated concentration-dependent toxicity. When O3D-LDH aggregated with microalgae, the chlorophyll level changed. It has been indicated that agglomeration of O3D-LDHs and microalgae affects the chlorophyll content and ultimately inhibits the growth of algae.[Qiu et al., 2022].

The toxicity of ZnFeAl LDHs in their nanoforms to *C. vulgaris* may also be linked to the presence of Zn and Fe ions in their structure. Algal chlorophyll content significantly decreased upon exposure to both ZnO and Fe₂O₃ nanoparticles, suggesting a disturbance in the photosynthetic process. Carotenoid content increased in algae exposed to ZnO and Fe₂O₃ nanoparticles. This increase suggests that algal cells utilize the antioxidant properties of carotenoids as a defense mechanism against metal stress [Saxena et al., 2021; Shariati et al., 2018].

Numerous biotic and abiotic stressors, including as low temperatures, salt, drought, malnutrition, pathogen assaults, and heavy metals, cause the production of anthocyanins [M. Kumar et al., 2020]. In the present study, anthocyanin content of both of the algae decreased with increasing concentrations of the nanoparticules, this might be caused by high toxicity of the nanoparticules on the cells. Peroxidation inhibition, metal chelation, and free radical scavenging are among the roles of phenols [Blokhina et al., 2003]. The phenolic content of the nanoparticles is larger at low concentrations than at intermediate ones, according to similar results from previous studies on the toxicity of CuFe LDH [Erdoğan et al., 2024] and CdSe nanoparticles [Movafeghi et al., 2019] to *C. vulgaris*.

Microalgae experience oxidative stress when they come into contact with environmental pollutants [Saxena et al., 2021]. As a result, antioxidant enzymes such as SOD and CAT become more active, indicating that the algae are under physiological stress. Studies [Melegari et al., 2013; Saxena et al., 2021; Suman et al., 2015] report dose-dependent increases in CAT activity in *C. vulgaris* treated with nanoparticles. It was demonstrated that this enzymatic defense system was inadequate to manage the oxidative stress that happened when high dosages of nanoparticles which are ZnO and FeO₃ were supplied.

In the literature, there is no ZnFeAl nanoparticles however there are toxicity studies of nanoparticles with similar composition. According to studies, the toxicity of Zinc-Iron Layered Double Hydroxides (Zn-Fe LDHs) or LDHs in general may vary depending on their composition, physical properties, exposure conditions and target organisms. The effects of Zn-Fe LDHs on aquatic creatures are dependent on a number of factors, including temperature, exposure duration, test organism type, physical or chemical breakdown, and radiation with biological heat temperature [Koba-Ucun et al., 2021]. ROS, the dissolution of hazardous components from nanoparticles (release of metal ions), and direct cell contact are the main ways that nanomaterials (NMs) can induce oxidative stress. Plant surface coatings can prevent photosynthesis or the absorption of nutrients [Buchman et al., 2019; Callaghan & MacCormack, 2017; Danabas et al., 2020].

Bioluminescence of the marine photobacterium *V. fischeri* was suppressed by Zn-Fe LDHs. The toxicity response was influenced by sample preparation time; a 1-hour preparation showed greater inhibition, whereas a 24-hour preparation showed structural alterations like the development of a thick white film and an elevation in pH (≈ 8.4) [Koba-Ucun et al., 2021; Rossetto et al., 2014]. EC₅₀ values for 3-hour preparation were 0.32 ± 0.03 g/L and 1-hour preparation were 0.11 ± 0.01 g/L at 30-minute exposure [Koba-Ucun et al., 2021]. This implies that toxicity is influenced by changes in the catalyst structure and the release of metal ions during interaction with the test media [Koba-Ucun et al., 2021]. Zn²⁺ and Fe³⁺ ions are known to be harmful to *V. fischeri* and may be a contributing factor in toxicity [F. Chen et al., 2020; Kurvet et al., 2017]. For freshwater microalgae, or *P. subcapitata* in particular, even at the lowest test concentration (0.10 g/L), Zn-Fe LDHs were found to be toxic to this microalgae and to significantly limit growth [Aruoja et al., 2009; Franklin et al., 2007; Koba-Ucun et al., 2021]. During the test, the Zn-Fe LDH catalyst showed signs of aggregation and morphological alterations [Dénél et al., 2019; Koba-Ucun et al., 2021].

For freshwater crustacean, *D. magna*, Zn-Fe LDHs resulted in immobility or death. The longer the exposure period, the greater the toxicity. For instance, at a dose of 0.05 g/L, 10% mortality was noted at 24 hours and 35% mortality at 48 hours. It was found that LDH particles stuck to the daphnids' antennae but not to their stomachs. It has been stated that the main cause of toxicity is the zinc ions released rather than particle

size [Baumann et al., 2014; Bundschuh et al., 2018; Dabrunz et al., 2011; Koba-Ucun et al., 2021]. For duckweed, or *Spirodela polyrhiza*, after 72 hours of treatment, it resulted in growth inhibition ($32 \pm 6\%$ at 0.075 g/L, $66 \pm 12\%$ at 2.00 g/L). The plants showed signs of morphological alteration and metal breakdown from the catalyst, as seen by their pale yellow hue. The determined EC50 value was 0.81 ± 0.19 g/L (72 hours) [Koba-Ucun et al., 2021; Mkandawire et al., 2014].

Effect of Cu-Mg-Fe LDHs on *Scenedesmus quadricauda*, after 72 hours of exposure, LDHs containing copper, magnesium, and iron significantly inhibited the growth of *S. quadricauda*, a comparable freshwater green alga. Growth inhibition is often associated with adverse effects on photosynthetic pigments [Ding et al., 2018; Koba-Ucun et al., 2021; H. Wu et al., 2023].

LDHs have a lot of potential for use in biomedicine; nevertheless, because of their propensity to aggregate and release metal ions, a thorough toxicity study is necessary. Techniques like surface modification are essential for controlling these hazards [Kiani et al., 2022; Yan et al., 2017].

In order to increase the safety of LDHs in biological applications, several tactics have been developed. Lipid membrane coating, by changing the distribution of nanoparticles within the body, lipid membrane coating decreases toxicity, improves colloidal stability, and stops them from aggregating. Though naked LDHs build up in the lungs, lipid-coated LDHs do the same in the liver [Yan et al., 2017]. PEG (polyethylene glycol) coating, improves the stability and biocompatibility of nanoparticles, extends their blood circulation period, and inhibits the reticuloendothelial system's quick clearance of them [Ye et al., 2022]. BSA (bovine serum albumin) coating, prevents aggregation and improves LDH colloidal stability [Keppel Hesselink & Kopsky, 2017; Ladewig et al., 2010; J. Zhang et al., 2024]. Using natural plant extracts such as plantago ovata for modification can greatly improve biocompatibility and decrease cytotoxicity to healthy cells, as well as make the production process more economical and ecologically friendly [Kiani et al., 2022; X. Wang et al., 2021].

There are methods for lowering toxicity of LDH during application to human body. Controlled release, by enabling tailored drug release to particular regions, such tumors, pH-sensitive release mechanisms help minimize systemic side effects [Kiani et al.,

2022; N et al., 2023; J. Zhang et al., 2024]. Targeted drug administration, drug availability to normal tissues can be decreased, lowering toxicity, by targeting administration to tumor cells through surface changes [**Ebadi et al., 2020; Keppel Hesselink & Kopsky, 2017; Ozkaynakci et al., 2015; Ye et al., 2022; J. Zhang et al., 2024].** Route of delivery, by delivering the medication straight to the target organ such as brain, techniques like intranasal delivery might lower systemic exposure and, consequently, systemic toxicity [**J. Zhang et al., 2024].**

LDHs offer a lot of potential for usage in many areas including biomedicine and cosmetic industry therefore a comprehensive toxicity investigation is required due to their tendency to agglomerate and release metal ions.



6. CONCLUSION

This study presents a comparative ecotoxicological evaluation of newly synthesized ZnFeAl LDH nanoparticles on freshwater and marine microalgae, providing insight into their environmental impact potential. The results suggest that two algae cells exhibit different antioxidative responses. In contrast to *C. cryptica*, which undergoes oxidative stress at extremely low nanoparticle concentrations, *C. vulgaris* is more resistant to LDH-induced growth suppression. This differential sensitivity implies that LDH nanoparticles may present a greater ecological danger in marine ecosystems, even the results cannot be extrapolated to all marine algae. The study emphasizes how crucial it is to consider a variety of trophic levels and habitat types when evaluating the toxicity of nanoparticles, especially those that are being utilized in environmental and industrial technologies. In the end, these findings advance our knowledge of how LDHs behave in the environment and support the need for regulatory frameworks that control their safe use and disposal. Overall, it is understood that ZnFeAl nanoparticles have potential toxic effects on algal species found in both freshwater and marine environments. These findings emphasize that algae species commonly found in aquatic environments are important bioindicators for ecosystem health and their sensitivity to environmental nanoparticle pollution should be considered.

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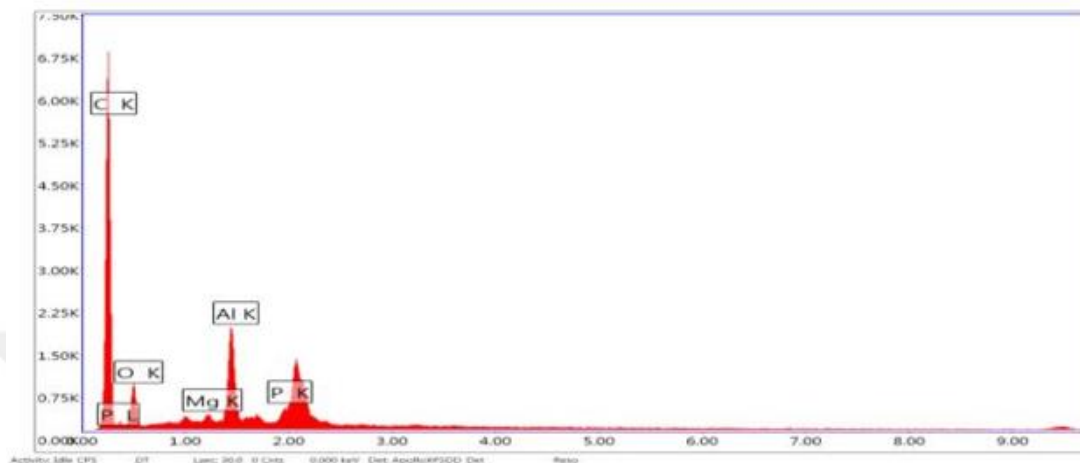
BIOGRAPHY

Zehra AKDAĞ completed her bachelor's degree at Gebze Technical University, Department of Molecular Biology and Genetics. During her undergraduate studies, she completed an Erasmus+ internship at the Universidad de Valladolid, Spain. After one year work experience as a R&D analyst, currently, she is a master student at Gebze Technical University, Institute of Biotechnology.

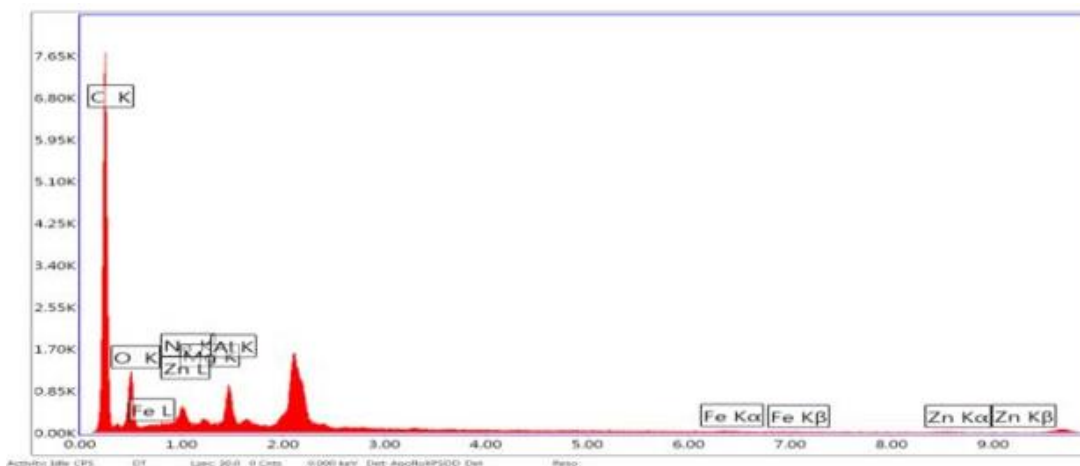


APPENDICES

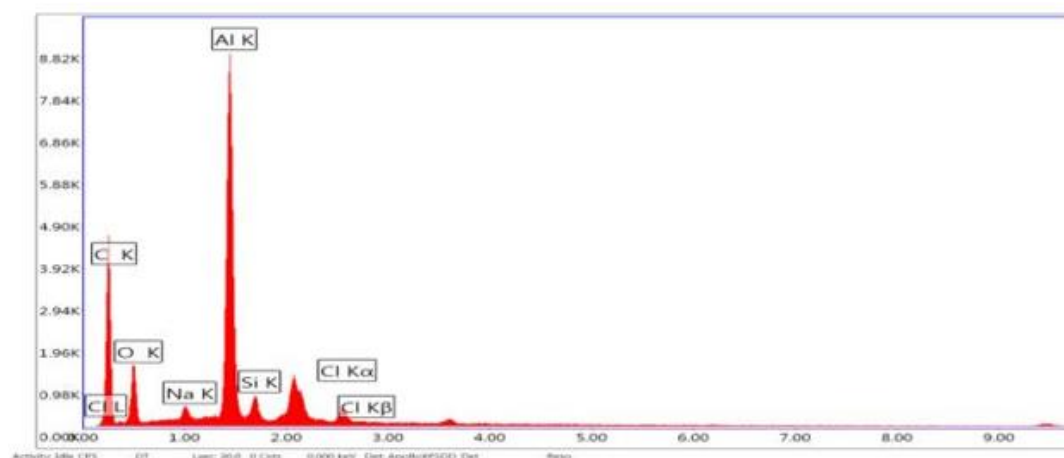
APPENDIX-A: EDS ANALYSIS OF *C. VULGARIS* EXPOSED TO 0 MG.L⁻¹ OF ZNFEAL LDH



APPENDIX-B: EDS ANALYSIS OF *C. VULGARIS* EXPOSED TO 250 MG.L⁻¹ OF ZNFEAL LDH



APPENDIX-C: EDS ANALYSIS OF C. CRYPTICA EXPOSED TO 0 MG.L⁻¹ OF ZNFEAL LDH



APPENDIX-D: EDS ANALYSIS OF C. CRYPTICA EXPOSED TO 250 MG.L⁻¹ OF ZNFEAL LDH

