Copper-Induced Changes in Growth and Physiology of *Tetraselmis indica*

A Dissertation for

Course Code and Course Title: MMI 651- Dissertation

Credits: 16

Submitted in partial fulfillment of Master's Degree

(M.Sc. In Marine Microbiology)

By

SEJAL SANJAY FADTE

SEAT NO: 22P0390013

ABC ID: 237587543842

PRN: 201910405

Under the Supervision of

DR. SANGEETA M. NAIK

School of Earth, Ocean & Atmospheric Sciences

Marine Microbiology

GOA UNIVERSITY
गोवा विद्यापीठ

GOA UNIVERSITY

DATE: MAY 2024

SEOAS, GOA UNIVERSITY TALEIGAO PLATEAU GOA

Examined By:

Seal of the School

DECLARATION OF THE STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "**Copper-Induced Changes in Growth and Physiology of *Tetraselmis indica***" is based on the results of investigations carried out by me in the Marine Microbiology at the School of Earth, Ocean & Atmospheric Sciences, Goa University under the Supervision of Dr. Sangeeta M. Naik and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the corrections of observations / experimental or other findings given the dissertation.

I hereby authorize the University authorities to upload this dissertation on the dissertation repository or anywhere else as the UGC regulations demand and make it available to any one as needed.

Ms. Sejal Fadte

22P0390013

Masters in Marine Microbiology

School of Earth, Ocean & Atmospheric Sciences

Date: 2nd May 2024

Place: Goa University

COMPLETION CERTIFICATE

This is to certify that the dissertation report **"Copper-Induced Changes in Growth and Physiology of *Tetraselmis indica*"** is a bonified work carried out by **Ms. Sejal Sanjay Fadte** under my supervision in partial fulfillment of the requirements for the award of the degree of **Masters** in the Discipline Marine Microbiology at the School of Earth, Ocean & Atmospheric Sciences, Goa University.

Dr. Sangeeta M. Naik
Marine Microbiology
Assistant Professor

Date: 2nd May 2024

SEOAS, GOA UNIVERSITY
TALEIGAO PLATEAU
GOA

School stamp

Sr. Prof. S. C. Ghadi
Dean of School of Earth, Ocean & Atmospheric Sciences
Date:
Place: Goa University

CONTENT

PREFACE

This research was carried out for the dissertation titled **"Copper-Induced Changes in Growth and Physiology of *Tetraselmis indica*".** Heavy metals, such as copper, pose a significant threat to the vitality of marine life. This dissertation delves into the intricate interplay between copper pollution and the growth and physiology of *T. indica*, a vital component of marine phytoplankton communities. As a primary producer, *T. indica* is crucial in marine food webs and biogeochemical cycles. Understanding how this unicellular green alga responds to copper exposure for assessing the broader implications of metal contamination on marine ecosystems. Through a comprehensive exploration of its growth dynamics, biochemical responses, and physiological adaptations, this dissertation aims to elucidate the mechanisms underlying *T. indica's* resilience or susceptibility to copper-induced stress.

ACKNOWLEDGEMENT

I would like to take this opportunity to express my sincere gratitude to my guide Dr. Sangeeta M. Naik, Assistant Professor, School of Earth, Ocean & Atmospheric Sciences, for her unwavering support, guidance and encouragement through the dissertation. Her invaluable feedback, insightful comments, and thoughtful suggestions have been instrumental in shaping this work.

I would also like to express my sincere gratitude towards our Dean Sr. Prof. S. C. Ghadi, and former Dean Sr. Prof. C.U. Rivonker, for allowing me to carry out my dissertation and availing all facilities of the school.

I like to thank our Lab Assistant Ms. Vaishali Merchant and Mr. Rohan Marshelkar, for providing, me with the required glassware's and chemicals. I extend my sincere thanks to the Applied Geology Department of School of Earth, Ocean, & Atmospheric Sciences and School of Chemical Sciences, Goa University for helping me with the requirements and for using their instruments.

I would like to extend a deep sense of gratitude towards all my friends, especially Sidhaee Gawande, Omkar Kamble, and Chetan Parab for always being there motivating and helping me throughout the dissertation.

Finally, I am also thankful to my parents for motivating me and for their immense support throughout the course of the project.

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Entity	Abbreviations
Copper	Cu
Copper sulphate	$CuSO_4$
Gram per liter	g/L
Heavy metals	HMs
Hydrochloric acid	HCl
Iron	Fe
Microgram/liter	µg/L
Micro molar	µM
Milligram/liter	mg/L
Milli molar	Mm
Sodium chloride	NaCl

ABSTRACT

Phytoplankton are the photosynthetic organisms found in both marine and freshwater ecosystems, are crucial for many aquatic food chains and generate approximately half of the earth's oxygen through photosynthesis. They are nevertheless, subject to anthropogenic pollution, such as heavy metal contamination. Copper is a common contaminant in aquatic systems and is hazardous to phytoplankton. The effects of copper content on *Tetraselmis indica,* a commonly studied species of marine phytoplankton, are summarized in this abstract.

Tetraselmis indica was subjected to various Cu concentrations to see how it affected growth rate, pigment, biochemical composition, and morphology. The results showed that the growth rate of *T. indica* decreased as the Cu concentration increased, and the pigment concentration of *T. indica* decreased with an increase in exposure time and Cu concentration. According to biochemical analysis, *T. indica's* cellular composition was altered by exposure to varied Cu concentrations. Morphology also changes with the increase in Cu concentration.

Overall, the findings indicate that *T. indica* is susceptible to Cu toxicity, which can cause considerable changes in its growth, pigment analysis, biochemical composition and morphology. These discoveries have significant implications for the well-being and productivity of marine ecosystems and industries such as aquaculture and fishing, which depend heavily on phytoplankton as a primary food source.

Keywords: Heavy metal, Biochemical composition, Adaptation, Phytoplankton

CHAPTER 1: INTRODUCTION

1.1 Background

Phytoplanktons are microscopic, photosynthetic organisms that form the base of many aquatic food webs. They are found in marine and freshwater environments are responsible for producing around 50% of the world's oxygen through photosynthesis (Falkowski et al., 1998). Phytoplankton's have a long evolutionary history that spans billions of years. One of the defining characteristics of phytoplankton is their size, which can range from less than one micrometer to several millimeters (Smetacek and Zingone, 2013). Phytoplankton plays a crucial role in the aquatic ecosystem and has significant global importance. They form the base of the aquatic food chain and provide the primary source of nutrition for zooplankton, which in turn supports higher trophic levels such as fish, marine mammals, and seabirds (Behrenfeld and Falkowski, 1997). The study of phytoplankton and their responses to environmental changes provides valuable insight into the impacts of climate change on marine and freshwater ecosystems (Behrenfeld and Falkowski, 1997). Algal ecotoxicological assessments utilize various physiological indicators like cell growth rate, biovolume, antioxidant response, pigment production, and photosynthetic rate to compare treated and untreated cells. In toxicological studies, cell number serves as a growth indicator, with environmental contaminants often altering cell size and shape through oxidative stress induction (Wang et al., 2017). Phytoplankton's have a range of growth requirements that influence their distribution and abundance in the aquatic ecosystem. Some key factors that influence phytoplankton growth include physical factors such as light, temperature, salinity, and nutrient availability, as well as biological factors such as competition and grazing pressure (Finkel et al., 2010).

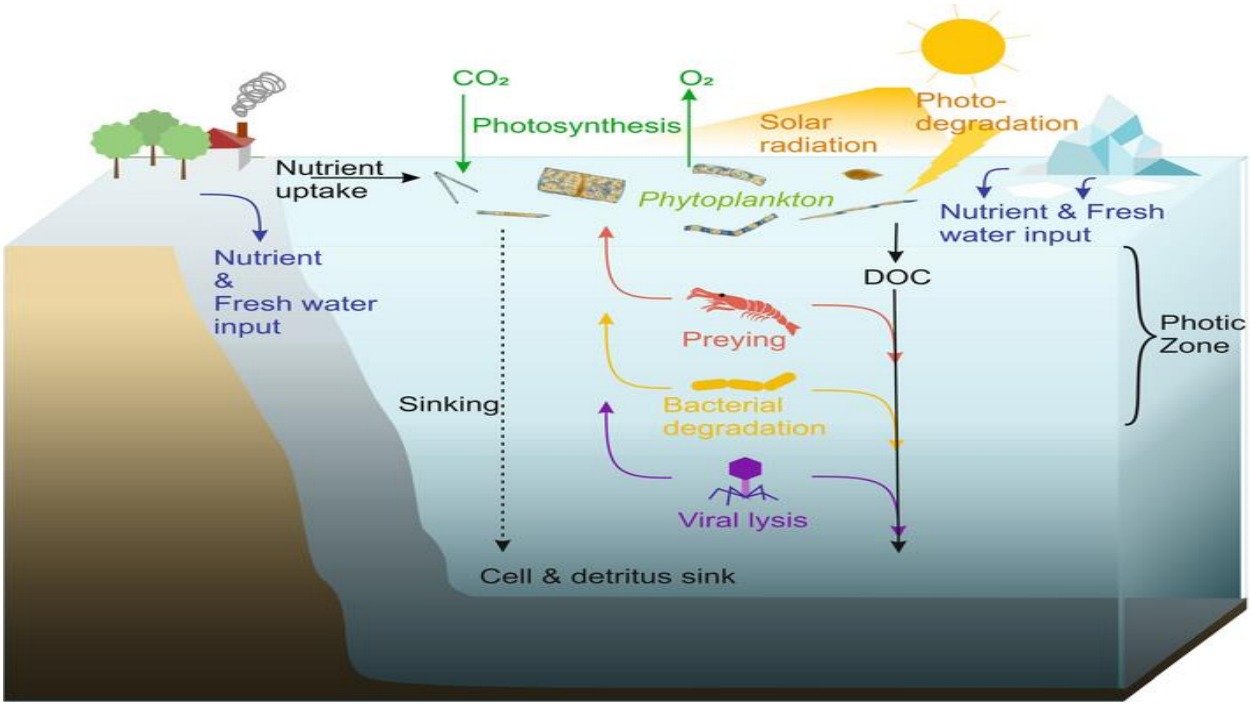

Figure 1.1: Significance of phytoplankton (Kase and Geuer, 2018)

1.1.1 Major groups of phytoplankton

Phytoplanktons are diverse and can be classified into various groups based on their size, shape, pigments, and habitat.

a. Cyanobacteria

The earliest photosynthetic organisms are thought to have appeared around 3.5 billion years ago and were likely similar to modern day cyanobacteria (Falkowski et al., 2008). They are also known as blue-green algae, are prokaryotic organisms that are capable of nitrogen fixation and play an important role in nutrient cycling (Paerl and Paul, 2012). They lack a nucleus and other membrane-bound organelles (Sanchez-Baracaldo et al., 2017). They are found in both freshwater and marine environments, and their blue-green color is due to the presence of phycobiliproteins (Field et al., 1998). Cyanobacteria can form large blooms that can harm aquatic organisms and

cause eutrophication, which can lead to hypoxic conditions in the water. Traditionally, cyanobacteria have been classified into five subsections based on their morphological features. These include the Chroococcales (unicellular and colonial forms), Oscillatoriales (filamentous forms with uniseriate or multiseriate filaments), Nostocales (filamentous form with specialized cells called heterocyst for nitrogen fixation), Stigonematales (filamentous forms with false branching), and Pleurocapsales (unicellular and colonial form with thick-walled cells) (Whitton and Potts, 2000).

b. Diatoms

Diatoms first appeared in the fossil record around 180 million years ago and have since diversified into a wide range of forms (Armbrust, E. V. 2009). They are known for their intricate silica shells that provide protection from predators and allow them to sink to deeper depths (Armbrust, E. V. 2009). They are unicellular, characterized by their ability to absorb blue and green light, and typically found in colder, nutrient-rich waters (Field et al., 1998). They are one of the most abundant and diverse groups of phytoplankton in the ocean, accounting for up to 40% of the primary production in the world's oceans (Nelson et al., 1995). They are known to be important food sources for many marine organisms, including zooplankton and benthic invertebrates (Bidle and Falkowski, 2004). They have chlorophyll *a, b,* and *c,* photo-protective and light-harvesting pigments (Wagner et al., 2006). Diatoms can be classified into two classes: centric and pennate. Centric diatoms have a radial symmetry, with their frustules having circular or elliptical shapes, while pennate diatoms have a bilateral symmetry, with their frustules having elongated shapes. According to Round et al., (1990) centric diatoms are further classified into five orders: Thalassiosirales, Coscinodiscales, Aulacoseirales, Biddulphiales, and Centrales.

Pennate diatoms, on the other hand are classified into two subclasses: Fragilariophyceae and Bacillariophyceae

c. Dinoflagellates

Dinoflagellates have evolved a unique system of flagella that allows them to swim and move in response to light and nutrients (Gustafson et al., 2000). They have a characteristic reddish-brown color due to the presence of pigments such as peridinin and fucoxanthin (Field et al., 1998). They can be found in both freshwater and marine environments and are known for their ability to produce bioluminescence, which can create stunning visual displays at night. Some dinoflagellates can form harmful algal blooms (HABs) that can negatively impact the environment and human health (Gilbert et al., 2005). Their cell wall is made up of cellulose plates (Dodge, J. D. 1989). Dinoflagellates can be classified into two main groups based on the type of photosynthetic pigments they contain: the autotrophic dinoflagellates, which contain chlorophyll *a* and *c*, and the heterotrophic dinoflagellates, which lack photosynthetic pigments and obtain their energy by consuming other organisms (Gomez, F. 2012). Another classification scheme divides dinoflagellates into three groups based on the shape and structure of their cellulose plates: the athecate, thecate, and naked dinoflagellates (Sournia et al., 1991).

d. Coccolithophores

Coccolithophores are thought to have evolved around 200 million years ago and have since become an important component of marine ecosystems (Smetacek and Zingone, 2013). They are eukaryotic phytoplankton characterized by calcium carbonate plates on their cell surface, which are important in global carbon cycling (Balch et al., 2011). They are typically found in warm, nutrient-poor waters and are characterized by their ability to reflect and scatter light due to the

presence of coccoliths (Field et al., 1998). They are unicellular and belong to the division Haptophyta (Von Dassow et al., 2014). They are further classified into the class Coccolithophyceae, which includes two orders: the Isochrysidales and the Coccolithales (Young et al., 2019).

e. Green algae

Green algae are a diverse group of photosynthetic organisms that play a crucial role in aquatic ecosystems by producing oxygen and serving as the base of the food web (Chisholm et al., 2016). They are single-celled or colonial organisms that can be found in both freshwater and marine environments. They are characterized by their green pigmentation, which is due to the presence of chlorophyll *a* and *b* (Guiry and Guiry, 2021). Green algae are classified into two main groups, Chlorophyta and Charophyta, with Chlorophyta being the most diverse group (Guiry and Guiry, 2021). These organisms can have a variety of shapes and sizes, ranging from tiny flagellated cells to large colonial forms. Some green algae species can also form harmful algal blooms under certain environmental conditions, which can negatively impact marine ecosystems (Hallegraeff, G. M. 2010).

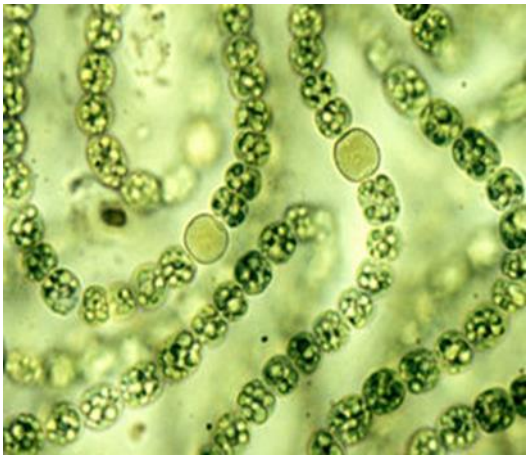

Figure 1.2 : Cyanobacteria

Figure 1.3: Diatoms (Dr. Robert Berdan)

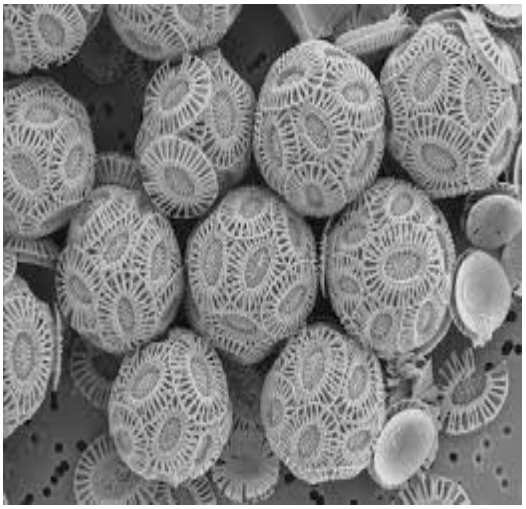

Figure 1.4: Coccolithohores

(Igor Siwanowicz)

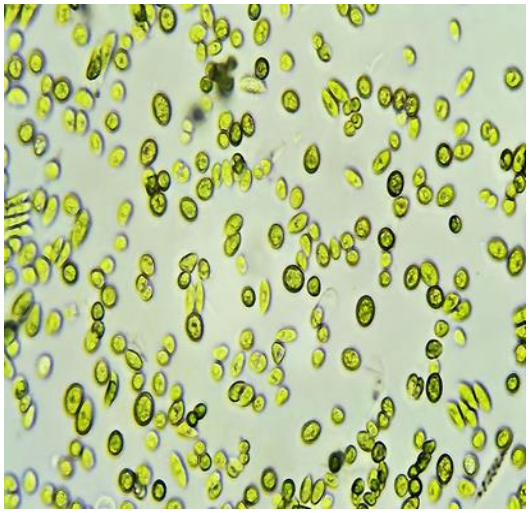

Figure 1.5: Green algae

(Gettyimage/Videologia)

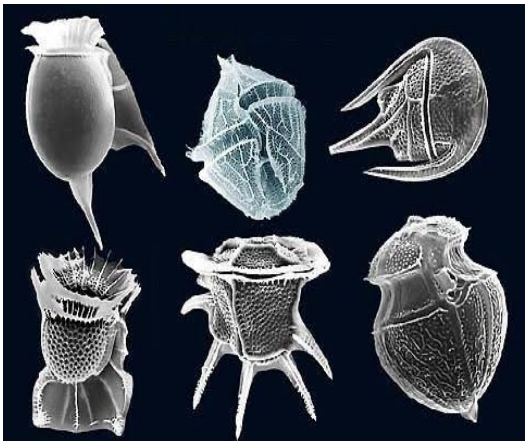

Figure 1.6: Dinoflagellates (fickleandfreckled, 2012)

1.1.2. Collection, preservation and enumeration of phytoplankton

According to Horner and Hargraves (1983) one of the most common methods for collecting phytoplankton is using a phytoplankton net. A phytoplankton net is a specialized mesh made of nylon or silk and has a small mesh size, usually between 20-100µm. The net is towed behind a boat at a constant speed, and the collected sample is then preserved in a fixative solution (Nishihara and Bower, 2006). Another method is using the Niskin sampler, which involves lowering the sampler into the water at a desired depth and collecting a discrete water sample (Nishihara and Bower, 2006).

According to a study by Litchman et al., (2000) the most effective preservation method for phytoplankton is to fix them with 1-2.5% of glutaraldehyde. The authors found that glutaraldehyde fixation resulted in minimal changes in cell size and shape, chlorophyll content, and cellular carbon content of phytoplankton samples. Another study by McManus et al., (2012) found that Formalin and Lugol's iodine was the most effective fixative for preserving cell morphology, while 1% glutaraldehyde was better for preserving fluorescence and 0.5% of paraformaldehyde is also effective (Vaulot et al., 1987).

Phytoplankton enumeration refers to quantifying the abundance and distribution of phytoplankton in aquatic systems. One common method is through microscopy, using either Sedgewick Rafter, which visually identifies and counts cells under the microscope (Parsons et al., 2013). Other methods include a flow cytometer, which uses lasers to measure the fluorescence emitted by individual cells, and DNA-based methods, which rely on the extraction and amplification of DNA from water samples (Vargas et al., 2015).

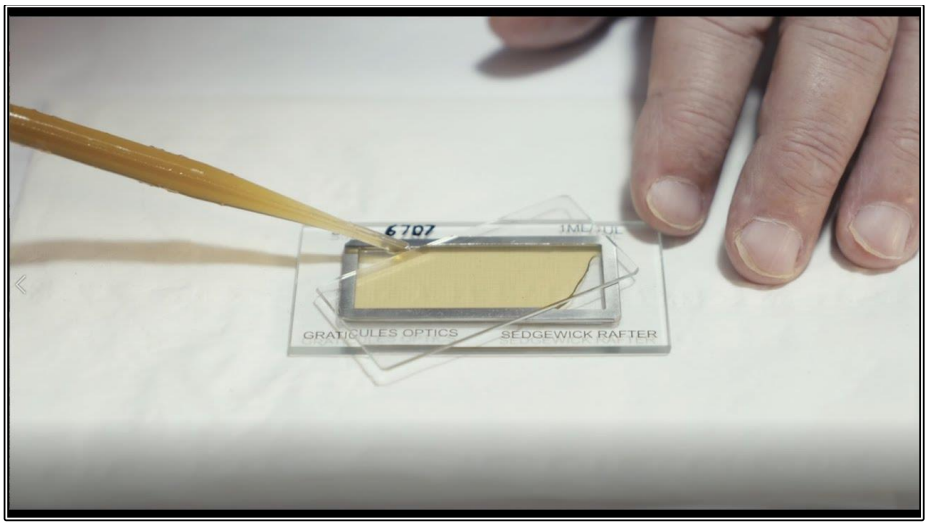

Figure 1.7: Sedgewick Rafter (LeGresley et al., 2010)

1.1.3. Effect of pollution and heavy metals on phytoplankton

Pollution can have significant impacts on phytoplankton. According to a study by Thangaradjou et al., (2018) pollution can affect the growth, reproduction, and survival of phytoplankton, as well as alter their species composition and abundance. Heavy metals are high-density metallic elements that are poisonous, even in small amounts. They are naturally present in the environment, but anthropogenic activities such as industrialization, mining, and agriculture have significantly increased their concentrations in the aquatic ecosystem. As primary producers phytoplankton are the first organisms to come into contact with heavy metals, and their responses have been widely studied (Mitra and Flynn, 2005). One way that pollution can affect the phytoplankton is by increasing nutrient levels in the water, which can lead to eutrophication. This can cause harmful algal blooms to form, which can release toxins that harm not only the phytoplankton but also other organisms in the ecosystem (Thangaradjou et al., 2018).

1.1.4 *Tetraselmis indica*

They belong to:

Division: Chlorophyta

Class: Chlorodendrophyceae

Order: Chlorodendrales

Family: Chlorodendraceae

Genus: *Tetraselmis*

Species: *indica*

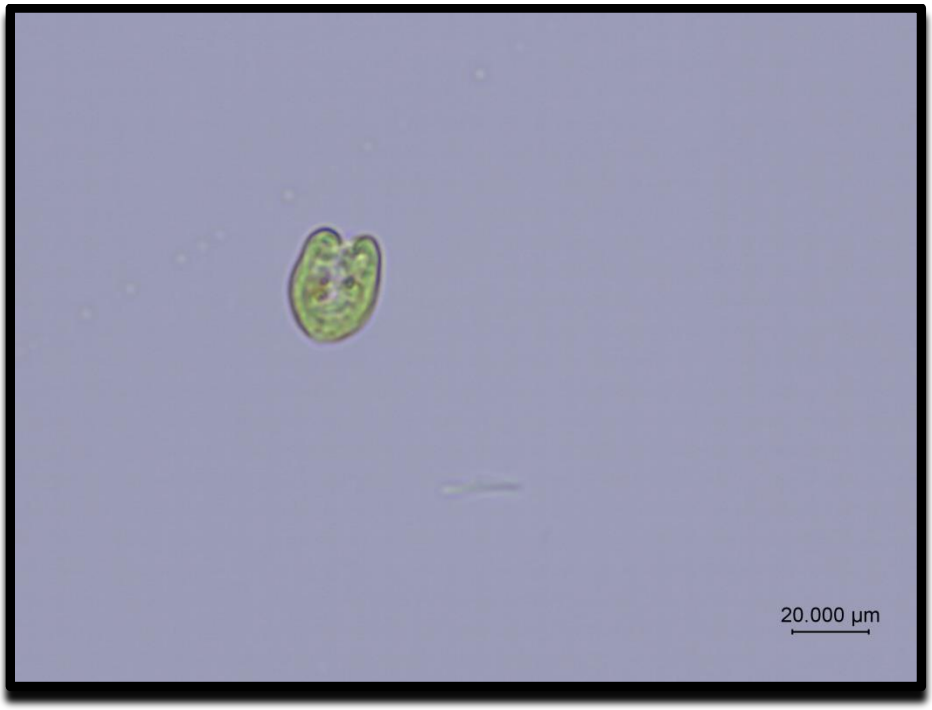

Figure 1.8: Morphology of *Tetraselmis indica*

In this study, *Tetraselmis indica* was as an organism. *Tetraselmis* species were once thought to belong to the Prasinophyceae family; new research has shown that they are actually related to the core Chlorophyta. *Tetraselmis* species even have implications for ballast water management programs. Due to such extreme environment of saltpans, this alga has to possess a remarkable physiology to adapt to these conditions (Naik and Anil, 2017). *Tetraselmis* strains offer several advantages, including their ability to thrive in diverse climates and aquatic environments, high tolerance to salt and eurythermal nature. They are extensively used in aquaculture as a nutrient source for various aquatic organisms such as shrimp, larvae, juvenile mollusks, and rotifers (Goswami et al., 2021). The best known members of the class are quadriflagellate unicells, but some species of *Tetraselmis* may form staked colonies during some stage of the life cycle. The four equal and homodynamic flagella that emerge from the cell's front pit are characteristic of the motile cells of the Chlorodendrophyceae family, which are often laterally compressed. The cells are typically covered by a theca, which is a thin cell wall formed by extracellular fusion of scales (Arora et al., 2013).

The biochemical composition of three *Tetraselmis* strains, highlighting their diverse biomolecules including proteins, lipids, carbohydrates, and amino acids. Specifically, *Tetraselmis* sp. CTP4, *T. chuii*, and *T. suecica* were found to contain proteins, lipids, and carbohydrates in varying concentrations per 100g of biomass (Goswami et al., 2021). Additionally, these strains exhibited a rich profile of amino acids, indispensable and non-indispensable, along with a sugar composition comprising arabinose, xylose, mannose, glucose, galactose, rhamnose, ribose, among others. Moreover, they contain various primary and secondary metabolites such as chlorophyll *a* and *b*, β-carotene, α-carotene, zeaxantine, neoxanthine, violaxanthin, and lutein (Goswami et al., 2021).

1.1.5. Impacts of copper on phytoplankton

Copper is an essential micronutrient for the growth and development of phytoplankton. Copper is required for various cellular processes in phytoplankton, including photosynthesis, respiration and nitrogen metabolism (Bowler et al., 2010). There have been several studies on the impact of copper on phytoplankton. For example, a study by Guo et al., (2019) investigated the effects of copper on growth, photosynthesis, and biochemical processes of marine phytoplankton. This study found that exposure to copper can decrease the growth and photosynthetic efficiency of phytoplankton, as well as alter their pigment content and biochemical processes. One study by Zhang et al., (2018) investigated the effect of copper on the growth and physiological characteristics of diatoms *Skeletonema costatum*. The researchers found that low concentration of copper (1-5 µM) promoted the growth and photosynthetic activity of the diatoms, while higher concentrations (10-50 µM) had inhibitory effects on the growth and photosynthesis. Additionally, copper has been shown to enhance the production of certain toxins in phytoplankton, such as domoic acid in the diatoms *Pseudo-nitzschia multiseries* (Bates et al., 1998). A study by Lu et al., (2020) investigated the impact of copper on the growth and antioxidant responses of freshwater phytoplankton. The study found that exposure to copper can decrease the growth and chlorophyll content of phytoplankton, as well as increase their production of reactive oxygen species, which can damage cells and impair physiological processes.

Copper can impact marine diatom *Thalassiosira oceanica* by affecting its photosynthesis efficiency due to a decreased availability of the copper containing protein plastocyanin. Exposure to copper can also later reduce the biochemical content, protein content, affect lipid

metabolism, changes structural organization, and modify ion balance in these microalgae, as they compete with nutrients for binding sites within the cell (Rocha et al., 2016).

1.1.6. The Role of Cu as Essential Metal in Micro algal Metabolism

Copper plays a vital role in the biology of organisms living in aquatic environments. Its importance to life dates back to the emergence of oxygenic photosynthesis driven by cyanobacteria metabolism. Before this, water-soluble ferrous iron was predominant in catalyzing redox reactions. However, with the advent of oxygenic photosynthesis, this iron was oxidized to insoluble Fe (III), making it unavailable for biological processes. Concurrently, the oxidation of non-bioavailable Cu (I) resulted in the formation of soluble Cu (II) (Chrichton et al., 2001). While evolution introduced chelators and storage proteins like ferritin to make iron accessible again for metabolism, Cu took on a new role leveraging the oxidizing power of oxygen due to its higher redox potential. Despite their distinct roles, Fe and Cu metabolisms are closely linked. These metals are components of analogous enzymes that participate in similar reactions. Organisms have evolved metabolic adaptations that enable them to switch between these metals based on their availability (Cerda et al., 2007). Many algae and cyanobacteria produce two soluble proteins crucial for electron transport in both photosynthesis and respiration: Fe-containing cytochrome C6 (Cyt) and Cu-containing plastocyanin (Pc). The production of these proteins depends on the availability of Cu. For instance, in *Chlamydomonas*, when Cu is scarce, the organism employs Cu-saving strategies. It replaces the more abundant plastocyanin with cytochrome C6, conserving Cu for the biosynthesis of Cyt oxidase (kropat et al., 2015).

In phytoplankton, copper plays essential roles in photosynthesis, respiration, and defense against reactive oxygen species (ROS) through superoxide dismutase (Wang et al., 2018). Often, Cu

availability limits the growth of these organisms. Additionally, in microalgae, Cu facilitates the trans membrane uptake of iron and may be crucial under Fe-limited conditions (Madonado et al., 2006). Exposure to Cu can alter algal cells size, density, and biochemical composition. For instance, in the green alga *Scenedesmus quadricauda*, exposure to 1 µM of Cu resulted in increased cell volume and elevated protein and carbohydrate content. However, a higher Cu concentration (2.5 µM) led to reduced cell abundance and chlorophyll-*a* content (Silva et al., 2018). El Agawany et al., (2021) observed a gradual increase in protein content in *Dunaliella tertiolecta* with low concentrations of essential heavy metals, including Cu (around 79 µM). Elevated levels of heavy metals inhibited protein synthesis to varying degrees. In the diatom *Thalassiosira oceanica*, Cu limitation reduced photosynthetic efficiency due to decreased plastocyanin (Pc) levels and maximum photosynthetic electron transport rates, leading to growth limitations. Cu limitation also up regulated enzymes involved in fatty acid metabolism, possibly aiming to boost lipid turnover to maintain membrane integrity in response to oxidative stress (Kong et al., 2020). Furthermore, sub-lethal concentrations of Cu affected both photochemical and non-photochemical quenching in the freshwater microalga *Selenastrum gracile*. This resulted in the inactivation of reaction centers and damage to photo protection mechanisms, ultimately compromising photosynthetic activity (Rocha et al., 2021).

1.1.7. Effects of copper at high concentration

When exposed to high concentrations of copper, microalgae experience various adverse effects. These include decreased growth rates, impaired photosynthesis and respiration, as well as alterations in cell and organelle size and morphology. Typically, metal toxicity arises from the binding of metals to the sulfhydryl groups of proteins. This binding can disrupt protein structure or displace essential elements, leading to functional impairments (Rocha et al., 2021). The

tolerance of microalgae to heavy metals (HMs), as well as the stress markers and mechanisms they employ to mitigate adverse effects, differ significantly among species. Cu stress has been observed to cause membrane damage in *Chlorella sorokiniana* and *Scenedesmus acuminatus*. Particularly in *Chlorella sorokiniana*, which accumulates higher levels of Cu and produces fewer antioxidants, such as peroxidase (POX), ascorbate peroxidase (APX), and glutathione reductase (GR) enzymes, as well as lower levels of proline, polyphenols, and ascorbate (ASC) (Hamed et al., 2017). Exposure to Cu has been shown to alter chlorophyll and carotenoid levels in two marine species: *Chaetoceros calcitrans* and *Nitzschia closterium*. Among them, *C. calcitrans* was the most sensitive, exhibiting increased activity of antioxidant enzymes like catalase (CAT) and ascorbate peroxidase (APX). Additionally, Cu can induce vacuolization in both the cytoplasm and chloroplast of algal cells. This was demonstrated in the diatom *Skeletonema costatum*, where Cu entered the cells and accumulated in spherical bodies located within the vacuoles (Nassiri et al., 1997).

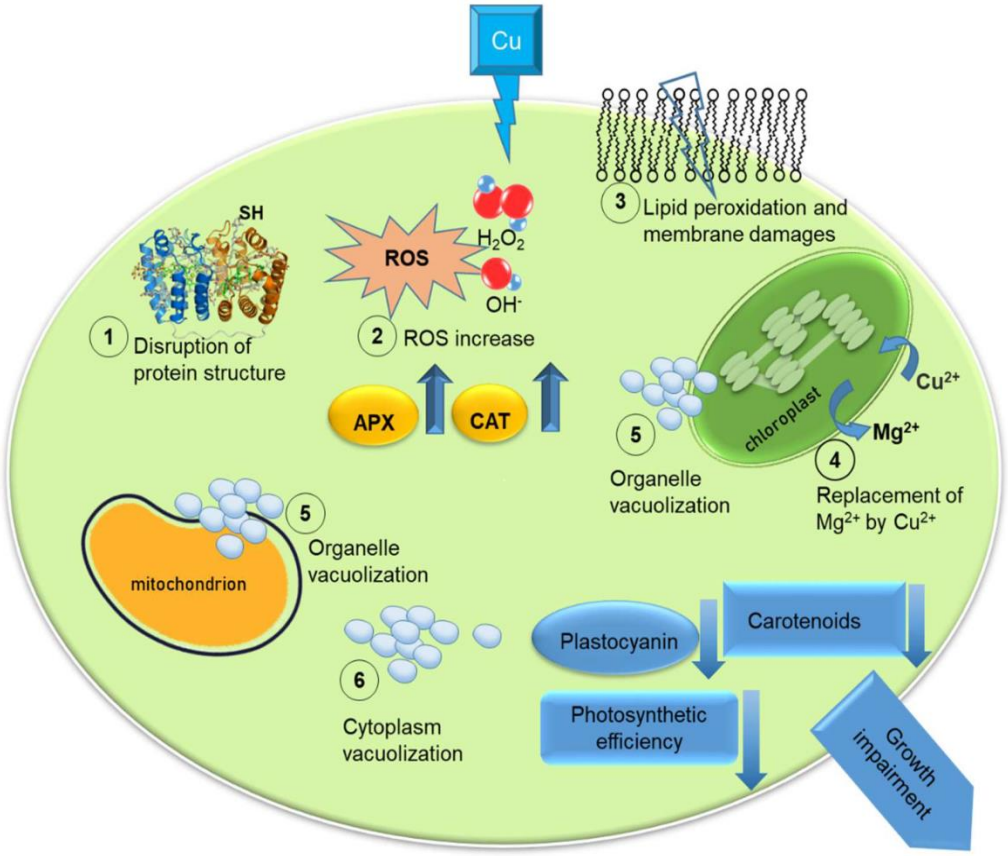

Figure 1.9: Illustration of the effects of high Cu concentration on micro algal cell (Cavetti et, al 2020).

1.2. Aims and Objective

This study aims to determine the impact of Cu on the growth of *Tetraselmis indica* and assess its influence on the accumulation of biomolecules such as carbohydrates, chlorophyll pigment (chlorophyll *a*, chlorophyll *b*, and total chlorophyll). Fourier Transform Infrared Spectroscopy (FT-IR) and Scanning Electron Microscopy (SEM) technique will also be employed to examine the organism's morphology, size, and surface characteristics.

1.3. Hypothesis

Exposure of phytoplankton to elevated copper concentrations will result in significant alterations in growth patterns, cellular morphology, and biomolecule accumulation, potentially negatively impacting overall physiological health.

1.4. Scope

Future studies need to be conducted on phytoplankton exposure to heavy metals. Some possible areas of focus include the determination of the precise mechanisms by which heavy metals affect phytoplankton and the impact on their physiology. This could entail examining the interplay between metals and essential cellular components like chlorophyll, photosynthesis, and respiration enzymes. While the effects of heavy metals on chlorophyll content and growth are well documented, metal exposure may impact other biological processes. Metals, for example, may influence the formation of secondary metabolites such as polyunsaturated fatty acids, which are essential for higher trophic-level nutrition. It is critical to develop effective solutions to minimize the effects of heavy metal contamination on phytoplankton. This research could look into the possibility for natural or manufactured remediation approaches like bioremediation or phytoremediation.

CHAPTER 2: LITERATURE REVIEW

National

According to a study by Kumar and Shin (2017) the interactions between heavy metals and algae indicate that initially, metals bind to the algal cell wall, which possesses a negatively charged external layer rich in extracellular polymeric substances. For instance, copper binds initially to protein carboxylic and amino residues at the cell wall. Subsequently, copper accumulates and moves through this layer to the plasma membrane, which binds to various sites, affecting cell membrane functions or entering the cell through facilitated diffusion or active uptake. Once inside the cell, copper influences photosynthesis, enzyme activity, and cell division. It competes for enzyme binding sites, inhibits enzymes involved in nitrogen metabolism and photosynthesis, and can cause changes in intracellular pH. Furthermore, copper impacts subcellular organelles like chloroplasts and mitochondria, leading to structural changes in thylakoid membranes and affecting chlorophyll fluorescence and photosynthesis.

The classification and physical characteristics of the *Tetraselmis* genus have been extensively researched. This taxonomic structure provides a thorough basis for comparing the physical and structural characteristics of the isolate from Goa with known *Tetraselmis* species. Examination of the water under the light microscope revealed that motile *Tetraselmis* cells and diatoms like *Amphora* and *Pseudo-nitzschia* were predominant. Although it cannot be ruled out that this species also exists in other marine habitats, the sampling location suggests that it prefers hypersaline conditions. *Tetraselmis* species is green algae planktonic in the sea that prefers high salinities. Motile cell are often compressed, measuring between 10 and 25 µM in thickness. They are bilaterally symmetrical, oval from the broad side, and elliptical from the narrow side. The species folds are unique (Arora et al., 2013).

Naik and Anil (2018) studied the fact that exposure to prolonged darkness had minimal impact on the chlorophyll levels of *T. indica* but resulted in enhanced carotenoid production. This underscores the importance of carotenoids for the survival of *T. indica* in dark conditions. In *T.indica*, the HPLC approach made pigment identification and quantification possible. Six pigments were able to be separated using the Heukelem method: lutein (Lut), violaxanthin (Vio), neoxanthin (Neo), β-carotene (β-car) and chlorophyll *a* (Chl *a*). While loroxanthin and loroxanthin esters were not found in *T. indica*, antheraxanthin (Anth) and the above six pigments were identified using a modified Zapata method. Additionally, the differing patterns observed between chlorophyll auto fluorescence and chlorophyll concentration highlight the limitations of using chlorophyll auto fluorescence as a reliable indicator of chlorophyll levels in this species.

This study aimed to examine the harmful effects of copper (Cu) and cadmium (Cd) on the growth and pigment levels (chlorophyll-*a* and carotenoid) of *Nitzschia* sp. phytoplankton. It involved four stages: phytoplankton cultivation, definitive testing, cell density assessment, and analysis of chlorophyll-*a* and carotenoid levels. Results demonstrated decreased cell density and pigment content with increasing heavy metal concentrations. The IC_{50}-96 h values for Cu and Cd on growth were 0.268 mgCu/L and 0.159 mgCd/L, respectively, while for chlorophyll-*a* content, they were 0.274 mgCu/L and 0.150 mgCd/L. The LOEC for *Nitzschia* sp. was 0.18 mgCu/L and 0.18 mgCd/L, with NOEC values of 0.1 mgCu/L and <0.18 mgCd/L. Additionally, toxic concentrations of 0.1 mgCu/L and 0.18 mgCd/L significantly impacted carotenoid content. (Hindarti and Larasati, 2019).

Sediment cores from the middle and upper Zuari Estuary were analyzed for grain size, organic carbon content, and the presence of metals like Fe, Mn, Zn, and Pb to assess metal enrichment and pollution levels. Additionally, the study examined the bioaccumulation of these metals in the

edible bivalve *Polymesoda erosa* to determine potential metal toxicity. The distribution of metals differed between the middle and upper estuary, likely due to variations in metal sources, hydrodynamics, and post-depositional processes. Pollution indices, including the contamination factor and pollution load index indicated metal pollution in both estuary sections. Metal speciation analysis revealed that Mn, Zn, and Pb were bioavailable in the sediments. Using a screening quick reference table and risk assessment code, it was inferred that Mn could pose potential harm to aquatic organisms. The concentration of metals found in *Polymesoda erosa* exceeded the standard permissible limits in both the middle and upper estuary, indicating potential metal toxicity to both the bivalve and human health (Cruz et al., 2020)

The study investigated the bioavailability of metals, including Fe, Mn, Zn, Cu, Co, and Ni, in sediment cores (K-1, K-2, K-3, and K-4) and their bioaccumulation in edible bivalves in the Kali Estuary, India, to assess metal toxicity. The Enrichment Factor (EF) identified anthropogenic sources for Zn, Co, and Ni, while the Geo-accumulation Index (Igeo) indicated pollution by Zn and Ni based on total metal analysis. The Pollution Load Index (PLI >1) supported the anthropogenic origin of metals in the estuary. Metal speciation analysis revealed the bioavailability of these metals in the sediments. Elevated Mn and Co levels, concentrations of metals in *Metetrix casta*, *Saccostrea cucullata*, and *Villorita cyprinoides* surpassed the permissible bioaccumulation limits, indicating metal toxicity in these bivalves and rendering them unsafe for human consumption. The Translocation Factor (TF > 1) suggested the potential use of *Kandelia candel* for phytoremediation of Fe, Zn, Cu, Co, and Ni at station K-3, and *Sonneratia caseolaris* for phytoremediation of Fe, Zn, and Ni at station K-4. (Desai et al., 2023).

Copper is essential but can become toxic at elevated levels, leading to widespread contamination. According to the Srivastsava et al., (2006) study aimed to investigate the effects of both low and

high doses of copper (ranging from 0.1 to 25 µM) on the aquatic macrophytes, *Hydrilla verticillata*, over 1 to 7 days. The plants accumulated significant amounts of copper, reaching a maximum of 770 μg^{-1} dry weight by day 7 at a concentration of 25 µM. Biomass and photosynthetic pigments remained relatively stable at concentrations up to 1 µM but declined noticeably at higher concentrations. Elevated malondialdehyde (MDA) levels and electrical conductivity (EC) at higher copper concentrations indicated oxidative stress. In response to copper exposure, there was a notable increase in the production of proteins and enzymes such as superoxide dismutase (SOD), ascorbate peroxidase APX), guaiacol peroxidase (GPX), catalase (CAT), and glutathione reductase (GR), but only up to moderate exposure levels. Total non-protein thiols (NP-SH) and cysteine levels rose significantly to 5 µM copper exposure but sharply declined at 25 µM. Reduced glutathione (GSH) levels decreased across all concentrations while oxidized glutathione (GSSG) levels increased simultaneously. Phytochelatin (PCs) were significantly induced at 1 and 5 microM concentrations on day 4 compared to the control. However, the low copper chelation efficiency indicated by the PC-SH to Cu ratio (6.5% at 1 µM and 2.4% at 5 µM) suggests that PCs only partially contribute to the integrated mechanisms of copper homeostasis and detoxification. The plants ability to tolerate moderate copper exposure and their high accumulation potential suggest their potential suitability for remediating moderately copper-contaminated water bodies.

According to a Mallick, M. (2003) exposure to copper led to an increase in lipid peroxidation, carotenoid levels, and superoxide dismutase activity in the green microalga *Chlorella vulgaris*. However, the activities of catalase, ascorbate peroxidase, and glutathione reductase, along with the cellular levels of GSH, ascorbate, and K^+, decreased. Interestingly, there was a notable

increase in intracellular proline content in cultures supplemented with copper. Despite the compromised antioxidant system in *C. vulgaris* under copper stress, the organism survived and grew even at a concentration of 3.0 µg/mL of copper, showing a 32% growth rate. Further research is required to understand the role of proline in regulating metal toxicity.

One study revealed that copper is a heavy metal frequently found in wastewater from industrial and mining activities due to its widespread industrial use. Some aquatic algal species, like the green alga *Desmodesmus* sp. AARLG074 can tolerate and accumulate copper, making them potentially valuable for bioremediation purposes in polluted waterways. This study aimed to understand how *Desmodesmus* can withstand significant changes in its external copper and pH levels. Specifically, we examined how these changes influenced the algal cells copper removal efficiency, growth, cellular structure, and metabolite composition. Our findings revealed that *Desmodesmus* could remove up to 80% of the copper from Jaworski's medium within 30 minutes of exposure. However, the efficiency of copper removal decreased at pH 4 compared to pH 6, indicating that both pH and copper concentration play roles in the removal process. Additionally, copper exposure negatively impacted algal growth and caused structural alterations in cells. Metabolite analysis using FT-IR and GC-MS showed that exposure to copper and acidic conditions primarily affected the algal cells levels of polysaccharides and amino acids. Specifically, fructose, lactose, and sorbose contents decreased significantly under both acidic and Cu conditions, while glycerol and melezitose levels increased at pH 4. Pathway analysis revealed that pH had a greater impact on alanine, aspartate, and glutamate metabolism, whereas copper predominantly affected arginine and proline metabolism. Both copper and pH influenced glutathione and galactose metabolism pathways (Buayam et al., 2019).

International

The study explored the potential of variable fluorescence, specifically the potential photochemical efficiency of photosystem II (Fv/Fm), measured using a fast repetition rate fluorometer (FRRF), as an indicator of metal pollution effects on natural phytoplankton communities. Phytoplankton samples were collected from a eutrophic embayment and exposed to various copper concentrations within the ppb range over 4 days. Initially, enhanced photosynthesis was observed at low copper concentrations (10 and 20 µg L^{-1}) with short exposure times (1 and 5 hours). However, after 72 hours, even at 10 µg L^{-1} of Cu, Fv/Fm values were significantly lower than the control's. The highest copper concentration tested (80 µg L^{-1}) led to a notable decrease in Fv/Fm after 5 hours. This response was contrasted with measurements of chlorophyll concentration, photosynthetic O_2 production rates, and changes in microplankton community composition and size structure. Reduction in overall particle size occurred at higher copper concentrations (80 and 40 µg L^{-1}), while an increase in flagellates and the diatom *Pseudonitzschia pungens* was observed at 20 µg L^{-1} of copper. The study highlights the effectiveness of this methodological approach as a rapid and nondestructive means of detecting trace-metal toxicity in natural phytoplankton (Perez et al., 2006).

This research aimed to optimize the biomass concentration, productivity, and biochemical composition of the marine microalga *Tetraselmis* species. The study utilized the Box-Behnken Design to analyze the impact of $NaNO_3$, NaH_2PO_4, metals, and vitamins in the f/2 medium on growth, total chlorophylls, carotenoids, and starch levels. Total chlorophylls were measured using spectrophotometry, while FT-IR spectroscopy assessed the biochemical composition of *Tetraselmis* sp. in both optimized and standard culture media. The optimized concentrations of

$NaNO_3$ (1.76 mM), NaH_2PO_4 (0.018 mM), metals (1500 µg/L^{-1}), and vitamins (312.5 µg/L^{-1}) led to a biomass concentration increase up to 5.72 g/L^{-1}, marking a 2.4-fold rise in productivity compared to standard conditions (408.57 mg/L^{-1}). The highest carotenoid content (0.3 mg/L^{-1}) was observed at peak levels of all factors. Maximum chlorophyll content (5.18 mg/L^{-1}) was achieved with elevated nitrate (1.76 mM), phosphate (0.054 mM), metals and vitamins concentrations. Meanwhile, starch content peaked at 42% DW with reduced nitrate and phosphate levels (0.58 mM and 0.027 mM) and restricted metals and vitamins. These limitations in nitrogen, phosphorous, metals, and vitamins shifted metabolism towards starch synthesis (Dammak et al., 2017).

Micro-Fourier Transform Infrared (FT-IR) Spectroscopy was employed to examine the spectral changes in three Cyanobacterium species (*Microcystis aeruginosa*, *Croooccus minutus*, and *Nostoc* sp.) and two Bacillariophyceae species (*Cyclotella meneghiniana* and *Phaeodactylum tricornutum*) under nutrient stress conditions. Over a 4-week starvation period, the cells physiological changes were tracked using key parameters and FT-IR spectroscopy. Variations in the FT-IR bands related to proteins, lipids, carbohydrates, and silicate were analyzed to estimate relative biomass composition. The findings indicate that short-term adaptations initially manifesting pigmentation and photosynthetic efficiency, while changes in biomass composition indicate longer term metabolic adjustments. Monitoring short- and long-term stress responses revealed that the metabolic strategies to manage increasing nutrient limitation vary significantly between species. FT-IR determines the single cell biomass composition in natural phytoplankton communities. FT-IR spectroscopy proves to be an effective and highly sensitive method for examining the species-specific alterations in the molecular composition of algae under various

growth-limiting conditions. Future efforts will focus on extracting data from FT-IR spectra to determine the actual C: N, C: P, or C: Si ratios per-cell basis (Stehfest et al., 2005).

According to a Gebhardt, K. A. (1976) study aims to discuss the impact of cadmium, copper and mercury on the brine shrimp, Artemia, found in the Great Salt Lake. Examined how these heavy metals affect the brine shrimp's susceptibility to toxicity, growth, reproduction, and hatching. The Great Salt Lake has higher concentrations of these metals compared to both freshwater and seawater. The experiments were conducted in salinities approximating the Great Salt Lake (150-320 g/L total dissolved solids). The study suggests that the toxic effects cadmium, copper, and mercury on brine shrimp may differ depending on the salt levels. When compared to other studies on heavy metals and marine creatures, the brine shrimp showed high resistance to cadmium and copper but only moderate resistance to mercury.

A study conducted by the Andrade et al., (2002) showed that copper compounds are frequently used to control algal blooms in fish and shrimp ponds. However, excessive use of copper can negatively impact the production of rotifers, subsequently affecting the overall yield. This study examined the combined effects of varying concentrations of copper on the growth of the saline water rotifer *B.plicatilis* at specific conditions. The population of *B.plicatilis* increased with higher algal levels. Rotifers exposed to 1 mg/L^{-1} of copper did not survive beyond 3 days. At 0.125 mg/L^{-1} of copper, there was a decrease in the *B.plicatilis* population only at low algal levels; at higher algal concentrations, growth was similar to the control, indicating that increased algal levels reduced copper toxicity. Copper presence reduced both growth and peak population densities of *B.plicatilis.* The findings suggest that algae are protective in mitigating heavy metal toxicity to rotifers.

Solene et al., (2011) investigated the impact of copper enrichment and changes in salinity on the growth, photosynthesis, and copper accumulation in two temperate brown seaweeds, *Ascophyllum nodosum* and *Fucus vesiculosus*. Increased copper concentration negatively affected photosynthesis and growth in both species, with 5 mg/L^{-1} causing severe inhibition and seaweed tip degradation. Copper toxicity was intensified under reduced salinity, impacting seaweed physiology sooner. Seaweed copper content correlated closely with water copper concentration, peaking after 1 day of exposure and increasing only with additional free copper. *A. nodosum's* copper content decreased at high water copper concentrations or low salinity, while *F. vesiculosus's* increased, indicating species-specific binding sites or uptake mechanisms. The findings highlight environmental changes direct and significant influence on algal physiology and metal binding capacity, emphasizing the importance of assessing algae's physiological status alongside thallus metal content for reliable biomonitoring.

The study examined the impact of heavy metal on the structure of various marine diatoms and a silicoflagellate. For *Thalassiosira aestivalis*, metals like Cu, Zn, Ge, and a metal mix caused granular, yellowed cytoplasm; disrupted chloroplasts, and delicate spines from the edges. These metals could be grouped based on their effects. Cu, Zn, Ge, and the metal mix increased chain length and prevented normal cell separation. They also hindered the formation of central chitinous threads. Metals like Hg, Cd, and Pb disrupted cell separation, forming clumps and elongated, bent cells, possibly affecting cell division. Metals Cr, Ni, Se, Sb, and AsIII causing cell lysis at higher concentrations. The similar effects of Cu, Zn, and Ge suggest they may impact a common biochemical pathway related to Si metabolism. Other diatoms and a silicoflagellate were affected by Cu and Hg (Hollibaugh et al., 1980).

The cupric ion (Cu^{2+}) serves as a nutrient and a toxic substance for freshwater and marine phytoplankton. It plays a role in various photochemical processes and can produce reactive oxygen species (ROS). Researchers have explored this dual nature to understand the effects of natural and human-induced copper releases on algal communities. Studies have examined how different organisms respond to varying copper levels, providing insights into the global distribution of significant phytoplankton species based on trace metal availability. This aims to consolidate data on the impact of copper levels on marine phytoplankton growth rates. Analysis suggests that the observed trends in phytoplankton sensitivity to copper largely depend on a single study. It explores the challenges in comparing these studies and discusses both consistent and conflicting results, focusing on taxonomic patterns and research methodologies. Additionally, introduce the first sensitivity measurements for marine unicellular diazotrophs using three strains of the nitrogen-fixing cyanobacterium, *Crocosphaera watsonii* (Lopez et al., 2019).

A Mowat and Reid (1977) study showed that the growth of *Phaeodactylum tricornutum*, Bohlin, *Tetraselmis spp* Stein, *Dunaliella primolecta* Butch., and *Hymenomonas elongata* (formerly *Cricosphaera elongate*) is not affected by the addition of copper, cadmium, or lead at concentrations below 10^{-4} M in batch culture using S88 medium. However, *Ditylum brightweii* undergoes osmotic disturbances and cell swelling when exposed to 10^{-3} M and 10^{-4} M copper. Phaeodactylum can withstand single doses of up to 10^{-3} M copper without growth reduction and shows significant copper uptake in continuous culture. On the other hand, *Hymenomonas elongata* can survive up to 10^{-4} M copper without a long term growth decrease but absorbs significantly less copper compared to *Phaeodactylum.*

Another study by Fanesi et al., (2019) they utilized Synchrotron FT-IR micro-spectroscopy combined with the partial least squares regression (PLSr) algorithm to measure protein, lipid, and carbohydrate levels at the individual cell level within a simulated phytoplankton community (consisting of a cyanobacterium, green alga, and diatom). The spectroscopic method employed in this study enables the quantification of macromolecules in individual phytoplankton cells. This approach suggests that population heterogeneity likely maintains a reservoir of non-acclimated cells ready to exploit new favorable environmental swiftly. Such insights challenge the average cell concept and highlight potential limitations in understanding phytoplankton population's dynamics and biogeochemical cycles in aquatic ecosystems.

Different microalgae species exhibit varying sensitivities to copper toxicity. Some species of the genus *Pseudo-nitzschia*, known for producing the phycotoxin domoic acid (DA), are believed to bind Cu and mitigate its toxic effects on cells. To better understand Cu impact on *Pseudo-nitzschia*, a toxic strain of *P. multiseries* and a non-toxic strain of *P. delicatissima* were exposed to varying concentrations of Cu (II) for 96 hours. Daily physiological assessments of the *Pseudo-nitzschia* cells were conducted using fluorescent probes and flow cytometry. These assessments included cell density measurements, lipid concentration, chlorophyll auto fluorescence, esterase activity, the percentage of dead algal cells, and counts of living and dead bacteria. Photosynthetic efficiency and oxygen consumption and production were also evaluated using pulse amplitude modulated fluorometry and an SDR Oxygen Sensor dish. Domoic acid content was quantified using ELISA kits. After 48 hours of Cu exposure, a significant increase in mortality was observed in *P. delicatissima*, whereas *P. multiseries* showed no change in survival compared to control cells. Esterase activity, chlorophyll autofluorescence, and lipid content in both strains increased significantly upon Cu exposure, with effects appearing earlier in *P. delicatissima* (24

hours) than in *P. multiseries* (up to 96 hours). Bacterial concentrations decreased significantly in *P. multiseries* when exposed to Cu, while bacterial levels remained similar between control and exposed *P. delicatissima* populations. Contrary to expectations, Cu exposure did not alter DA concentrations in *P. multiseries*. Furthermore, adding DA to non-toxic *P. delicatissima* did not improve cell survival, indicating that extracellular DA does not shield *Pseudo-nitzschia* spp. from copper toxicity. The findings suggest that *P. delicatissima* is more susceptible to copper toxicity than *P. multiseries*. This difference may not be attributed to DA production in *P. multiseries* but could be due to inherent species-specific differences in copper sensitivity or variations in bacterial communities associated with these algal species (Lelong et al., 2012).

Copper plays a crucial role in various physiological and metabolic processes, but its effects on phytoplankton cells are tightly regulated within a narrow range. Even slightly elevated concentrations can be toxic, impacting photosynthesis and respiration. In a study with the freshwater microalga *Selenastrum gracile*, cells exposed to different concentrations of free copper ions showed decreased cell density and increased production of total proteins and lipids. Interestingly, there was no significant effect on total carbohydrates. Additionally, copper exposure increased phospholipids and sterols, while there was a decrease in saturated fatty acids (Rocha et al., 2016).

Hyung et al., (2021) discovered a new euryhaline microalga, *Tetraselmis jejuensis* sp. thriving in supralittoral tide pools with salinities ranging from 0.3% to 3.1%. They isolated fifteen strains of *T. jejuensis* from Daejeong (DJ) and Yongduam (YO) and established clonal cultures in the lab. Morphological analysis showed that these cells have a flattened shape, four flagella arranged in two opposite pairs emerging from a depression near the apex, a cup-shaped chloroplast with a single pyrenoid surrounded by starch, and eyespot regions not positioned near the flagellar base.

These cells exhibit unique features distinct from other *Tetraselmis* species. Specifically, they display a honeycomb-like structure on the cell body surface and have a pyrenoid invaded by cytoplasmic channels containing electron-dense material and various small cytoplasmic channels in different directions. In phylogenetic analysis based on small subunit (SSU) rDNA sequences, the 15 strains from DJ and YO formed a distinct clade within the Chlorodendrophyceae, positioned at the base of clades containing *T. carteriiformi/subcordiformis, T. chuii/suecica,* and *T. striata/convolutae.* The strains in this unique clade were identified as belonging to the same species. The SSU rDNA sequences of these strains differed by a maximum of 1.53% and 1.19% from *Tetraselmis suecica* (MK541745), the closest species identified through phylogenetic analysis. Based on these morphological, molecular, and physiological characteristics, they propose a new species within the *Tetraselmis* genus, naming it *Tetraselmis jejuensis*, with "jejuensis" reflecting its discovery location on Jeju Island, Korea.

CHAPTER 3: METHODOLOGY

3.1. Materials and tools

The materials used in the experiment is the culture of *Tetraselmis indica*, distilled water, 10% HCl, $CuSO_4$, NaCl, seawater, components of f/2 media, 0.25% glutaraldehyde, 90% acetone, 5% phenol, conc H_2SO_4, 1mL glucose solution, 4% glutaraldehyde, ethanol and phosphate buffer solution. Tools used in the experiment are filtrations unit and GF/F filter paper, 100mL conical flasks, 500mL conical flasks, 250mL conical flasks, measuring cylinder, aluminium foil, 25mL and 100mL volumetric flasks, beakers, dropper, tissue paper, stoppered tubes, glass pipette, Eppendorf tubes, refractometer, Sedgwick rafter, microscope, cuvettes and spectrophotometer.

3.2. Disinfection procedure

Disinfecting the flasks and beakers is necessary to disable unwanted organisms in the equipment. The flasks and other equipment are washed with soap water thoroughly, then washed with tap water, rinsed and soaked in10% HCl overnight. The next day, it was washed with distilled water and dried before autoclaving.

3.3 Analysis of phytoplankton exposed to heavy metals

a. 6 flasks having Cu concentrations of 0.5 µM, 2.5 µM, 5 µM, 25 µM, 50 µM, and control was selected and subculture after every week till the start of the main experiment.

b. A seawater sample was collected and filtered using GF/F filter paper, autoclaved, and cooled down under running tap water to avoid crystal formation.

c. 16 flasks of 500mL were covered with aluminium foil and autoclaved.

d. 300 mL of f/2 media was prepared in each flask, and Cu sulphate of the above concentration was added, respectively. The experiment was conducted in triplicates along with control flasks.

e. From the 6 subculture flasks, the cells were counted from each flask using Sedgewick Rafter and inoculum volume was calculated to get 2000 cells/mL.

$C= (N\times 1000mm^3)/ (L\times D\times W\times S)$

Where: N= number of cells/ colonies counted

L= length of transect strip (mm)

W= width of transect strip (mm)

D= chamber depth (mm)

S= number of transects counted

f. Appropriate volume of inoculum was added in each flask, and the experimental flask was subjected to 16 hours of light and 8 hours of dark cycle for 21 days.

g. Samples were analyzed every 3 days for growth rate, carbohydrates, and pigments.

3.3.1 Growth rate

a. 980 µL of sample from respective flasks were taken in Eppendorf tube and fixed with 0.25% glutaraldehyde and stored in a refrigerator, and cells were counted using Sedgewick Rafter. The graph was plotted of cell count v/s number of days

b. The growth rate, generation time and doubling time were calculated using the formula:

Growth rate (μ) = 1n (N_t/N_0) × 1/t

Where N_t = final number of cells

N_0 = initial number of cells

t = time

Generation time = 1n (2) / μ

Doubling time = μ / 1n (2)

3.3.2 Total Carbohydrate

a. 1mg/mL of standard glucose solution was prepared for the standard plot. Different concentration of glucose solution from the standard solution was prepared, having concentrations 20, 40, 80, 150, 200 and 500 µg/mL. 2 mL of each concentration was taken in stoppered tubes respectively, 0.5 mL of 5% phenol, and 5 mL of concentrated H_2SO_4 was added, incubate for 15 mins and check absorbance at 490nm. Similarly, prepare blank using distilled water. Plot a standard graph of absorbance v/s concentrations.

b. 2 mL of the sample was taken from each respective flasks in stoppered tubes and 0.5 mL of 5% phenol was added and 5 mL of concentration H_2SO_4 was added. Tubes were incubated for 15 minutes and absorbance was taken at 490nm.

c. The amount of carbohydrate of the sample was calculated from the standard graph.

d. Samples were taken for analysis every 3 days from day 0 to day 21.

3.3.3 Pigment analysis

a. 2 mL of sample was centrifuged at 5000*g for 10 minutes in order to measure the pigments.

b. The pellet was suspended in 2 mL of 90% acetone and again the solution was centrifuged at 5000*g for 5 minutes.

c. Absorbance at A_{666}, A_{653}, and A_{470} were measured to quantify pigments using the equations.

d. Samples for pigment analysis were taken on every 3 days from day 0 to day 21.

1. [Chlorophyll *a*] (mg/L^{-1})=$15.65*A_{666}$ --$7.340*A_{653}$
2. [Chlorophyll *b*] (mg/L^{-1}) = $27.05*A_{653} – 11.21*A_{666}$
3. [Total Chlorophylls] (mg/L^{-1}) = [Chlorophyll *a*] + [chlorophyll *b*] (Dammak et al., 2017).

3.3.4 Scanning Electron Microscopy

a. Samples were taken from 5 sub cultured flasks for scanning electron microscopy to analyze the biofilms associated with the polymers.

b. 1670 µL samples were fixed with 330 µL of 4% Glutaraldehyde for 2-23 hours.

c. Subsequently samples were transferred to a 50% ethanol and phosphate buffer saline solution and kept at -20^0c until analysis.

d. Samples were dehydrated on the ice through an ethanol series 50%, 70%, 85%, 95% (10 min in each concentration) followed by 3 cycles (15 minutes each) in 100% ethanol; air dried sputter coated with gold, and observed with Scanning Electron Microscope (Davidov et al., 2020).

3.3.5 Fourier Transform Infrared Spectroscopy (FT-IR)

a.1.5-2 mL of the cell suspension centrifuged at 5000 g for 10 minutes, pellet was then resuspended in distilled water to remove salts and cell debris.

b. The sample was then centrifuged again (8000 g for 8 min) and resuspended in 10–20 μL of distilled water.

c. 2 μL of the suspension were deposited on a silicon micro plate (384 wells, Bruker) and dried in a cabinet drier.

d. Spectra were recorded in transmission mode with 32 scans co-added and averaged in the spectral range 4000–700 cm^{-1} with a resolution of 4 cm^{-1}.

e. Background signal was measured scanning an empty well using the same acquisition settings (Fanesi et al., 2019)

CHAPTER 4: ANALYSIS AND CONCLUSION

Result

4.1 Growth curve and growth rate of *Tetraselmis indica*

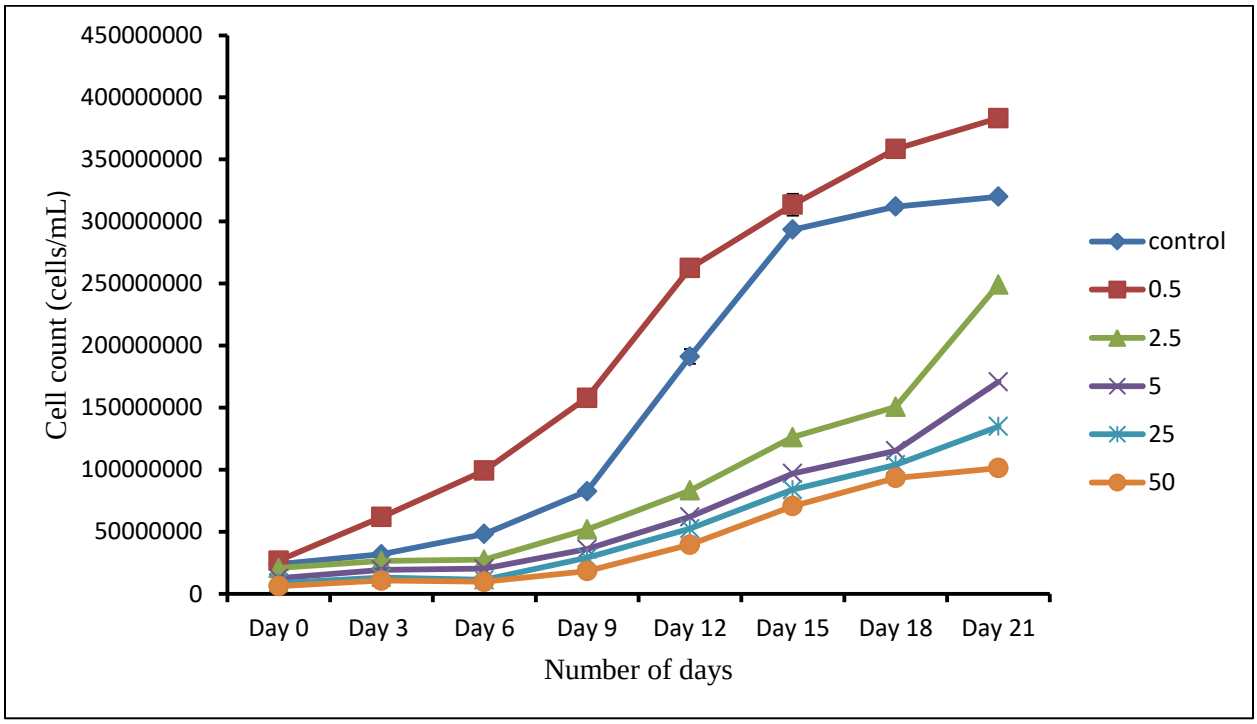

Figure 4.1: Growth curve of *Tetraselmis indica* exposed to different Cu concentration

The above figure showed that *T.indica* had a typical growth phase, with a very short or no lag phase followed by an exponential phase and reaching a stationary phase. In the presence of 0.5µM Cu concentration, cells thrived from day 3 to day 21, while in the control flask, cells reached the stationary phase by day 15. However, in the presence of 2.5 µM Cu concentration, cells grew slower compared to the control flask. Growth patterns were similar for 5 µM and 25 µM Cu concentration, and growth was slower in 50 µM Cu concentration. Overall, T.indica showed the ability to grow under exposure to different Cu concentrations, but the growth rates decreased with increased Cu concentration.

Cu concentration	Growth rate (d^{-1})	Doubling time (d)	Generation time ($1/d^{-1}$)
Control	0.784	0.392	2.549
0.5 µM	0.742	0.371	2.693
2.5 µM	0.638	0.319	3.108
5 µM	0.571	0.285	3.498
25 µM	0.101	0.050	1.974
50 µM	0.085	0.042	3.207

Table 4.1: Growth rate, doubling time and generation time of *T. indica*

The growth rate of *T. indica* decreased with an increase in Cu concentration, thus showing the adverse effects of Cu on phytoplankton (table 4.1).

Figure 4.2: *Tetraselmis indica* culture

4. 2. Total Carbohydrate

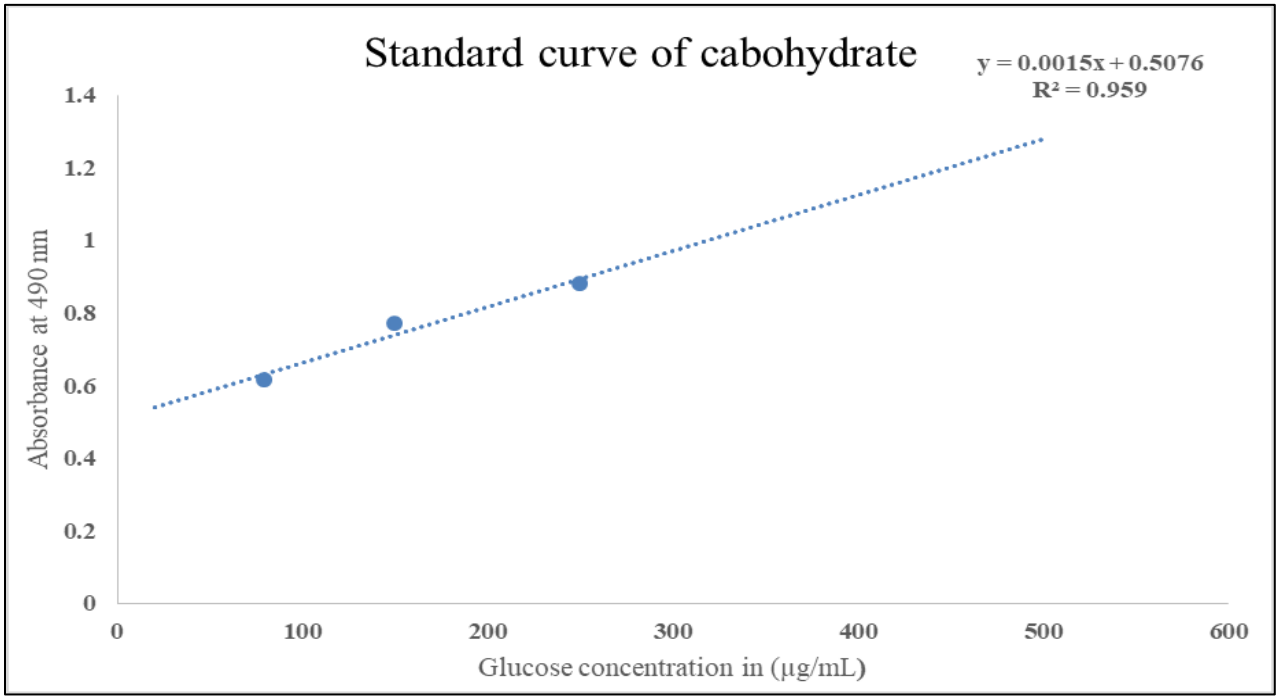

Figure 4.3: Standard plot of carbohydrate

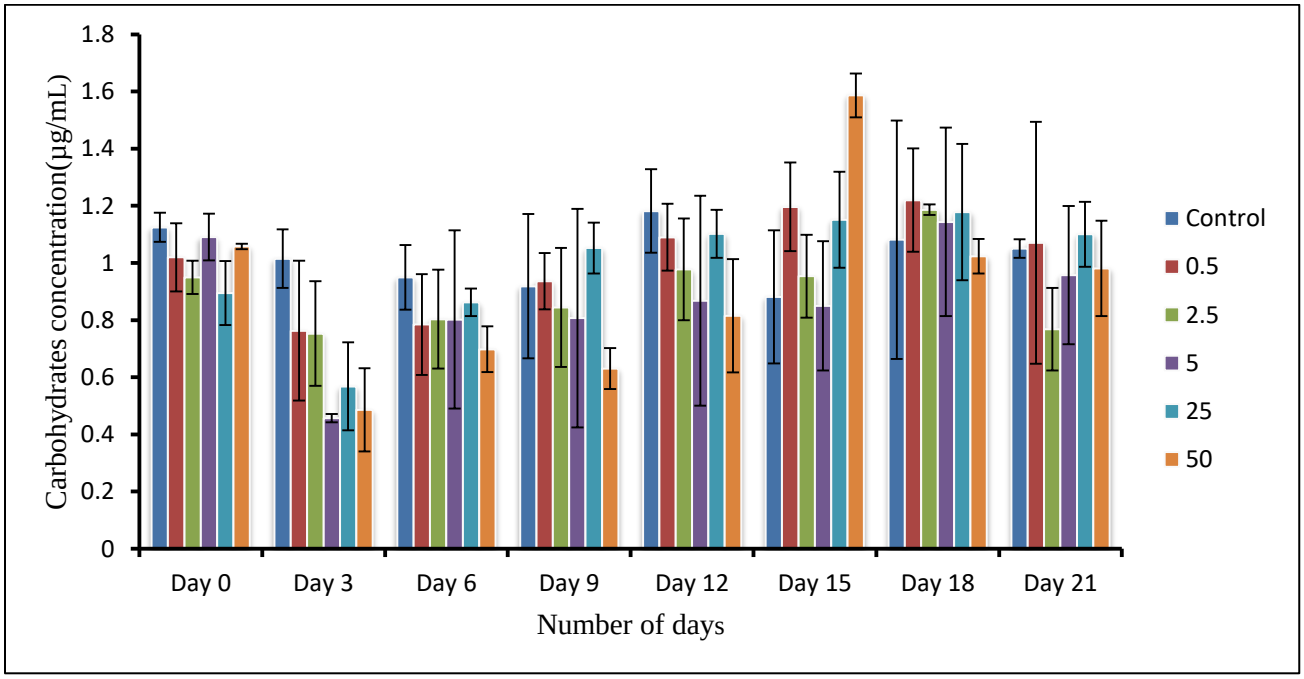

Figure 4.4: Effect of Cu on the total carbohydrate of *T. indica*

The graph illustrates that the total carbohydrate levels increase with exposure time. From day 3 to day 12, the amount of carbohydrates was more in control, but on day 15, the amount of carbohydrate in the Cu-exposed flask increased, showing a significant increase in 50 µM Cu concentration. At first, the total carbohydrate in 25 µM and 50 µM Cu concentrations was less but increased as the number of days of exposure increased, showing that even in high Cu concentration, the phytoplankton was showing high levels of total carbohydrate production as a mechanism for bioremediation.

4.3 Effect of Cu on pigments

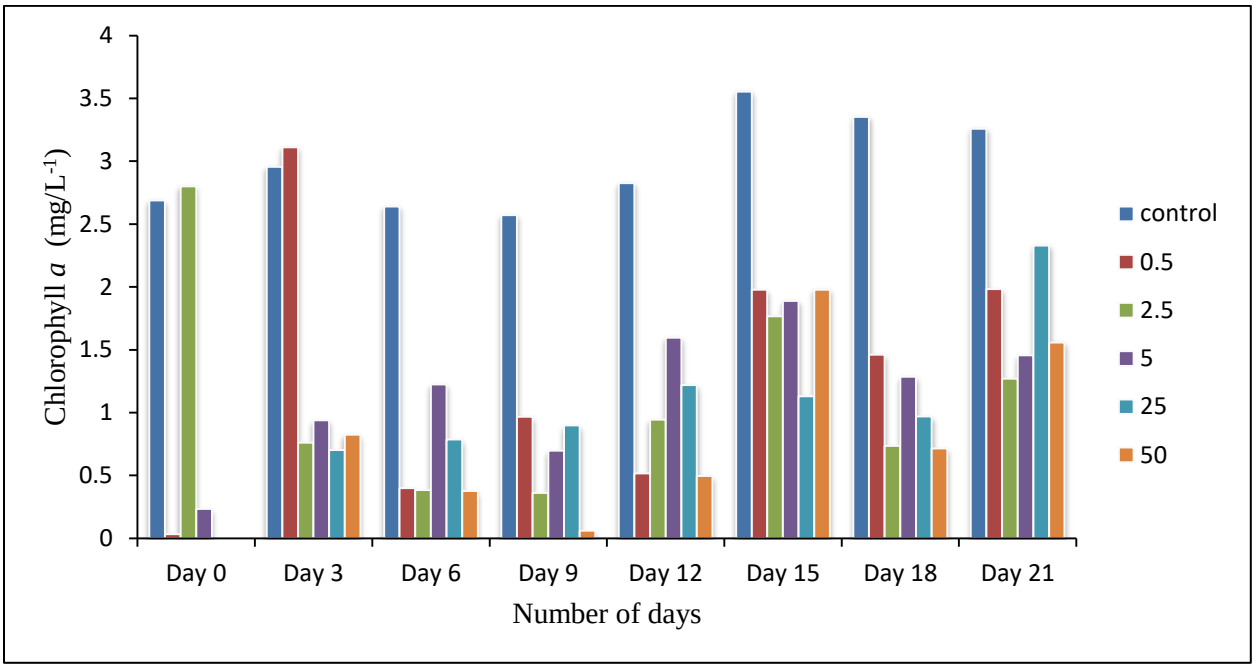

Figure 4.5: Effect of Cu on chlorophyll *a* content of *Tetraselmis indica*

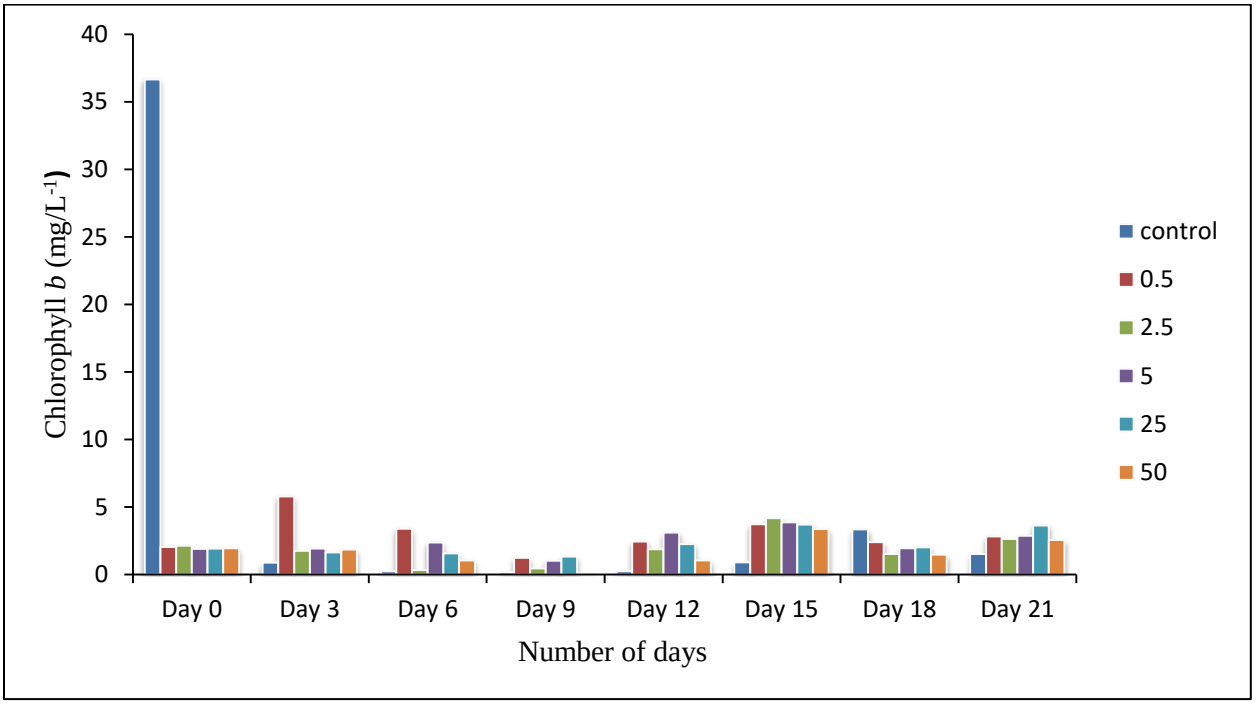

Figure 4.6: Effect of Cu on chlorophyll *b* content of *T. indica*

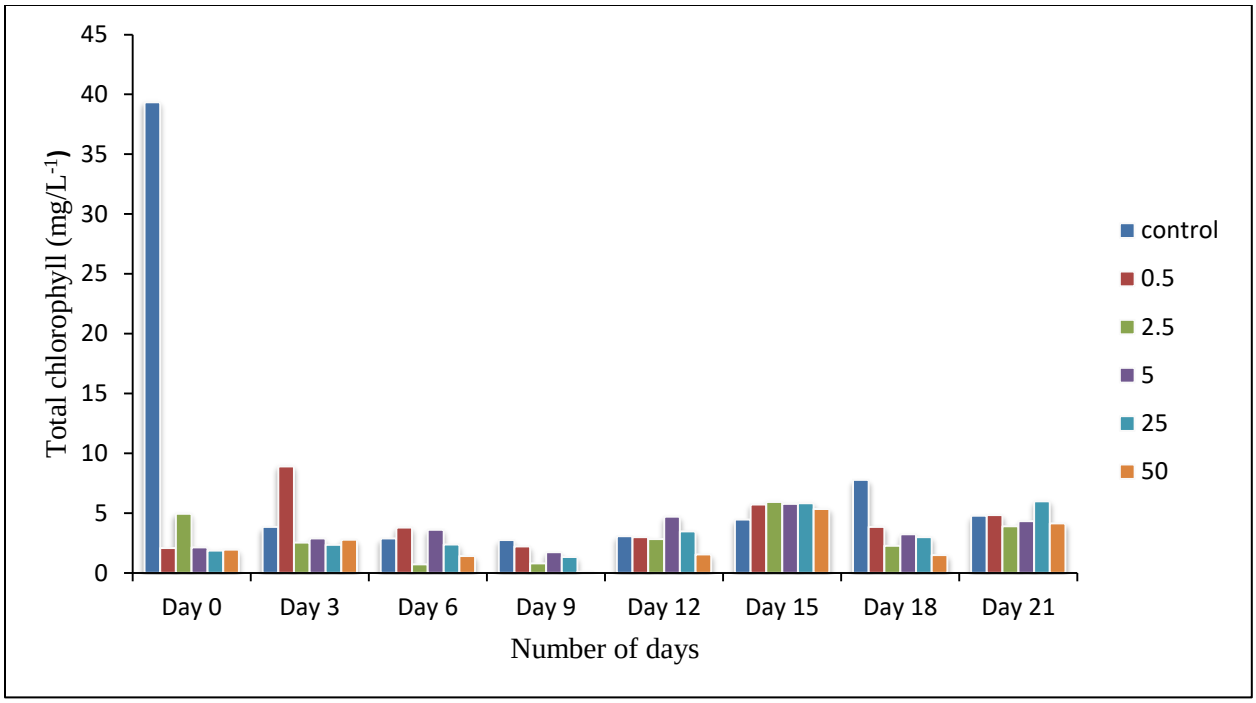

Figure 4.7: Effect of Cu on total chlorophyll of *Tetraselmis indica*

From figure 4.5-4.7 it was noted that the chlorophyll *a*, chlorophyll *b* and total chlorophyll content in *T. indica* decreased with increase in exposure time and with an increase in Cu concentration but in chlorophyll *a* only control flask is increasing with increase in exposure time.

4.4 Scanning Electron Microscopy

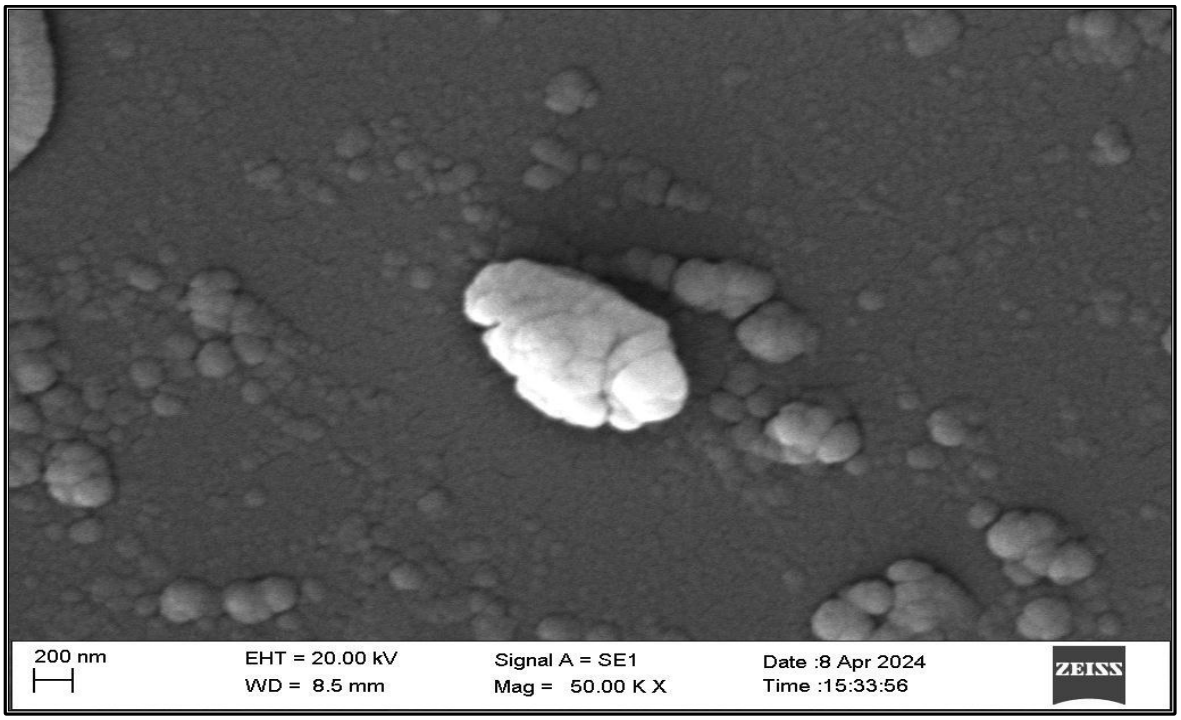

Figure 4.8: Scanning electron microscopy of *Tetraselmis indica*

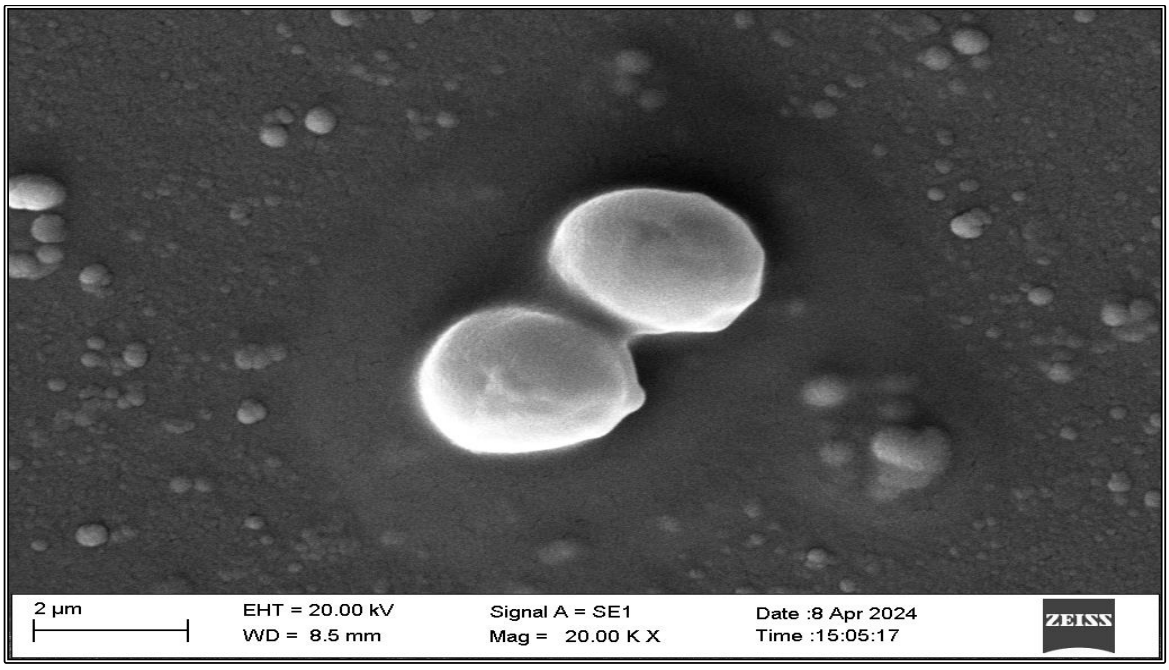

Figure 4.9: *Tetraselmis indica* in 0.5 µM Cu concentration

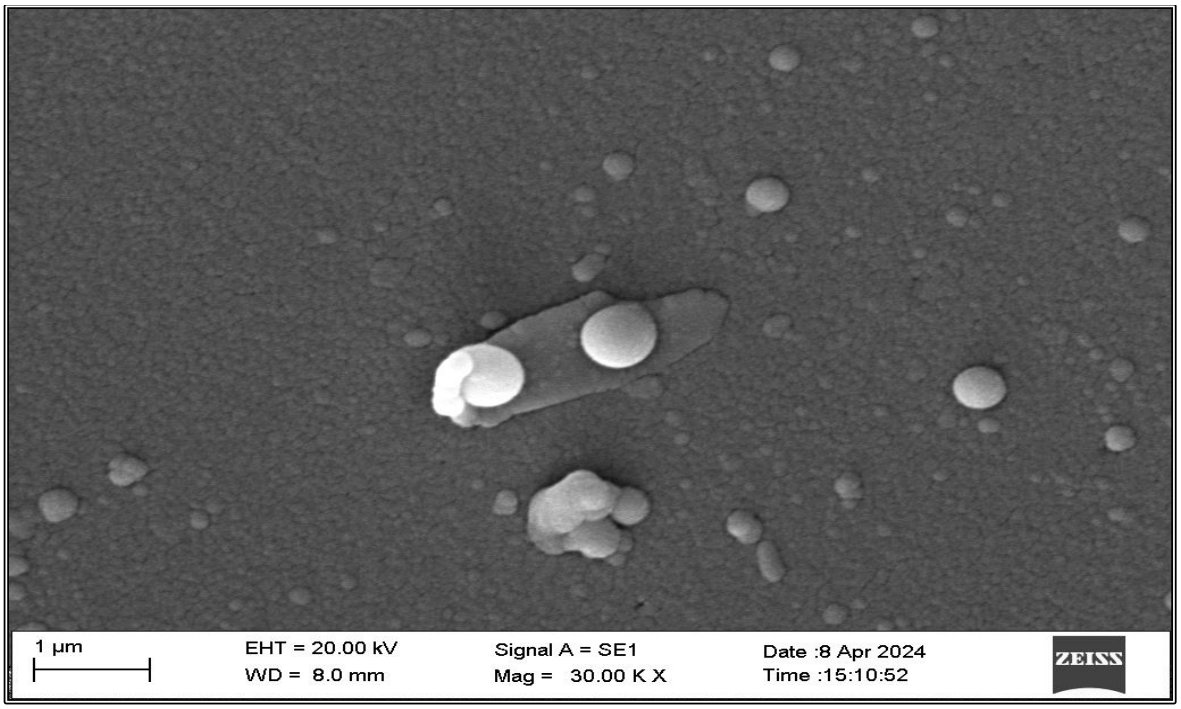

Figure 4.10: *Tetraselmis indica* in 2.5 µM Cu concentration

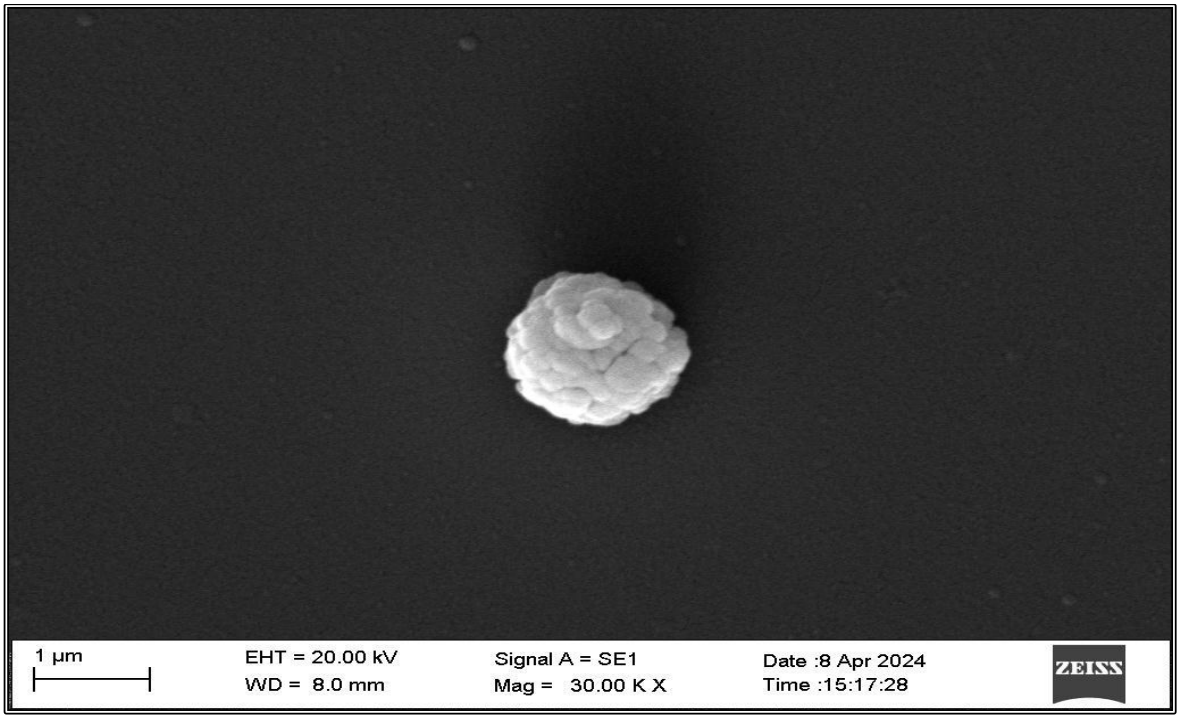

Figure 4.11: *Tetraselmis indica* in 5 µM Cu concentration

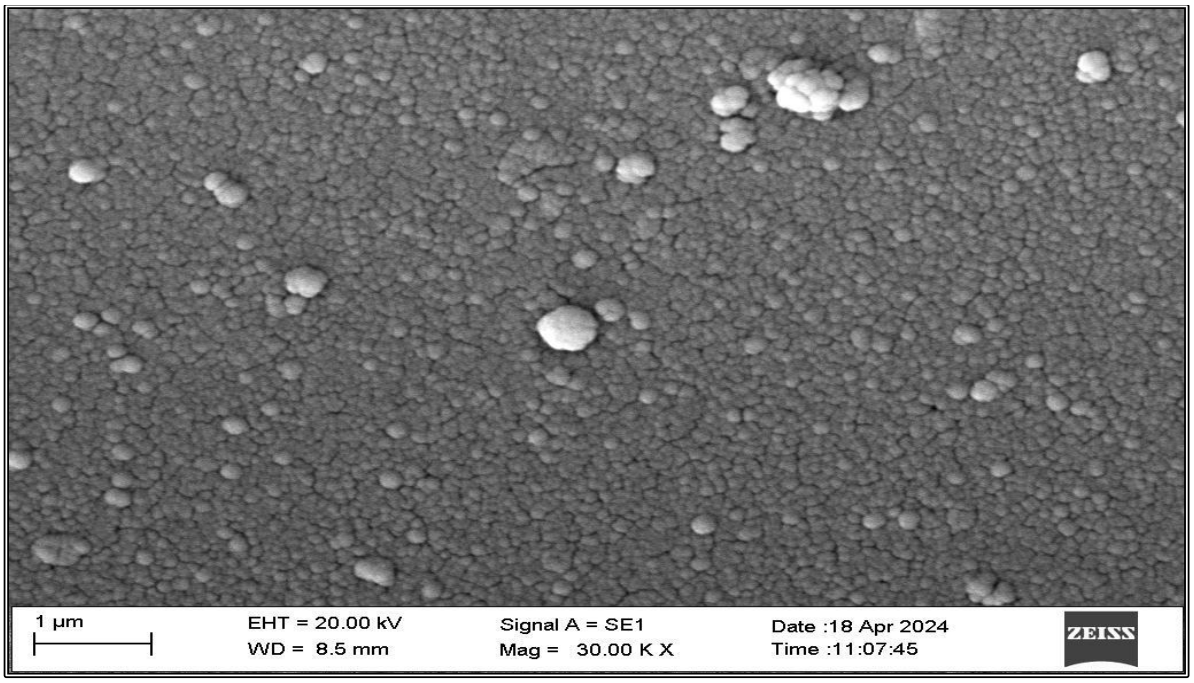

Figure 4.12: *Tetraselmis indica* in 25 µM Cu concentration

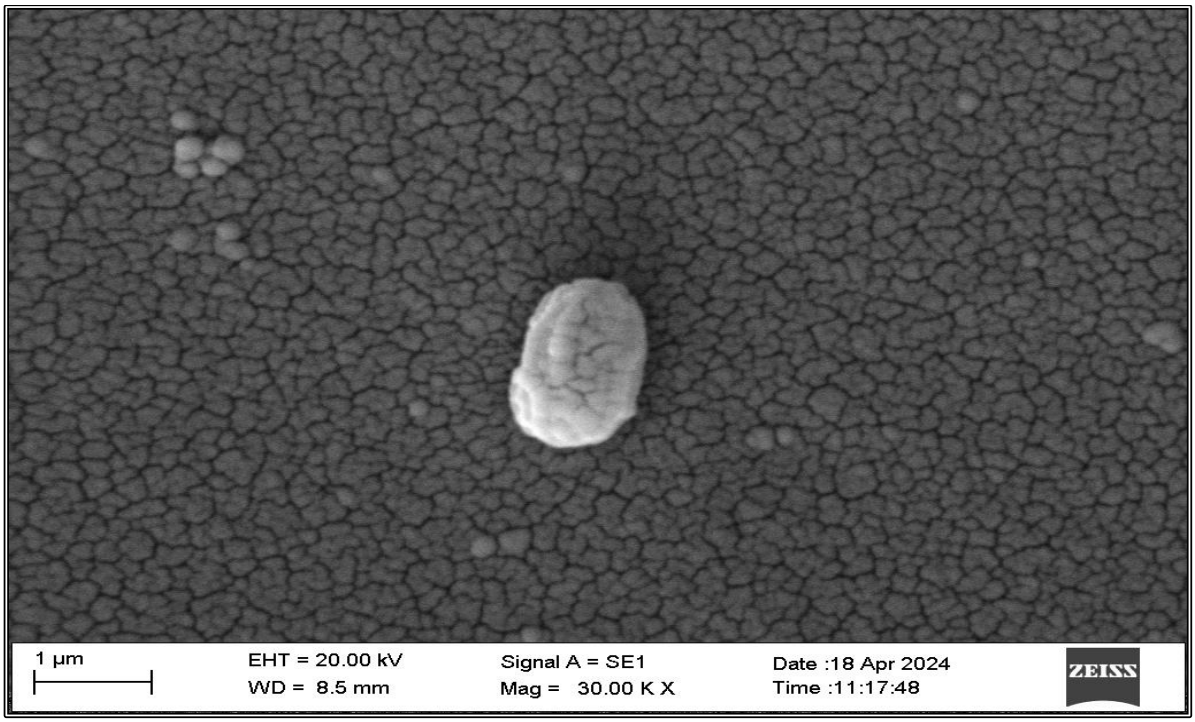

Figure 4.13: *Tetraselmis indica* in 50 µM Cu concentration

From figure 21-26 SEM of *Tetraselmis indica,* **4.8.** Narrow lateral view. **4.9.** Dividing cells that are still enclosed by the parent cell wall. **4.10**. Individual cells. **4.11**. Broad lateral view. **4.12**. Scatter of cells. **4.13**. Posterior view with scales.

4.5 FT-IR (Fourier transform-infrared spectroscopy)

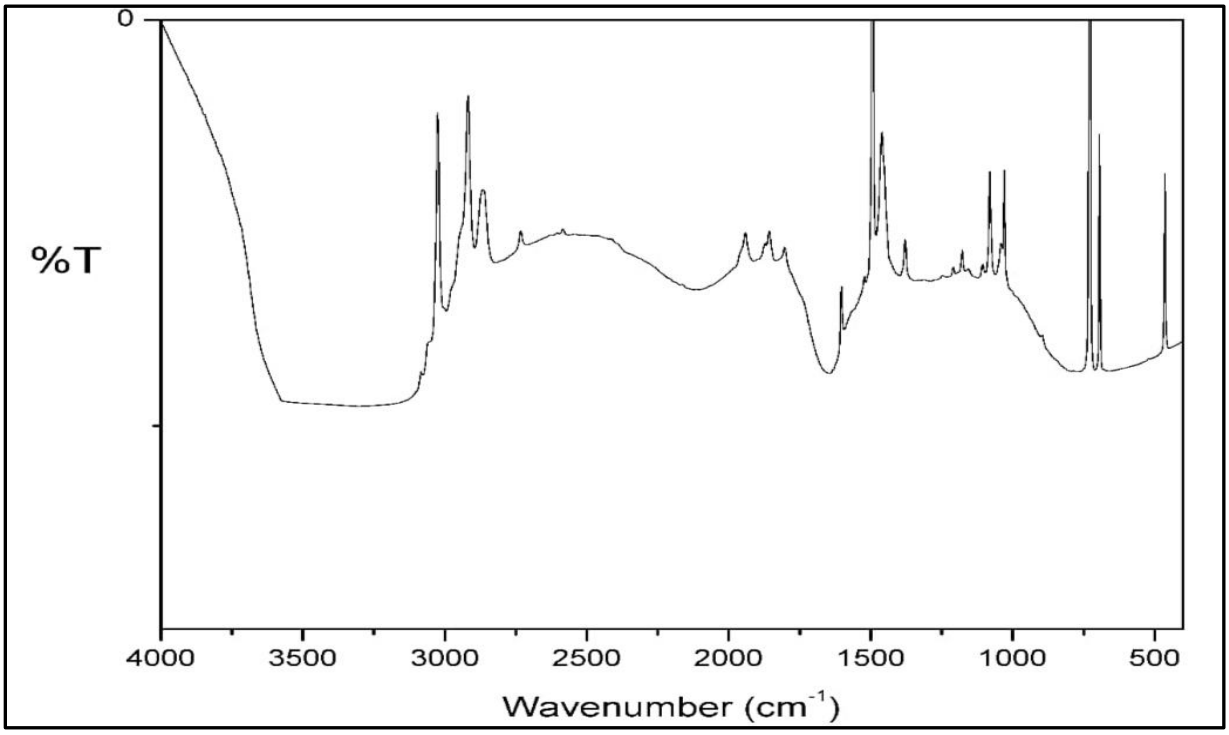

Figure 4.14: FT-IR spectra of *Tetraselmis indica*

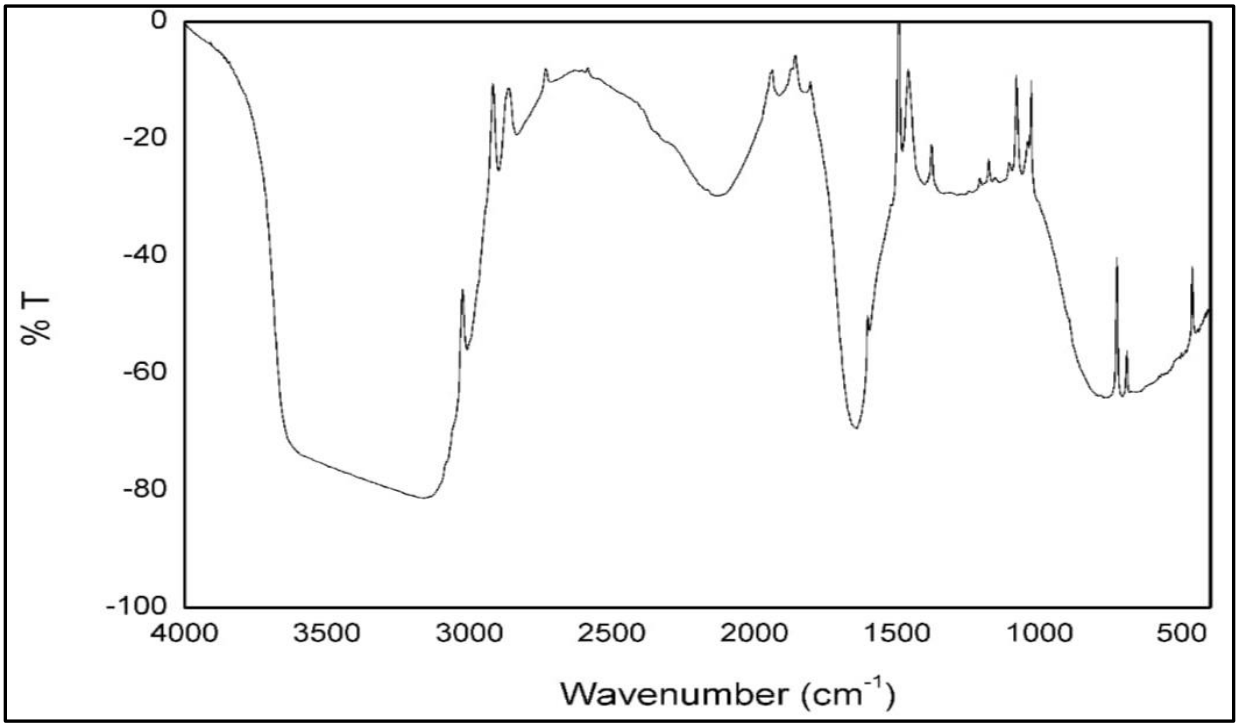

Figure 4.15: FT-IR spectra of *Tetraselmis indica* with 0.5 µM Cu concentration

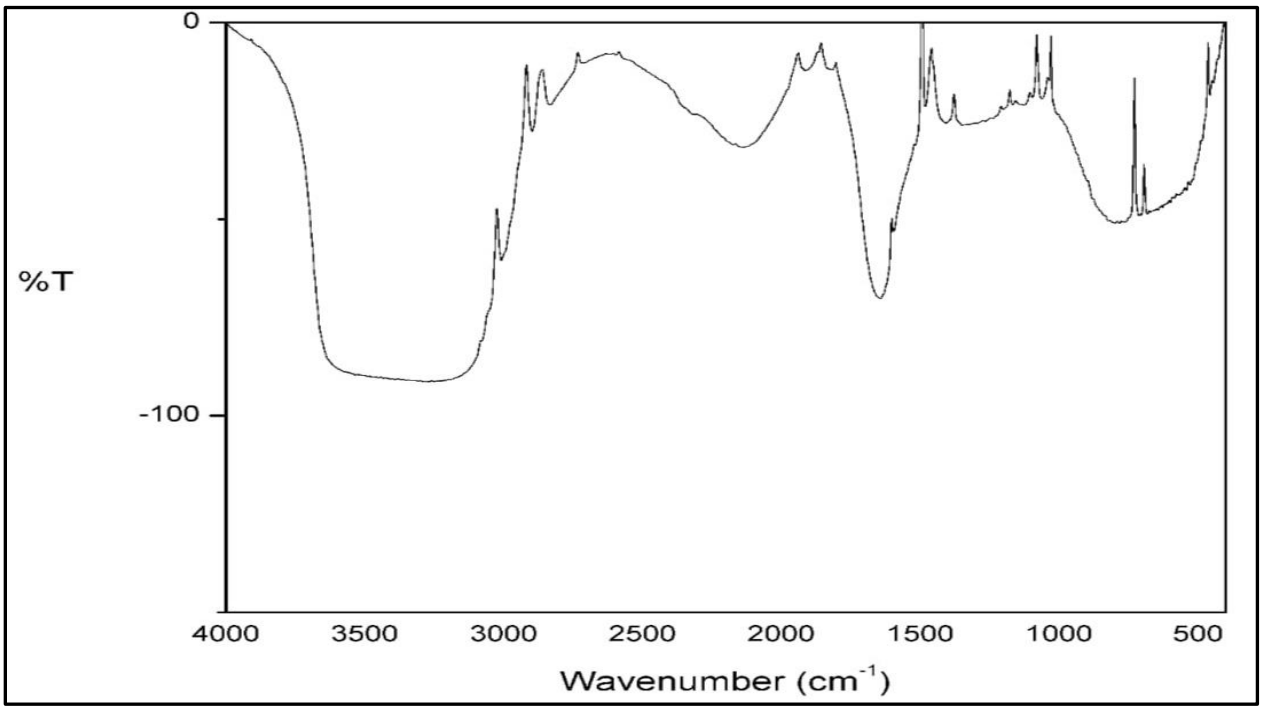

Figure 4.16: FT-IR spectra of *Tetraselmis indica* with 2.5 µM Cu concentration

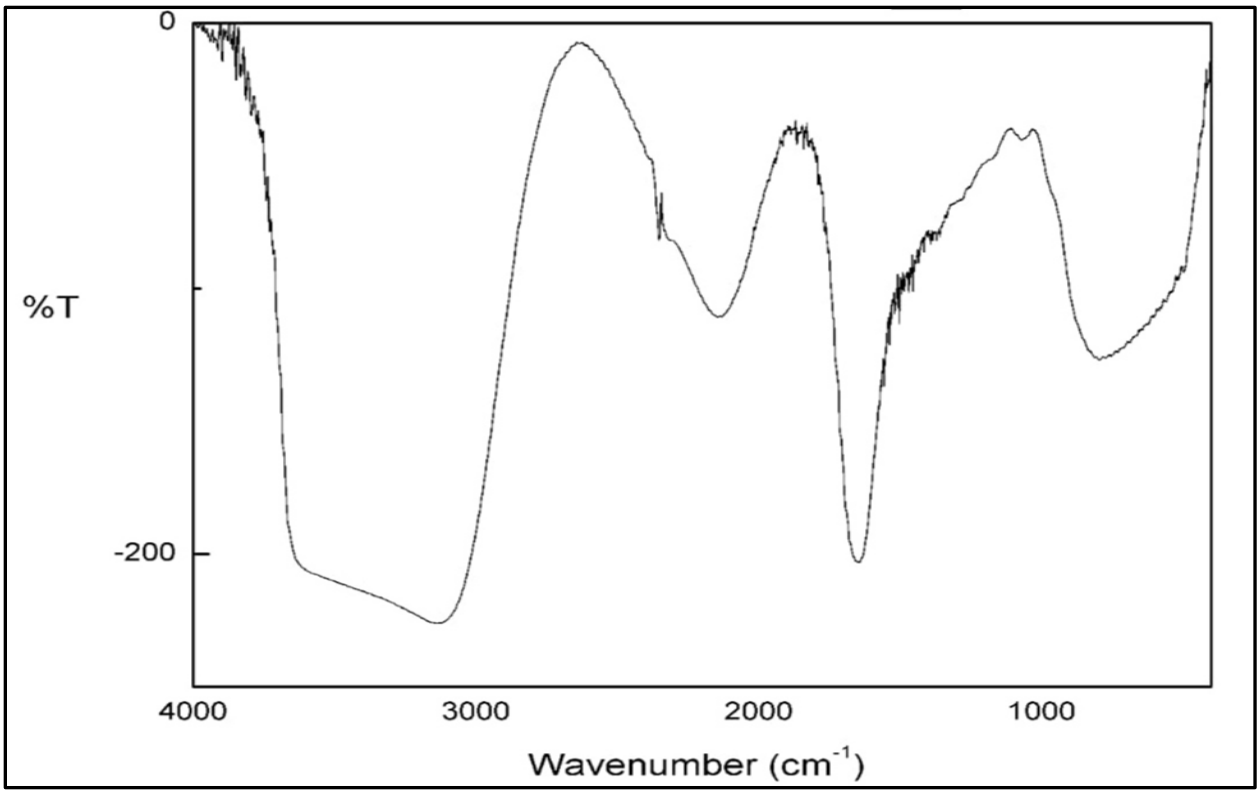

Figure 4.17: FT-IR spectra of *Tetraselmis indica* with 5 µM Cu concentration

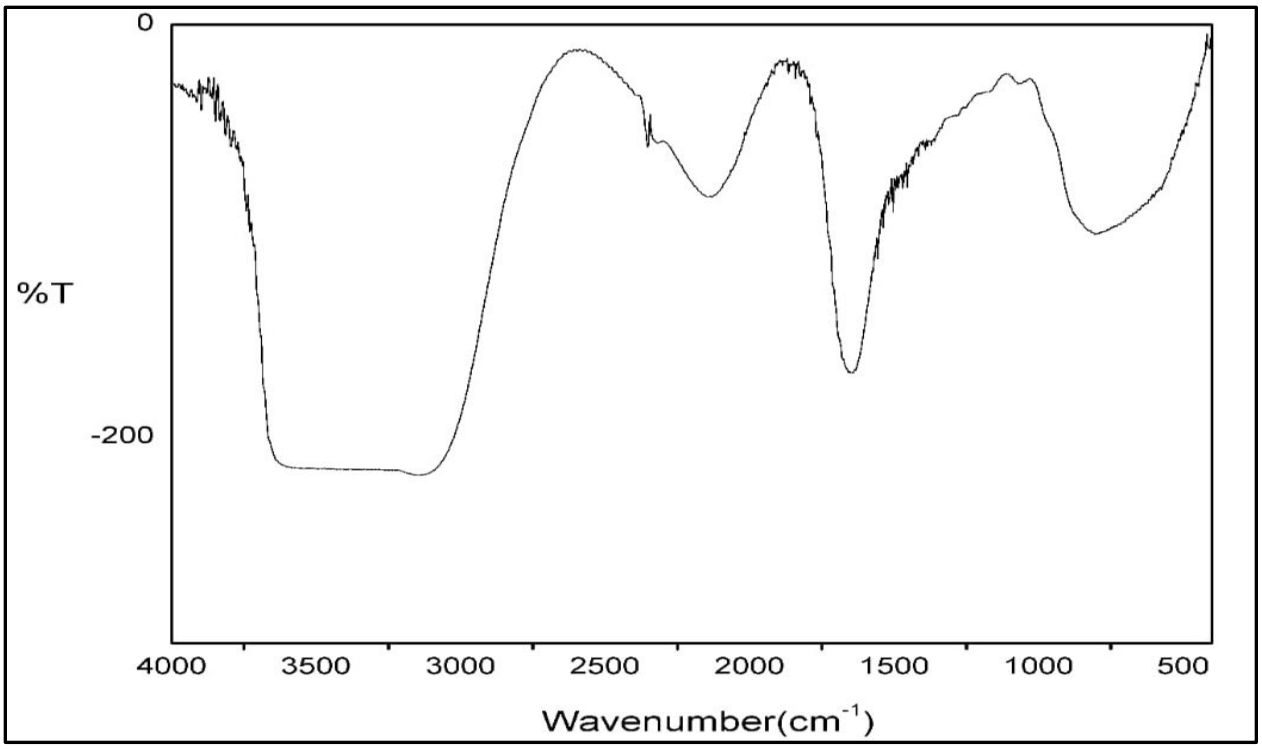

Figure 4.18: FT-IR spectra of *Tetraselmis indica* with 25 µM Cu concentration

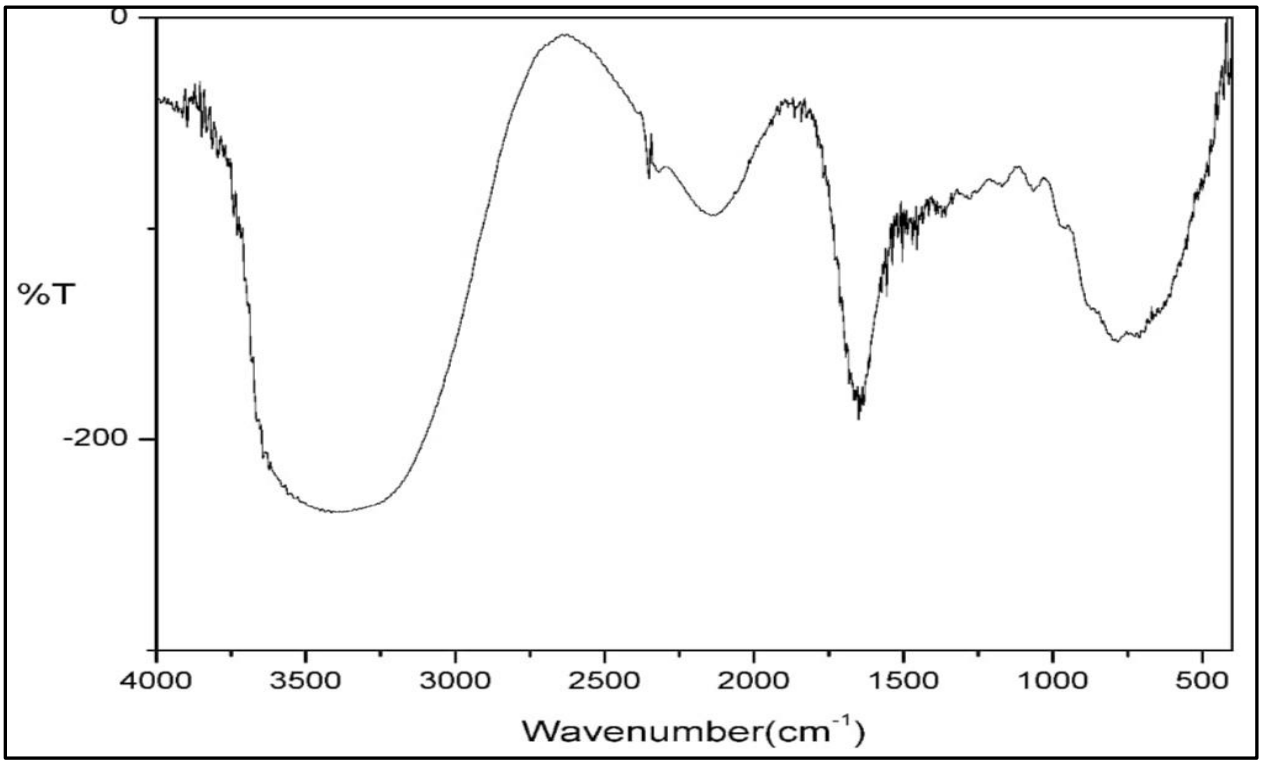

Figure 4.19: FT-IR spectra of *Tetraselmis indica* with 50 µM Cu concentration

The FTIR graph (figure 4.14) indicates various absorption peaks corresponding to different functional groups within the phytoplankton sample. These include O-H stretching vibrations around 3300 cm^{-1}, C-H stretching vibrations around 2800-3000 cm^{-1}, amide bands indicative of proteins between 1650-1550 cm^{-1}, C-O stretching vibrations around 1200-1000 cm^{-1}. In an FTIR spectrum (figure 4.15), peaks on the graph correspond to molecular bond vibrational frequencies, aiding in identifying functional groups. For phytoplankton cultures, expect broad peaks (3200-3600 cm^{-1}) indicating O-H groups, sharp peaks (2800-3000 cm^{-1}) suggesting C-H stretching, and peaks around 1650 cm^{-1} indicating C=O stretching. Additionally, peaks at 1500-1600 cm^{-1} could relate to N-H bending vibrations, while those below 1500 cm^{-1} offer intricate structural insights in the fingerprint region. Figure 4.16 indicates 3000-2800 cm^{-1}: peaks from C-H stretching vibrations in lipids. 1700-1600 cm^{-1}: peaks linked to C=O stretching vibrations, indicating proteins, lipids, and nucleic acids. 1500-1200 cm^{-1}: peaks due to N-H bending and C-N stretching vibrations, typical of proteins and nucleic acids. 1200-900 cm^{-1}: peaks associated with C-O and C-C stretching vibrations, often found in carbohydrates and polysaccharides. In figure 4.17 these could include broad peaks around 3200-3600 cm^{-1} indicating O-H stretching from alcohols, phenols, or water, peaks near 2800-3000 cm^{-1} associated with C-H stretching in lipids, a strong peak around 1650 cm^{-1} corresponding to C=O stretching in proteins or lipids, peaks around 1500-1600 cm^{-1} indicating N-H bending or C=C stretching in amino acids or other nitrogen-containing compounds, and peaks in the region of 1200-1000 cm^{-1} due to C-O stretching in carbohydrates or phospholipids. Figure 4.18 shows 3000-2800 cm^{-1}: this region often shows peaks due to C-H stretching vibrations in lipids. 1700-1600 cm^{-1}: this area may display peaks associated with protein amide I bands (C=O stretching) and amide II bands (N-H bending). 1500-1200 cm^{-1}: this region can show peaks due to various vibrations, including those

from phospholipids and nucleic acids. 1200-900 cm^{-1}: this is known as the fingerprint region and can contain many peaks that are specific to the molecular structure of the compounds present. The peaks in the graph (figure 4.19) correspond to the vibrational frequencies of the bonds within the molecules of the sample. Each type of bond (e.g., C-H, N-H, O-H, C=O) has a characteristic absorption range, which can be used to identify the presence of certain functional groups in the sample. Broad peaks around 3200-3600 cm^{-1}, which could indicate the presence of O-H groups, possibly from alcohols, phenols, or water. Sharp peaks near 2800-3000 cm^{-1}, which could be indicative of C-H stretching vibrations from aliphatic compounds. Peaks around 1650 cm^{-1}, which might suggest C=O stretching vibrations, possibly from proteins, lipids, or other organic compounds. Peaks in the region of 1500-1600 cm^{-1} could be associated with N-H bending vibrations from amine groups in proteins or other nitrogen-containing compounds. Peaks below 1500 cm^{-1}, which are often referred to as the fingerprint region, can provide very specific information about the molecular structure.

Discussion

The result in figure 4.1 showed that *Tetraselmis indica* had a typical growth phase, with very short or no lag phase followed by an exponential phase and reaching a stationary phase. In the presence of 0.5µM Cu concentration, cells thrived from day 3 to day 21, while in the control flask, cells reached the stationary phase by day 15. However, in the presence of 2.5 µM Cu concentration, cells grew slower than the control flask. Growth patterns were similar for 5 µM and 25 µM Cu concentrations; growth was slower in 50 µM Cu concentration. Overall, *T.indica* showed the ability to grow under exposure to different Cu concentrations, but the growth rates decreased with increased Cu concentration. The study by Hindarti and Larasati (2019) demonstrated a decrease in cell density with increased heavy metal concentration in *Nitzschia* species. In another study, high Cu concentration slowed down growth and peak population densities in *B. plicatilis* (Andrade et al., 2002). The study Solene et al., (2011) investigated with Ascophyllum nodosum and Fucus vesiculosus showed that the increased Cu concentration negatively affected photosynthesis and growth in both species. In another study, with *Skeletonema costatum* exposed to Cu found that the phytoplankton had a longer growth phase than *S. costatum* growing in media without Cu, indicating that it was trying to adapt in the environment with Cu and aiding in bioremediation (Pratama et al., 2020).

The growth rate of *T. indica* decreased with an increase in Cu concentration, thus showing the negative effects of Cu on phytoplankton (table 4.1). According to Tavares et al., (2017), a decline in phytoplankton growth rates can trigger alterations in the oceanic food web and nutrient biogeochemical cycles. In a study by Rani et al., (2020), the impact of Cu on the growth of *T. indica* was investigated using a batch culture system. The research revealed that *T. indica* experienced a notable decrease in growth rate when exposed to Cu concentrations exceeding 0.5

mg/L. Additionally, these conditions resulted in an extended lag phase and reduced maximum biomass yield for *T. indica*. Similarly, a study conducted by Basha et al., (2019) examined the effects of Cu on *T. indica* growth in a semi-continuous culture setup. The findings indicated that *T. indica* exhibited a marked reduction in growth rate with Cu concentrations above 0.2 mg/L. Moreover, Cu exposure induced alterations in cell morphology and photosynthetic pigment levels.

From the graph (figure 4.4), we can see that total carbohydrate levels increase with an increase in the exposure time. From day 3 to day 12 the amount of carbohydrates was more in control, but on day 15, the amount of carbohydrates in the Cu-exposed flask increased, showing a major increase in 50 µM Cu concentration. At first, the total carbohydrate in 25 µM and 50 µM Cu concentrations was less but increased as the number of days of exposure time increased, showing that even in high Cu concentration, the phytoplankton was showing high levels of total carbohydrate production as a mechanism for bioremediation. In one study, the amount of total carbohydrate produced in the diatom *Cylindrotheca fusiformis* and the dinoflagellates *Gymnodinium* species rise with increased copper concentration. Research on the precise mechanisms by which Cu exposure affects the production of total carbohydrates in *T. indica* is limited. Some studies have proposed potential explanations for this observed phenomenon. One possible explanation is that exposure to copper can alter the composition of the culture medium, impacting total carbohydrate production. For instance, a study by Rani et al., (2020) discovered that copper exposure reduced the pH of the culture medium, potentially affecting the availability of carbon sources necessary for carbohydrate synthesis.

In the study by Silva et al., (2018), it showed that Cu changed the chemical composition of biomolecules in *Scenedesmus quadricauda*, even in 1.0 µM Cu concentration, there was a 10-

fold increase in carbohydrate synthesis that in control. Another possible explanation is that Cu exposure might enhance the activity of enzymes associated with carbohydrate metabolism. For instance, a study by Li et al., (2017) revealed that Cu exposure resulted in elevated activity of enzymes related to pentose sugars and NADPH, a reducing agent essential for many biosynthetic reactions, including carbohydrate synthesis.

From figure 4.5-4.7 it was noted that the chlorophyll *a*, chlorophyll *b* and total chlorophyll content in *T. indica* decreased with an increase in exposure time and with an increase in Cu concentration but in chlorophyll *a* only control flask increased with increase in exposure time. A study by Basha et al., (2019) investigated the effect of Cu on the chlorophyll content of *T. indica* in a semi-continuous culture system. The study found that Cu exposure significantly reduced the chlorophyll a, b and total chlorophyll content of T. indica at Cu concentration above 0.2 mg/L.

If the amounts of chlorophyll *a, b,* and *c* in phytoplankton exposed to heavy metals are reduced, this may indicate a detrimental effect of the metal exposure on the photosynthetic system of the phytoplankton (Gao et al., 2018). Cu has been shown in another study to reduce photosynthetic activity by interfering with the electron transport chain and substituting magnesium in the center of chlorophyll molecules, which harms the antenna complex and photosystems (Silva et al., 2018). The exact method of elevated Cu concentration influencing *T. indica's* chlorophyll content is still unknown. Still, a number of studies have proposed mechanisms. One theory on potential mechanisms is that exposure to copper may cause oxidative stress, which can harm the photosynthetic machinery and lower the amount of chlorophyll. Rani et al., (2020) discovered that *T. indica* exposed to Cu had higher amounts of reactive oxygen species, which can oxidative damage proteins, lipids, and pigments. In the end, this damage may cause the amount of chlorophyll to decrease. The findings indicated that *Nitzschia* sp. intracellular pigment content

decreased as the concentration of heavy metals increased. According to the study by Hindarti and Larasati (2019), on chlorophyll-*a* content. For *Nitzschia* sp., the LOEC was 0.18 mg Cu/L and 0.18 mg Cd/L, but the NOEC was 0.01 mgCu/L and less than 0.18 mg Cd/L. Copper and cadmium toxicity had a substantial impact on the carotenoid content of toxicant concentrations of 0.1 mg Cu/L and 0.1mg Cd/L. Another possible mechanism is that exposure to Cu may also interfere with chlorophyll biosynthesis. Cu is a necessary cofactor for a number of the enzymes involved in the production of chlorophyll, and elevated Cu concentrations may hinder this process. This interference may cause the amount of chlorophyll to decrease.

From figure 4.8-4.13 SEM of *T. indica***,** 4.8. Narrow lateral view. 4.9. Dividing cells that are still enclosed by the parent cell wall. 4.10. Individual cells. 4.11. Broad lateral view. 4.12. Scatter of cells. 4.13. Posterior view with scales.

CONCLUSION

Understanding how copper impacts the growth and physiology of *Tetraselmis indica* indicates that it likely inhibits growth and disrupts physiological functions. This knowledge is crucial for safeguarding the health of our oceans and the diverse life forms relying on them. The effects of heavy metals, like copper, on phytoplankton can be complex and diverse, influenced by factors such as metal type, concentration, phytoplankton species, and physiological conditions. Our recent investigation observed typical growth patterns in *T. indica*, characterized by a short or nonexistent lag phase, followed by exponential growth and reaching a stationary phase. With prolonged exposure, there was an increase in total carbohydrate production, aiding in the organism's defense mechanisms. However, heavy metal exposure can adversely affect phytoplankton, including decreased growth rates and reduced chlorophyll content. Further research is necessary to fully comprehend the scope of these effects and explore potential mitigating strategies.

REFERENCES

Anderson, D. M., Glibert, P. M., & Burkholder, J. M. (2002). Harmful algal blooms and eutrophication: nutrient sources, composition, and consequences. *Estuaries*, *25*, 704-726.

Armbrust, E. V. (2009). The life of diatoms in the world's oceans. *Nature*, *459*(7244), 185-192.

Arora, M., Anil, A. C., Burgess, K., Delany, J., & Mesbahi, E. (2015). Asymmetric cell division and its role in cell fate determination in the green alga *Tetraselmis indica.* Journal of biosciences, 40, 921-927.

Arora, M., Anil, A. C., Leliaert, F., Delany, J., & Mesbahi, E. (2013). *Tetraselmis indica* (Chlorodendrophyceae, Chlorophyta), a new species isolated from salt pans in Goa, India. *European Journal of Phycology*, *48*(1), 61-78.

Balch, W. M., Drapeau, D. T., Bowler, B. C., Lyczskowski, E., Booth, E. S., & Alley, D. (2011). The contribution of coccolithophores to the optical and inorganic carbon budgets during the Southern Ocean Gas Exchange Experiment: New evidence in support of the “Great Calcite Belt” hypothesis. *Journal of Geophysical Research: Oceans*, *116*(C4).

Behrenfeld, M. J., & Falkowski, P. G. (1997). A consumer's guide to phytoplankton primary productivity models. *Limnology and oceanography*, *42*(7), 1479-1491.

Bentley-Mowat, J. A., & Reid, S. M. (1977). Survival of marine phytoplankton in high concentrations of heavy metals, and uptake of copper. *Journal of experimental marine biology and ecology*, *26*(3), 249-264.

Bidle, K. D., & Falkowski, P. G. (2004). Cell death in planktonic, photosynthetic microorganisms. *Nature Reviews Microbiology*, *2*(8), 643-655.

Bowler, C., Vardi, A., & Allen, A. E. (2010). Oceanographic and biogeochemical insights from diatom genomes. *Annual review of marine science*, *2*, 333-365.

Buayam, N., Davey, M. P., Smith, A. G., & Pumas, C. (2019). Effects of Copper and pH on the Growth and Physiology of Desmodesmus sp. AARLG074. Metabolites, 9(5), 84.

Connan, S., & Stengel, D. B. (2011). Impacts of ambient salinity and copper on brown algae: 1. Interactive effects on photosynthesis, growth, and copper accumulation. Aquatic Toxicology, 104(1-2), 94-107.

Dammak, M., Hadrich, B., Miladi, R., Barkallah, M., Hentati, F., Hachicha, R., ... & Abdelkafi, S. (2017). Effects of nutritional conditions on growth and biochemical composition of *Tetraselmis* sp. *Lipids in Health and Disease*, *16*, 1-13.

Dammak, M., Hlima, H. B., Tounsi, L., Michaud, P., Fendri, I., & Abdelkafi, S. (2022). Effect of heavy metals mixture on the growth and physiology of *Tetraselmis* sp.: Applications to lipid production and bioremediation. *Bioresource Technology*, *360*, 127584.

De Vargas, C., Audic, S., Henry, N., Decelle, J., Mahé, F., Logares, R., ... & Velayoudon, D. (2015). Eukaryotic plankton diversity in the sunlit ocean. *Science*, *348*(6237), 1261605.

Dodge, J. D. (1989). Records of marine dinoflagellates from North Sutherland (Scotland). *British Phycological Journal*, *24*(4), 385-389.

Fanesi, A., Wagner, H., Birarda, G., Vaccari, L., & Wilhelm, C. (2019). Quantitative macromolecular patterns in phytoplankton communities resolved at the taxonomical level by single-cell Synchrotron FTIR-spectroscopy. *BMC plant biology*, *19*, 1-14.

Field, C. B., Behrenfeld, M. J., Randerson, J. T., & Falkowski, P. (1998). Primary production of the biosphere: integrating terrestrial and oceanic components. *science*, *281*(5374), 237-240.

Finkel, Z. V., Beardall, J., Flynn, K. J., Quigg, A., Rees, T. A. V., & Raven, J. A. (2010). Phytoplankton in a changing world: cell size and elemental stoichiometry. *Journal of plankton research*, *32*(1), 119-137.

Gebhardt, K. A. (1976). Effects of heavy metals (cadmium, copper, and mercury) on reproduction, growth, and survival of brine shrimp (Artemia salina) from the Great Salt Lake.

Glibert, P. M., Berdalet, E., Burford, M. A., Pitcher, G. C., & Zhou, M. (2018). Harmful algal blooms and the importance of understanding their ecology and oceanography. *Global ecology and oceanography of harmful algal blooms*, 9-25.

Hallegraeff, G. M. (2010). Ocean climate change, phytoplankton community responses, and harmful algal blooms: a formidable predictive challenge 1. *Journal of phycology*, *46*(2), 220-235.

Hindarti, D., & Larasati, A. W. (2019, March). Copper (Cu) and Cadmium (Cd) toxicity on growth, chlorophyll-a and carotenoid content of phytoplankton *Nitzschia* sp. In IOP

Conference Series: Earth and Environmental Science (Vol. 236, p. 012053). IOP Publishing.

Kapkov, V. I., Belenikina, O. A., & Fedorov, V. D. (2011). Effect of heavy metals on marine phytoplankton. *Moscow University Biological Sciences Bulletin*, *66*, 32-36.

Kumar, K. S., & Shin, K. H. (2017). Effect of copper on marine microalga *Tetraselmis suecica* and its influence on intra-and extracellular iron and zinc content. *Korean Journal of Ecology and Environment*, *50*(1), 16-28./

Litchman, E., de Tezanos Pinto, P., Edwards, K. F., Klausmeier, C. A., Kremer, C. T., & Thomas, M. K. (2015). Global biogeochemical impacts of phytoplankton: a trait-based perspective. *Journal of ecology*, *103*(6), 1384-1396.

Lopez, J. S., Lee, L., & Mackey, K. R. (2019). The toxicity of copper to *Crocosphaera watsonii* and other marine phytoplankton: a systematic review. *Frontiers in Marine Science*, *5*, 511.

Luna-Andrade, A., Aguilar-Duran, R., Nandini, S., & Sarma, S. S. S. (2002). Combined effects of copper and microalgal (*Tetraselmis suecica*) concentrations on the population growth of Brachionus plicatilis Müller (Rotifera). Water, Air, and Soil Pollution, 141, 143-153.

Mallick, N. (2004). Copper-induced oxidative stress in the chlorophycean microalga *Chlorella vulguris*: response of the antioxidant system. *Journal of Plant Physiology*, 165 (5), 591-597.

Mitra, A., & Flynn, K. J. (2005). Predator–prey interactions: is 'ecological stoichiometry'sufficient when good food goes bad?. *Journal of Plankton Research*, *27*(5), 393-399.

Naik, S. M., & Anil, A. C. (2017). Long-term preservation of *Tetraselmis indica* (Chlorodendrophyceae, Chlorophyta) for flow cytometric analysis: Influence of fixative and storage temperature. *Journal of microbiological methods*, *139*, 123-129.

Naik, S. M., & Anil, A. C. (2018). Influence of darkness on pigments of *Tetraselmis indica* (Chlorodendrophyceae, Chlorophyta). *Journal of Photochemistry and Photobiology B: Biology*, *186*, 17-22.

Nowicka, B. (2022). Heavy metal–induced stress in eukaryotic algae—mechanisms of heavy metal toxicity and tolerance with particular emphasis on oxidative stress in exposed cells and the role of antioxidant response. *Environmental Science and Pollution Research*, *29*(12), 16860-16911.

Paerl, H. W., & Otten, T. G. (2013). Harmful cyanobacterial blooms: causes, consequences, and controls. *Microbial ecology*, *65*, 995-1010.

Pérez, P., Estévez-Blanco, P., Beiras, R., & Fernández, E. (2006). Effect of copper on the photochemical efficiency, growth, and chlorophyll *a* biomass of natural phytoplankton assemblages. Environmental Toxicology and Chemistry: An International Journal, 25(1), 137-143.

Rocha, G. S., Parrish, C. C., Lombardi, A. T., & da GG Melão, M. (2016). Copper affects biochemical and physiological responses of *Selenastrum gracile* (Reinsch). *Ecotoxicology*, *25*, 1468-1477.

Saadaoui, W., Gamboa-Rosales, H., Sifuentes-Gallardo, C., Durán-Muñoz, H., Abrougui, K., Mohammadi, A., & Tarchoun, N. (2022). Effects of lead, copper and cadmium on bioaccumulation and translocation factors and biosynthesis of photosynthetic pigments in Vicia faba L.(Broad Beans) at different stages of growth. *Applied Sciences*, *12*(18), 8941.

Sánchez-Baracaldo, P., Raven, J. A., Pisani, D., & Knoll, A. H. (2017). Early photosynthetic eukaryotes inhabited low-salinity habitats. *Proceedings of the National Academy of Sciences*, *114*(37), E7737-E7745.

Silva, J. C., Echeveste, P., & Lombardi, A. T. (2018). Higher biomolecules yield in phytoplankton under copper exposure. *Ecotoxicology and environmental safety*, *161*, 57-63.

Smetacek, V., & Zingone, A. (2013). Green and golden seaweed tides on the rise. *Nature*, *504*(7478), 84-88.

Smith, M. A. (1983). The effect of heavy metals on the cytoplasmic fine structure of Skeletonema costatum (Bacillariophyta). *Protoplasma*, *116*, 14-23.

Stehfest, K., Toepel, J., & Wilhelm, C. (2005). The application of micro-FTIR spectroscopy to analyze nutrient stress-related changes in biomass composition of phytoplankton algae. *Plant Physiology and biochemistry*, *43*(7), 717-726.

Thangaradjou, T., & Bhatt, J. R. (2018). Status of seagrass ecosystems in India. *Ocean & coastal management*, *159*, 7-15.

Thomas, W. H., Hollibaugh, J. T., & Seibert, D. L. (1980). Effects of heavy metals on the morphology of some marine phytoplankton. *Phycologia*, *19*(3), 202-209.

Von Dassow, P., John, U., Ogata, H., Probert, I., Bendif, E. M., Kegel, J. U., ... & De Vargas, C. (2015). Life-cycle modification in open oceans accounts for genome variability in a cosmopolitan phytoplankton. *The ISME journal*, *9*(6), 1365-1377.

Wagner, H., Jakob, T., & Wilhelm, C. (2006). Balancing the energy flow from captured light to biomass under fluctuating light conditions. *New Phytologist*, *169*(1).

Wang, H., Ebenezer, V., & Ki, J. S. (2018). Photosynthetic and biochemical responses of the freshwater green algae *Closterium ehrenbergii* Meneghini (Conjugatophyceae) exposed to the metal coppers and its implication for toxicity testing. *journal of microbiology*, *56*, 426-434.

Whitton, B. A. (Ed.). (2012). *Ecology of cyanobacteria II: their diversity in space and time*. Springer Science & Business Media.

Yang, K., Chen, Q., Zhang, D., Zhang, H., Lei, X., Chen, Z., ... & Xu, H. (2017). The algicidal mechanism of prodigiosin from Hahella sp. KA22 against Microcystis aeruginosa. *Scientific reports*, *7*(1), 7750.

APPENDIX

Preparation of f/2 media

The seawater was collected from Siridao Beach in cans and it was filtered through GF/F filter paper. The salinity was checked using refractometer and was found out to be 35. The filtered seawater was then autoclaved and cooled down under running tap water to avoid the formation of crystals. Take 950mL of filtered autoclaved seawater and add the following components.

Composition of f/2 media

Components	**Stock solution**	**Quantity**
$NaNO_3$	75 g/L dH_2O	1 mL
$NaH_2PO_4.H_2O$	5 g/L dH_2O	1 mL
Trace metal solution	See below	1 mL
Vitamin solution	See below	0.5 mL

f/2 trace metal solution

Components	**Stock solution**	**Quantity**
$FeCl_3.6H_2O$	---	3.15g
$Na_2EDTA.2H_2O$	---	4.36g
$CuSO_4.5H_2O$	9.8g/L.DH_2O	1 mL
$Na_2MoO_4.2H_2O$	6.3g/L.DH_2O	1 mL
$Zn_2SO_4.2H_2O$	22.0g/L.DH_2O	1 mL
$CoCl_2.6H_2O$	10.0g/L.DH_2O	1 mL
$MnCl_2..4H_2O$	180.0g/L.DH_2O	1 mL
Autoclave		

f/2 vitamin solution

Components	**Stock solution**	**Quantity**
Thiamine HCl (vit. B_1)	----	200mg
Biotin (vit. H)	0.1g/L DH_2O	10 mL
Cyanocobalamin (vit. B_{12})	1.0g/L DH_2O	1 mL
Filter sterilized using 0.2mm filter paper		

Preparation of 100 mL conc. NaCl

To the 100 mL of distilled water add NaCl until the solution becomes saturated.

Preparation of 10% HCl

To the 900 mL distilled water add 100 mL of HCl.

Preparation of 5% phenol

To the 100 mL of distilled water add 5 g of crystalline phenol.

Preparation of 90% acetone

To the 90 mL of acetone add 10 ml of distilled water.

Preparation of 1L Phosphate Buffered Saline (PBS) (1x)

Components	**g/L**
Sodium chloride (NaCl)	8
Potassium chloride (KCl)	0.2
Disodium hydrogen phosphate (Na_2HPO_4)	1.44
Potassium dihydrogen phosphate (KH_2PO_4)	0.24

Adjust pH to 7.4

Preparation of 100 mL 50% ethanol in Phosphate Buffered Saline (PBS)

Add 50 mL of ethanol in 50 mL PBS

Preparation of ethanol series

50%

Add 50 mL ethanol and 50 mL distilled water.

75%

Add 75 mL ethanol and 25 mL distilled water.

85%

Add 85 mL ethanol and 15 mL distilled water.

95%

Add 95 mL ethanol and 5 mL distilled water.