Copper-induced stress and its effect on *Tetraselmis indica* (Chlorodendrophyceae, Chlorophyta)

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by

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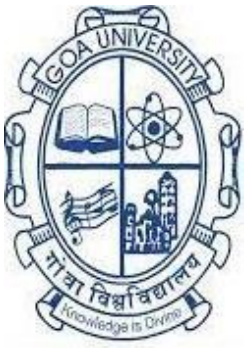

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I hereby declare that the data presented in this Dissertation entitled, "Copper- induced stress and its effect on *Tetraselmis indica* (Chlorodendrophyceae, Chlorophyta)" is based on the results of investigations carried out by me in the Marine Microbiology 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 be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation **"Copper- induced stress and its effect on *Tetraselmis indica* (Chlorodendrophyceae, Chlorophyta)"** is a bonified work carried out by **Ms. Janica Sequeira** under my supervision in partial fulfilment 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.

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LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Phytoplankton are important primary producers in aquatic ecosystems and are required for the survival of marine life. They are, nevertheless, subject to anthropogenic pollution, such as heavy metal contamination. Copper (Cu) 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.

T. indica was subjected to various Cu concentrations to see how it affected growth, pigment, and biochemical composition. The results showed that Cu considerably lowered *T. indica's* growth rate and pigment concentration. *T. indica's* cellular composition was altered by exposure to varied Cu concentrations, according to biochemical analyses.

Overall, the findings indicate that *T. indica* is susceptible to Cu toxicity, which can cause considerable changes in its growth, pigment synthesis, and biochemical composition. These discoveries have ramifications for the health and productivity of marine ecosystems and the aquaculture and fishing sectors that rely on phytoplankton for food. As a result, it is critical to create effective solutions for reducing Cu pollution and protecting the health of marine ecosystems.

1. INTRODUCTION

Phytoplankton are microscopic, photosynthetic organisms that form the base of many aquatic food webs. They are found in both marine and freshwater environments and 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 diversity is vast, with over 5,000 species identified to date (Falkowski et al, 1998). These organisms exhibit a wide range of shapes, sizes, and pigmentation, which allows them to occupy different niches within the water column (Litchman et al, 2007). 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). They can regulate global climate by sequestering carbon dioxide from the atmosphere. They also have important applications in biotechnology, aquaculture, and climate change research. For example, some phytoplankton species produce bioactive compounds that have potential applications in developing new drugs and medicines, while others are used as food sources in aquaculture. Additionally, the study of phytoplankton and their responses to environmental changes provide valuable sights into the impacts of climate change on marine and freshwater ecosystems (Behrenfeld and Falkowski, 1997).

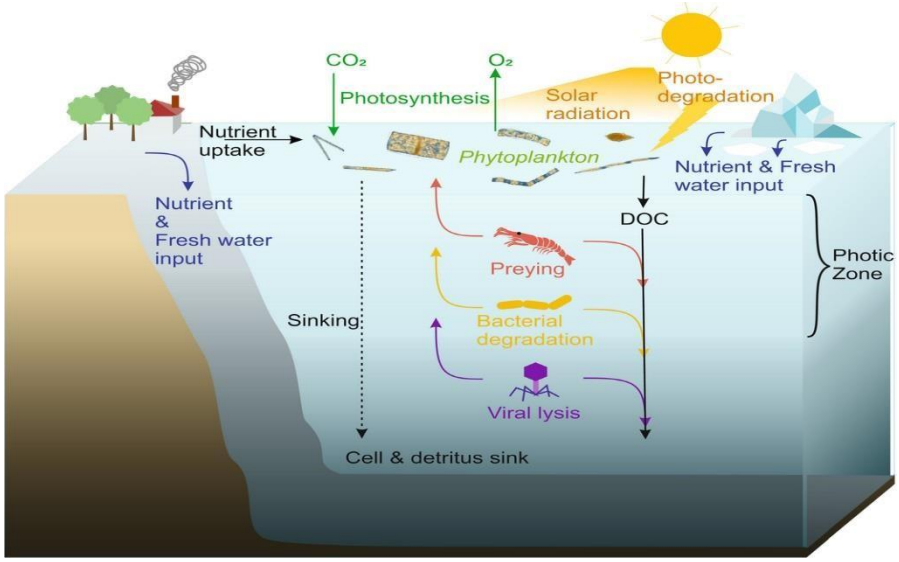

Figure 1: Significance of phytoplankton (Kase and Geuer, 2018)

1.1. Major Groups of Phytoplankton

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

1.1.1. 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 can 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 forms with specialized cells called heterocyst's for nitrogen fixation), Stigonematales (filamentous forms with false branching), and Pleurocapsales (unicellular and colonial forms with thick-walled cells) (Whitton and Potts,2000).

1.1.2. Diatoms

Diatoms first appeared in the fossil record around 180 million years ago and have since diversified into a wide range of forms (Armbrust, 2009). They are known for their intricate silica shells that provide protection from predators and allow them to sink to deeper depths (Armbrust, 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* and photoprotective and light-harvesting pigments (Wagner et al, 2006). Diatoms can be classified into two major 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.

1.1.3. 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 a stunning visual display 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, 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, 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, 1991).

1.1.4. 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 the presence of 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).

1.1.5. 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 have negative impacts on marine ecosystems (Hallegraeff, 2010).

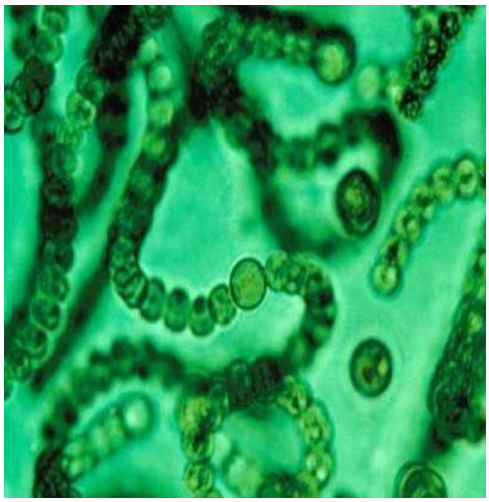

Figure2: Cyanobacteria

Figure 3: Diatoms

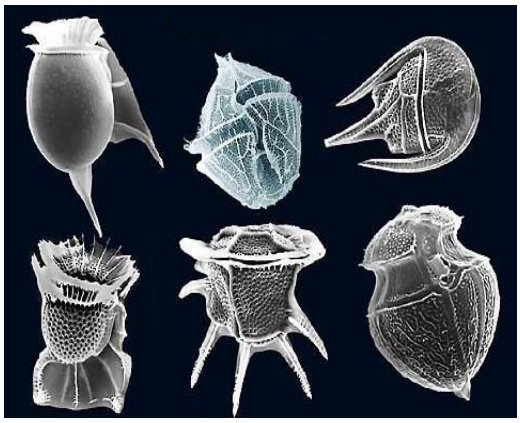

Figure 4: Dinoflagellates

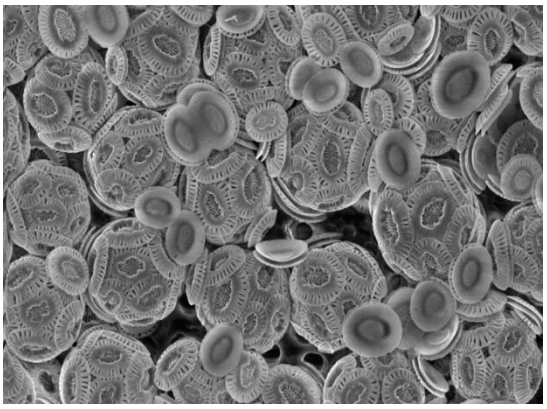

Figure 5: Coccolithophores

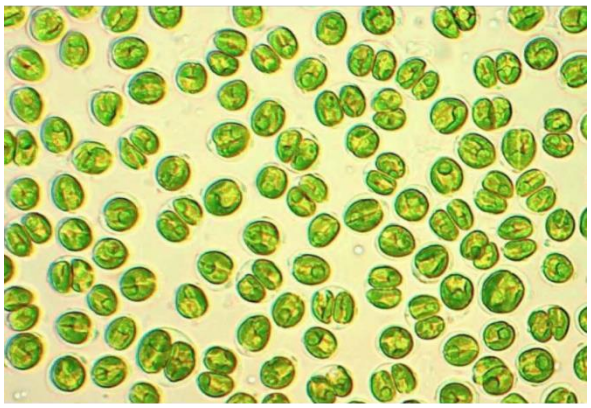

Figure 6: Green algae

1.2. Factors affecting the growth of phytoplankton.

Phytoplankton's have a range of growth requirements that influence their distribution and abundance in the aquatic ecosystem. Some key factors that influence the growth of phytoplankton include physical factors such as light, temperature, salinity, and nutrient availability, as well as biological factors such as competition and grazing pressure.

1.2.1. Light

Light is a critical factor for phytoplankton growth, as photosynthesis is the primary means by which they obtain energy. They require different levels of light depending on their

size and pigmentation, with smaller phytoplankton typically requiring higher light levels than larger phytoplankton (Finkel et al, 2010). However, excessive light levels can also be harmful to phytoplankton, leading to photoinhibition and oxidative stress (Hanelt, 1998).

1.2.2. Temperature

Temperature is another important physical factor that can influence phytoplankton growth. phytoplankton's have optimal temperature ranges for growth, with different species having different temperature preferences (Raven and Geider, 1988). Temperature changes can also influence nutrient availability, as warmer waters can promote stratification and reduce nutrient mixing from deeper waters (Boyd et al, 2013).

1.2.3. Nutrient

Nutrient availability is a key factor that limits phytoplankton growth in many aquatic ecosystems. Nitrogen, phosphorus, and iron are among the most important nutrients for phytoplankton growth, these nutrients are often limited in the open ocean (Falkowski et al, 1998). Nutrient availability can be influenced by a range of factors, including atmosphere deposition, runoff from land, and upwelling from deep waters. The exact amount of nutrients required by phytoplankton can also depend on the level of other environmental factors, such as light and temperature. For example, some studies have shown that phytoplankton requires higher levels of nutrients when light levels are low (Behrenfeld et al, 2006), while others have found that warmer temperatures can increase the nutrient requirements of some species of phytoplankton (Li et al, 2017).

A study by Moore et al, (2013) found that nitrogen and phosphorus concentrations in the ocean were strongly correlated with phytoplankton growth rates. The authors estimated that the minimum concentrations of nitrogen and phosphorus required to support phytoplankton growth were 2.5 μmol/L and 0.08 μmol/L, respectively. Other studies have

also found that iron can be a limiting nutrient for phytoplankton growth in certain regions of the ocean (Boyd et al, 2007). In these areas, iron fertilization experiments have been carried out in an attempt to stimulate phytoplankton growth and sequester carbon dioxide from the atmosphere (Boyd and Hutchins, 2012).

1.2.4. Salinity

Phytoplankton are adapted to a wide range of salinities, but some are more tolerant of changes in salinity than others. Changes in salinity can also affect the availability of nutrients and the quality of light availability to phytoplankton, which can, in turn, affect their growth rates (Mackey et al, 2012).

1.2.5. Biological factors

Biological factors such as competition and grazing pressure can also influence phytoplankton growth. Competition for nutrients and light can limit the growth of the phytoplankton population, with more competitive species often dominating environments with limited resources (Litchman et al, 2007). Grazing pressure from zooplankton and other predators can also influence phytoplankton growth, with high grazing pressure leading to reduced phytoplankton biomass and changes in community composition (Sommer et al, 2012). Overall, the growth of phytoplankton is influenced by a complex interplay of physical and biological factors.

1.3. 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 20-100 μm. 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 by using Niskin Sampler, it 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 and colleagues (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 and colleagues (2012) found that formalin and Lugol's iodine solution were also effective in preserving phytoplankton samples. 2% 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 and colleagues, 1987).

Phytoplankton enumeration refers to the process of quantifying the abundance and distribution of phytoplankton in aquatic systems. One common method is through microscopy, using either Sedgewick Rafter or Hemocytometer, visually identifying and counting the cells under microscope (Parsons et al, 2013). Other method includes 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 (de Vargas et al, 2015).

Figure 7: Phytoplankton net ("A simple plankton met with 20-micron mesh size.jpg" by Warichrb29 is licensed under CC SA 4.0)

Figure 8: A Hemocytometer (Yung et al, 2018)

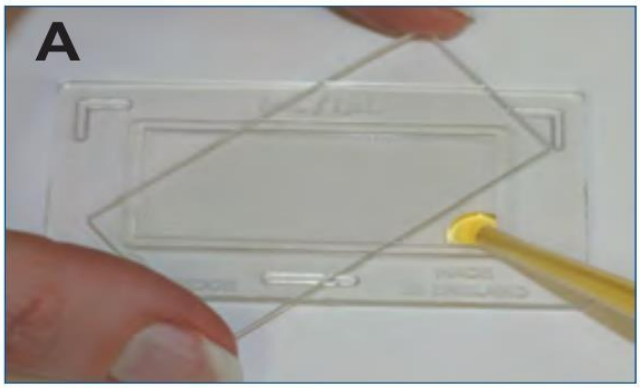

Figure 9: Sedgewick Rafter (LeGresley et al, 2010)

1.4. Isolation of phytoplankton.

Identification and isolation of phytoplankton can be challenging due to their small size, diverse morphology, and complex ecological interactions. To overcome these challenges, researchers use various media for the cultivation, identification, and isolation of phytoplankton. These media are designed to provide the necessary nutrients, minerals, and environmental conditions for the growth of specific types of phytoplankton.

1.4.1. f/2 media

The f/2 medium is a widely used medium for the cultivation and identification of marine phytoplankton (Guillard and Ryther, 1962). It contains a balanced mix of inorganic salts and trace elements that supports the growth of a wide range of marine algae.

1.4.2. BG-11 media

The BG-11 medium, on the other hand, is specifically designed for the isolation and cultivation of cyanobacteria, which are a type of photosynthetic bacteria often found in the aquatic environment (Rippka et al, 1979). It contains a mix of inorganic salts, trace elements, and vitamins.

1.4.3. WC media

Another medium commonly used for the isolation of freshwater algae is the WC medium, which contains a mix of inorganic salts, trace elements, and organic compounds like glucose and yeast extract (Andersen, 2005). Identification of phytoplankton can be accomplished through various methods, such as morphological, biochemical, and molecular techniques (Hasle, 1978; Medlin et al, 1997).

1.5. 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. 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). One study in the Gulf of Mexico found that oil pollution from the Deepwater Horizon oil spill significantly reduced the abundance and diversity of phytoplankton in the affected areas (Liu et al, 2012). The oil had a toxic effect on the phytoplankton, hindering their ability to photosynthesize and leading to reduces growth and reproduction. Pollution can also reduce the amount of light that reaches phytoplankton, which can inhibit their photosynthesis and growth. In addition, pollution can increase the acidity of water, which can affect the ability of phytoplankton to form their shells or skeletons, potentially leading to decreased survival rates (Thangaradjou et al, 2018).

Heavy metals are metallic elements with high density 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). Heavy metals can also disrupt the balance of essential nutrients in phytoplankton, which can lead to nutrient imbalances and decreased growth rates. For example, exposure to cadmium can reduce the uptake of iron, which is an essential nutrient for phytoplankton growth (Dong et al, 2020). Mercury exposure can lead to the formation

of methylmercury, which is a highly toxic compound that can accumulate in the food web and ultimately harm higher tropic levels (Dong et al, 2020).

Heavy metals can have significant adverse effects on the biomolecules of phytoplankton. For example, a study conducted by Malaviya et al, (2020) showed that exposure to cadmium caused a decrease in chlorophyll *a* and protein content in the diatom *Thalassiosira pseudonana*. Another study conducted by Mishra et al, (2020) showed that mercury exposure decreased the carbohydrate content of the green alga *Chlorella vulgaris*. Similarly, lead exposure has been shown to decrease the chlorophyll content and photosynthetic rate of the diatom *Navicula pelliculosa* (Vijayaraghavan and Ashokkumar, 2014). These studies suggest that heavy metals can interfere with the normal metabolic processes of phytoplankton, potentially reducing their ability to produce organic matter through photosynthesis. Finally, heavy metals can also affect the cellular structure of phytoplankton. For example, lead has been shown to induce morphological changes in the diatoms *Thalassioira weissflogii*, including the formation of abnormal siliceous frustules (Jin et al, 2014). Similarly, cadmium exposure has been shown to induce the formation of vacuoles and other cellular abnormalities in the green algae *Scenedesmus quadricauda* (Gao et al,2012). These studies suggest that heavy metals can disrupt the normal cellular structure of phytoplankton, potentially leading to cellular damage and dysfunction. These effects can lead to a decline in phytoplankton populations, which can have cascading effects on aquatic ecosystems, therefore, it is important to continue studying the effects of heavy metals on phytoplankton, both to understand their ecological implications and to develop strategies for mitigating their negative effects.

1.6. Impacts of copper on phytoplankton

Copper (Cu) is an essential micronutrient for the growth and development of phytoplankton. Cu is required for various cellular processes in phytoplankton, including photosynthesis, respiration and nitrogen metabolism (Bowler et al, 2010). One study by Zhang and colleagues (2018) investigated the effect of Cu on the growth and physiological characteristics of diatoms *Skeletonema costatum*. The researchers found that low concentration of Cu (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, Cu 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).

There have been several studies on the impact of Cu on phytoplankton. For example, a study by Guo et al, (2019) investigated the effects of Cu on the growth, photosynthesis, and biochemical processes of marine phytoplankton. This study found that exposure to Cu can decrease the growth and photosynthetic efficiency of phytoplankton, as well as alter their pigment content and biochemical processes. Another study by Zhang et al, (2010) investigated the impact of Cu on the growth and physiological responses of a marine diatom, which is a type of phytoplankton. The study found that exposure to Cu can reduce the growth and photosynthetic efficiency of the diatom, as well as alter its fatty acid composition. 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 Cu 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. Chen et al, (2018) investigated the mechanism by which Cu affects the growth of the marine diatom *Skeletonema costatum*. They found that Cu inhibited the activity of key enzymes involved in photosynthesis and energy metabolism, leading to decreased growth rates and

biomass production. Another study with *Phaeodactylum tricornutum* found that Cu-induced oxidative stress affected the activity of enzymes involved in energy metabolism and nutrient uptake (Wang et al, 2019).

In a study by Baruah and Kurian (2010), Cu was found to significantly reduce the growth rate of the green algae *Chlorella vulgaris*, with higher Cu concentrations leading to greater growth inhibition. Cu has been shown to reduce the photosynthetic activity of the green algae *Tetraselmis suecica* (Leyva et al, 2015). Cu can inhibit the activity of photosystem II, which can reduce the efficiency of photosynthesis (Qian et al, 2020).

Therefore, understanding the impacts of Cu on phytoplankton can inform management and conservation strategies for aquatic ecosystems, and help ensure their longterm health and sustainability.

1.7. Tetraselmis indica

They belong to: Division:

Chlorophyta

Class: Chlorodendrophyceae

Order: Chlorodendrales

Family: Chlorodendraceae

Genus: *Tetraselmis*

Species: *indica*

According to a study by Tomas (1997), these species are a relatively small and specialized group of green algae. They are unicellular alga that ranges from 9 to 12 µm in

diameter and has a smooth cell wall. They are motile cells generally laterally compressed and quadriflagellate. The cells are typically covered by a theca, which is a thin cell wall formed by the extracellular fusion of scales (Arora et al, 2013). They are known for its high biomass productivity, rapid growth rate, and ability to tolerate a wide range of environmental conditions (Sharma et al, 2019). It has been shown to have probiotic properties and can improve the growth and survival of shrimp larvae when used as a feed supplement (Sivagnanavel murugan et al, 2019).

Furthermore, the high lipid content of *T. indica* makes it a promising source for biofuel production (Sharma et al, 2019). They contain chlorophyll *a*, chlorophyll *b*, and carotenoid pigments (Naik and Anil, 2018).

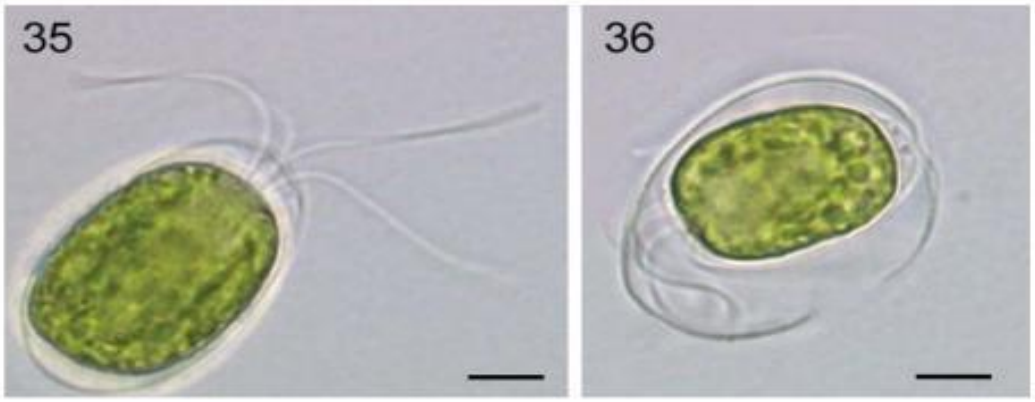

Figure 10: *Tetraselmis indica* transformation from motile to resting stage. (Arora et al, 2013).

1.8. Objective.

This study aims to determine the effect of Cu on the growth of *T. indica* and determine its effects on biomolecule accumulation such as carbohydrates and chlorophyll pigments (chlorophyll *a*, chlorophyll *b*, chlorophyll *c* and total chlorophyll).

2. METHODOLOGY

2.1. 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, Lugol's iodine, 0.25% glutaraldehyde, glucose solution, 5% phenol, concentrated H_2SO_4, and 90% acetone. Tools used in the experiment are filtrations unit and GF/F filter paper, 100mL conical flasks, 500mL conical flasks, measuring cylinder, aluminum foil, 50mL and 100mL volumetric flasks, beakers, dropper, tissue paper, stoppered tubes, glass pipette, Eppendorf tubes, refractometer, Sedgwick Rafter, Hemocytometer, microscope, cuvettes and spectrophotometer.

2.2. Disinfection procedure

Disinfecting the flasks and beakers is necessary in order to disable unwanted organisms in the equipment's. The flasks and other equipment's are washed with soap water thoroughly, then washed with tap water, rinsed, and soaked in 10% HCl overnight. The next day, it was washed with distilled water and dried before autoclaving.

2.3. Preparation of f/2 media

The Seawater was collected from Cacra Beach in cans and it was filtered through GF/F filter paper. The salinity was checked using a refractometer and was found out to be 15 so the salinity was fixed to 35 by adding NaCl. The filtered seawater was then autoclaved and cooled down under running tap water to avoid the formation of crystals. This was used for f/2 media preparation (given in the table1, 2, 3).

Take 950mL of filtered autoclaved seawater and add the following components:

Components	**Stock solution**	**Quantity**
$NaNO_3$	75 g/L dH_20	1mL
$NaH_2PO_4.H_2O$	5g/L dH_2O	1mL
$Na_2SiO_3.9H_{2O}$	30g/L dH_2O	1mL
Trace metal solution	See below	1mL
Vitamin solution	See below	0.5mL

Table 1: Composition of f/2 media

f/2 trace metal solution

Components	**Stock solution**	**Quantity**
$FeCl_3.6H_20$	---	3.15g
$Na_2EDTA.2H_2O$	---	4.36g
$CuSO_4.5H_2O$	9.8g/L DH_2O	1mL
$Na_2MoO_4.2H_2O$	6.3g/L DH_2O	1mL
$ZnSO_4.7H_2O$	22.0g/L DH_2O	1mL
$CoCl_2.6H_2O$	10.0 g/L DH_2O	1mL
$MnCL_2.4H_2O$	180.0 g/L DH_2O	1mL
Autoclave		

Table 2: Composition of f/2 trace metal solution

f/2 vitamin solution

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

Table 3: Composition of f/2 vitamin solution

2.4. Preliminary test

a. 50 mL of the above media was poured in 16 autoclaved 100 mL conical flasks by using autoclaved measuring cylinder.

b. Standard Cu sulphate solutions of 5000 µM and 100 µM was prepared in standard volumetric flasks.

c. From the standard Cu sulphate solutions different concentration of Cu sulphate i.e., 0.5 µM, 2.5 µM, 5 µM, 10 µM, 25 µM, 50 µM and 100 µM was calculated and added to each media flasks respectively. The experiment was conducted in duplicates and 2 control flasks were kept with just the media and no Cu solution was added.

d. Phytoplankton culture of *T. indica* was provided. 980 µL of the culture was taken in 1mL Eppendorf tube and fixed with 20 µL of Lugol's iodine. The phytoplankton number was counted using Sedgewick Rafter and volume for 2000 cells/mL was calculated using the formula:

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

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

e. 500 µL of culture was inoculated in each of the 16 flasks. The flasks were subjected to 16hours of light and 8 hours of dark condition. Phytoplankton were allowed to grow in media containing heavy metal. Room temperature was measured every 3 days.

f. In every 3 days 980 µL of sample from the flasks were taken in Eppendorf tubes and fixed with 20 µL of Lugol's iodine, starting from day 0 to day 30.

g. The cells were counted using Hemocytometer using the formula:

Cell/mL = total number of cells × dilution factor × 10^4/ number of squares counted

h. The growth rate, doubling time and generation time was determined for 30 days.

i. From the preliminary experiment, four concentration of Cu sulphate showing good growth was selected along with control for further analysis.

2.5. Analysis of phytoplankton exposed to heavy metals

a. 5 flasks having Cu concentrations of 2.5µM, 10µM, 50µM, 100µM and control was selected and subculture after every week till the start of the main experiment.

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

c. 15 flasks of 500mL were covered with aluminum foil and autoclaved.

d. 400 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 5 sub cultured flasks, the cells were counted from each flask using Sedgewick Rafter and volume of inoculum was calculated to get 2000 cells/mL.

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

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

2.5.1. Growth rate

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

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

Growth rate (μ) = $\ln (N_t / N_0) \times 1/t$ where N_t = final number of cells

N_0 = initial number of cells

t = time

Generation time = $\ln (2) / \mu$

Doubling time = $\mu / \ln (2)$

2.5.2. Carbohydrate analysis

a. For the Standard plot: 1mg/mL of standard glucose solution was prepared. Different concentration of glucose solution from the standard solution was prepared, having concentrations 20, 40, 80, 150, 250 and 500 µg/mL. 2mL of each concentration was taken in stoppered tubes respectively, 0.5mL of 5% phenol, and 5mL of concentrated $H_2S\ O_4$ 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. 2mL of the sample was taken from each respective flasks in stoppered tubes and 0.5mL of 5% phenol was added and 5mL of concentrated 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.

2.5.3. Pigment analysis

a. Filter 10mL-15mL of experimental sample on GF/F filter paper and store it Eppendorf tube at -20˚C until analysis

b. Put the filter paper in centrifuge tube and add 5mL of 90% acetone and vortex for 30 sec.

c. Cover the tubes with foil and keep at 4˚C overnight.

d. Next day centrifuge at 5000rpm at 4˚C for 5min and take the absorbance at 750nm, 664nm, 647nm and 630nm.

e. Samples for pigment analysis was taken on day 0, day 6, day 12 and day 21

f. Calculate the chlorophyll as follows: subtract the absorbance value of 750nm from all other individual absorbance (A)

$(chl.\ a)_{extract} = 11.85A_{664} / I – 1.54A_{647} /I – 0.08A_{630}/I$

$(chl.\ b)_{extract} = 21.03A_{647} /I – 5.43A_{664} /I -2.66_{630} /I$

$(chl.\ c)_{extract} = 24.52A_{630} /I – 1.67A_{664} /I – 7.60A_{647}/I$

Where A = corrected absorbance.

I = path length in cm.

The concentration of each chlorophyll in the sample in µg/mL is obtained by the following equation:

$(chl.x)_{sample} = (chl.x)_{extract} \times v/V) v$

= volume of ethanol in mL

V = volume of sample filtered in mL

The total concentration of chlorophyll in the sample in µg/ mL is obtained by the following equation:

$(chl.)_{total} = (chl.\ a)_{sample} + (chl.\ b)_{sample} + (chl.\ c)_{sample}$

µg/ml is converted to pg/mL

3. RESULT AND DISCUSSION

3.1. Preliminary growth curve of *T. indica*

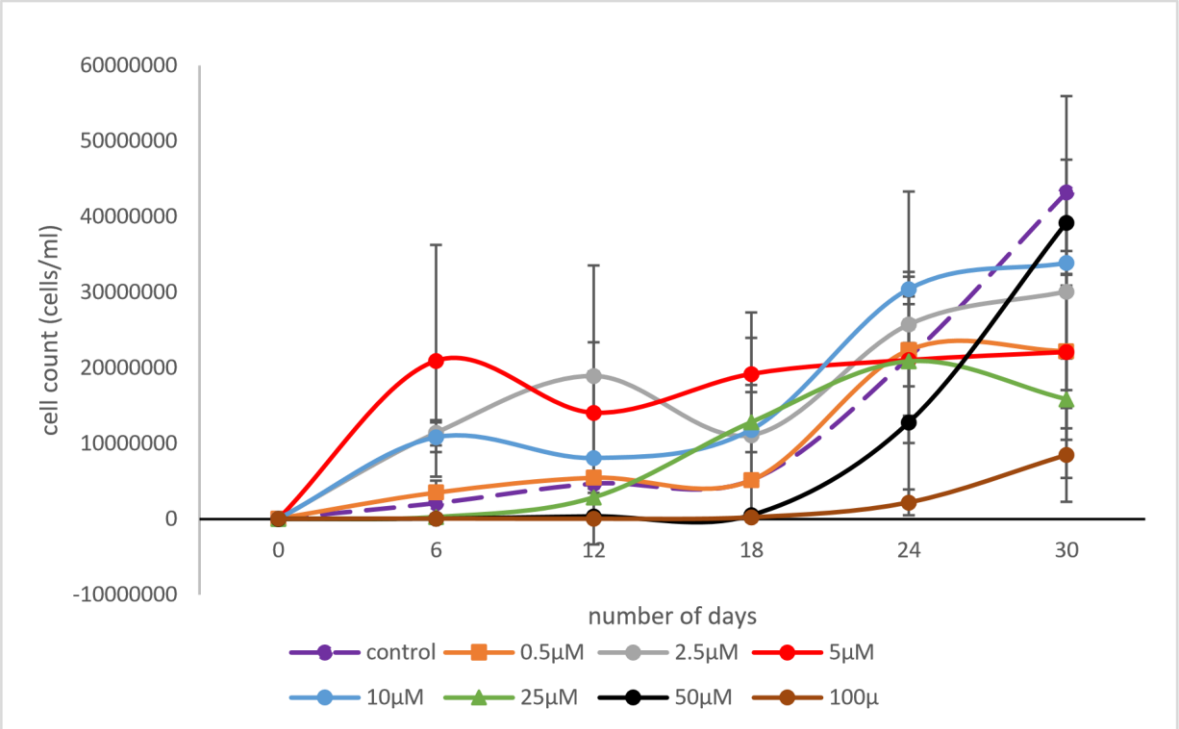

Figure 11: Preliminary growth curve of *Tetraselmis indica*

It was seen that in 50 µM and100 µM *T. indica* had a longer lag phase. That implies that phytoplankton at these concentrations took a long time to adjust to the change of environment and entered the exponential phase from day 18. One study by Rani et al, (2020) examined the effect of Cu and zinc on the growth of *T. indica*, it was shown that the phytoplankton had a longer lag phase when exposed to above 0.5 mg/L and 5 mg/L of Cu

and Zn concentrations respectively. In concentration 2.5 µM the phytoplankton cell count was decreased from day 24. In 0.5 µM, the cells entered into stationary phase on day 30. From these concentrations 2.5 µM, 10 µM, 50 µM and 100 µM was selected for further experiment.

3.2. Growth curve and growth rate of *T. indica*

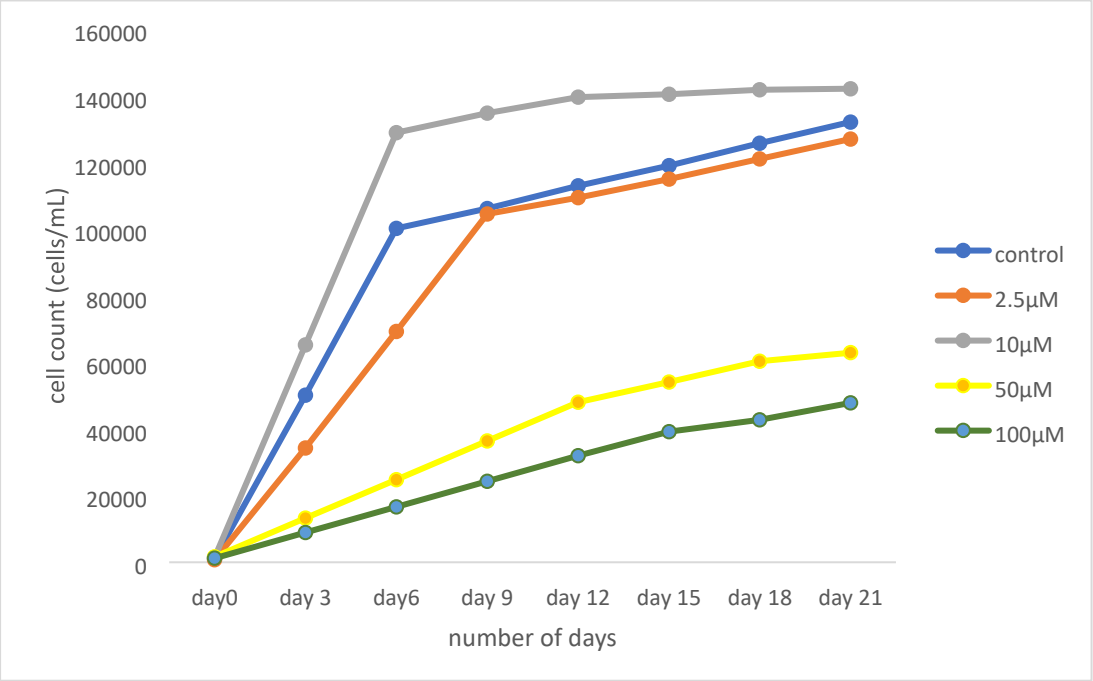

Figure 12: Growth curve of *Tetraselmis indica* exposed to different Cu concentrations.

The result in figure10 showed that *T. indica* had a typical growth phase, with very short or no lag phase and followed by an exponential phase and reaching to stationary phase. The cells grew more vigorously in 10 µM Cu concentration from day 3 to day 9 and reached into the stationary phase. 2.5 µM and control flask showed similar growth patterns and cells in 50 µM and 100 µM Cu concentrations grew slowly. This showed that *T. indica* was able to grow under exposure to different Cu concentrations but the growth was slowed down as the concentration of Cu was increased. In the study by Hindarti and Larasati (2019) similar growth pattern was seen in *Nitzschia* species, where the growth was decreased as the time exposure of heavy metals increased. In another study, with *Skeletonema costatum* exposed

to Cu found out 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).

Cu concentration	**Growth rate (d^{-1})**	**Doubling time (d)**	**Generation time ($1/d^{-1}$)**
Control	0.224647	3.085498	0.324097
2.5µM	0.2451	2.828018	0.353605
10µM	0.217778	3.182814	0.314187
50µM	0.173812	3.987915	0.250758
100µM	0.175237	3.955493	0.252813

Table 4: Growth rate, doubling time and generation time of *Tetraselmis indica*

The growth rate of *T. indica* decreased with increase in Cu concentration, thus showing negative effect of Cu on the phytoplankton (table 4).

According to a study by Tavares et al (2017), a decrease in the growth rate of phytoplankton can lead to changes in the food web and biogeochemical cycling of nutrients in the ocean. One such study by Rani et al. (2020) examined the effect of Cu on the growth of *T. indica* in a batch culture system. The study found that *T. indica* showed a significant reduction in growth rate when exposed to Cu concentrations above 0.5 mg/L. The study also found that *T. indica* had a longer lag phase and a lower maximum biomass yield under these conditions. Similarly, another study by Basha et al. (2019) investigated the effect of Cu on the growth of *T. indica* in a semi-continuous culture system. The study found that *T. indica* showed a significant reduction in growth rate when exposed to Cu concentrations above 0.2

mg/L. The study also found that Cu exposure led to changes in the cell morphology and photosynthetic pigments.

3.3. Total carbohydrate

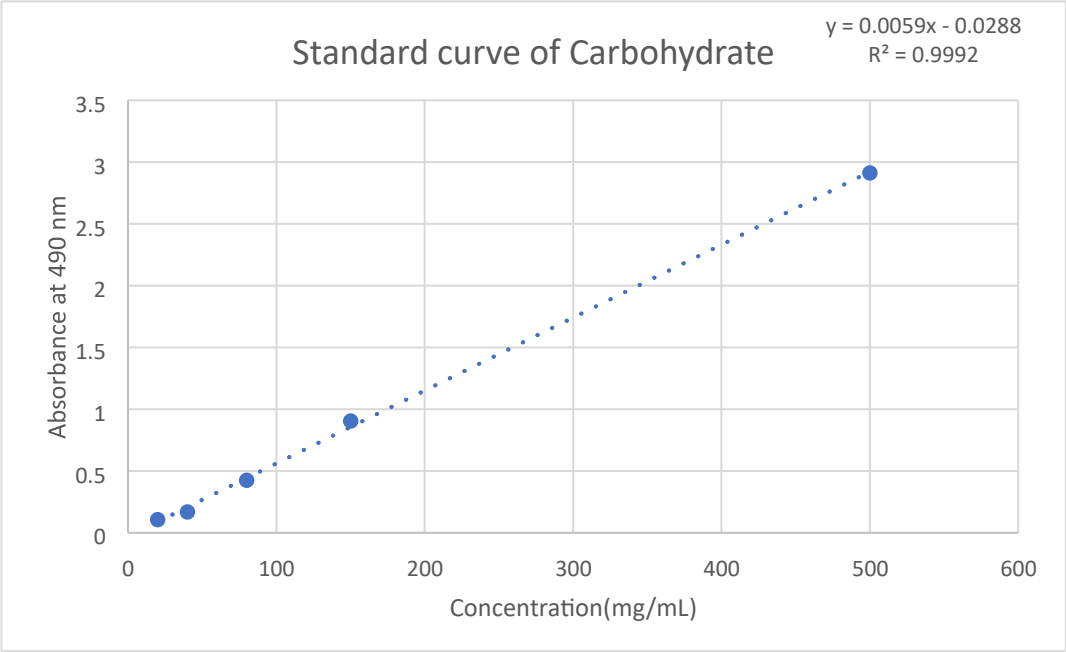

Figure 13: Standard plot of carbohydrate.

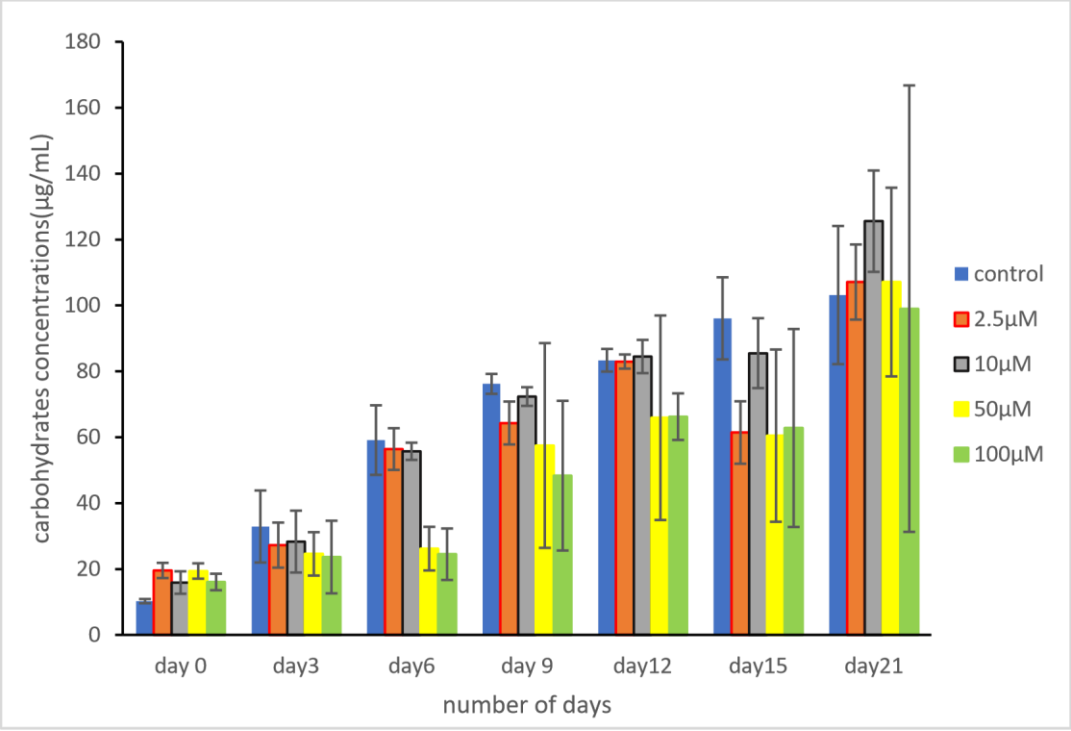

Figure 14: Effect of Cu on the total carbohydrate of *Tetraselmis indica*.

From the graph, we can see that total carbohydrate levels increases with increase in the exposure time. From day 3 to day 15 the amount of carbohydrate was more in control, but on day 21, the amount of carbohydrates in the Cu exposed flask increased, showing a major increase in 10 µM Cu concentration. At first, the total carbohydrate in 50 µM and 100 µM Cu concentration 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. Similar results were observed 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 than in control. In another study, the production of total carbohydrate in the diatom *Cylindrotheca fusiformis* and dinoflagellate *Gymnodinium* species increased with Cu concentration (Pistocchi et al, 1996). Phytoplankton can synthesis carbohydrate as a mechanism to protect them against toxic metals in the ocean as carbohydrate can bind to Cu and reduce its bioavailability (Silva et al, 2018).

There is limited research on the specific mechanisms by which Cu (Cu) exposure affects the production of total carbohydrate in *T. indica*. However, some studies have suggested possible explanations for this phenomenon.

One possible explanation is that Cu exposure may increase the activity of enzymes involved in carbohydrate metabolism. For example, a study by Li et al. (2017) found that Cu exposure led to an increase in the activity of enzymes involved in the pentose phosphate pathway, which is a pathway that generates pentose sugars and NADPH, a reducing agent required for many biosynthetic reactions, including carbohydrate synthesis.

Another possible explanation is that Cu exposure may lead to changes in the composition of the culture medium, which can affect the production of total carbohydrate. For example, a study by Rani et al. (2020) found that Cu exposure led to a decrease in the pH of the culture medium, which can influence the availability of carbon sources for carbohydrate synthesis.

Finally, it is also possible that Cu exposure may lead to changes in the expression of genes involved in carbohydrate metabolism, which can ultimately affect the production of total carbohydrate.

3.4. Effect of Cu on pigments

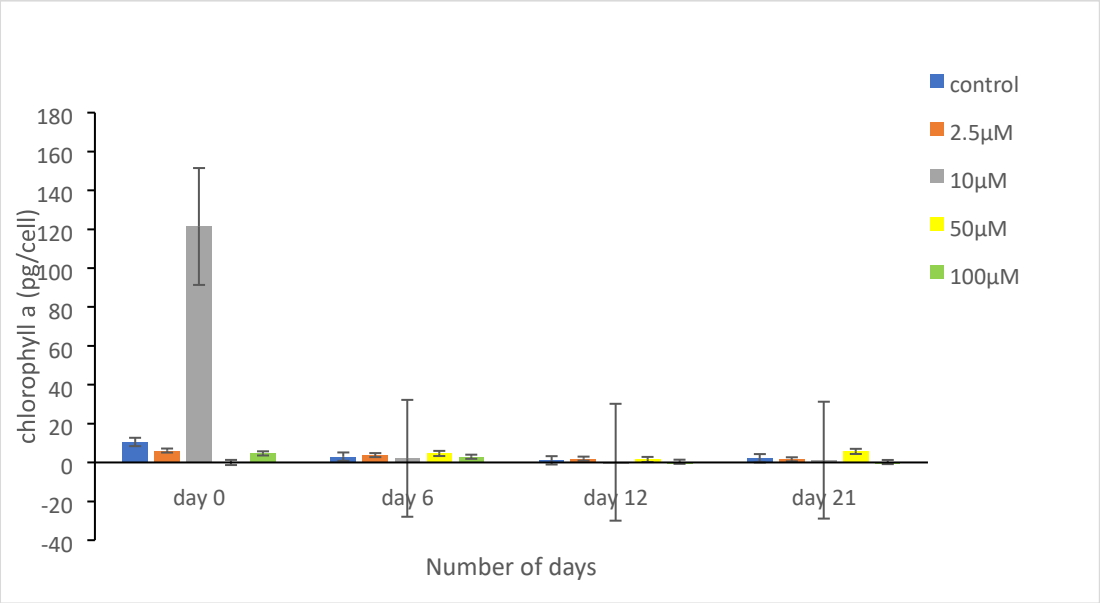

Figure 15: Effect of Cu on chlorophyll *a* content of *Tetraselmis indica*.

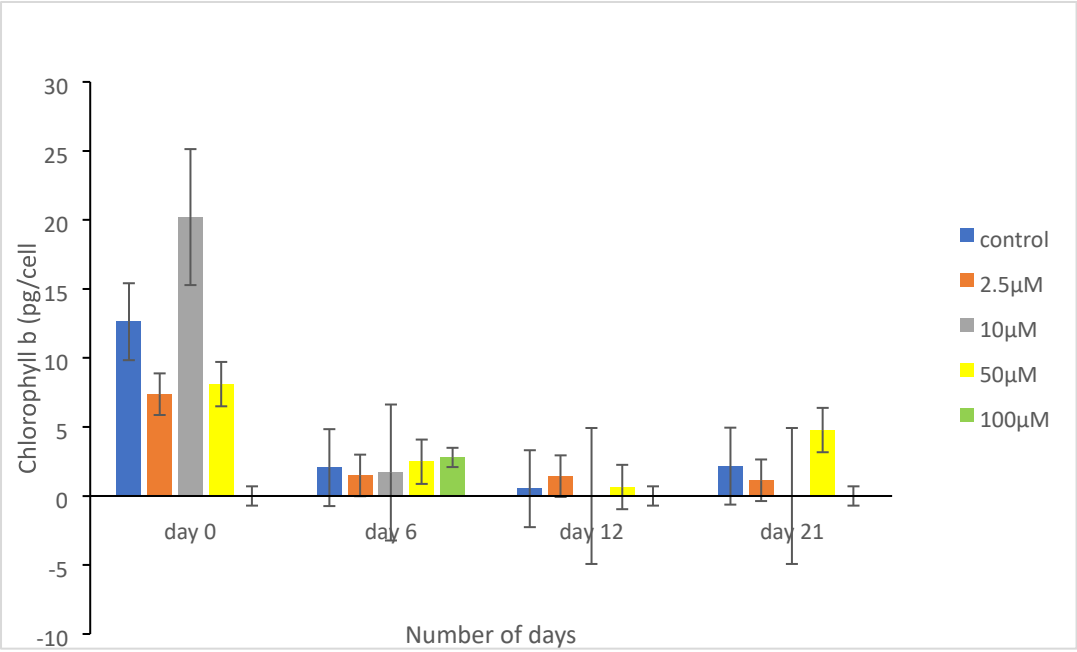

Figure 16: Effect of Cu on chlorophyll *b* content of *Tetraselmis indica*

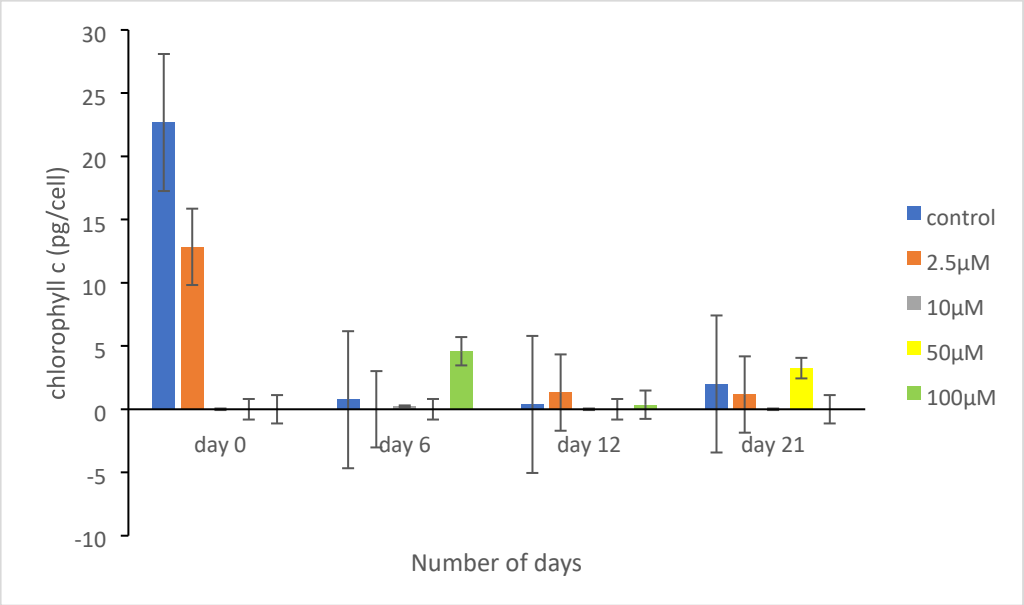

Figure 17: Effect of Cu on chlorophyll *c* of *Tetraselmis indica*

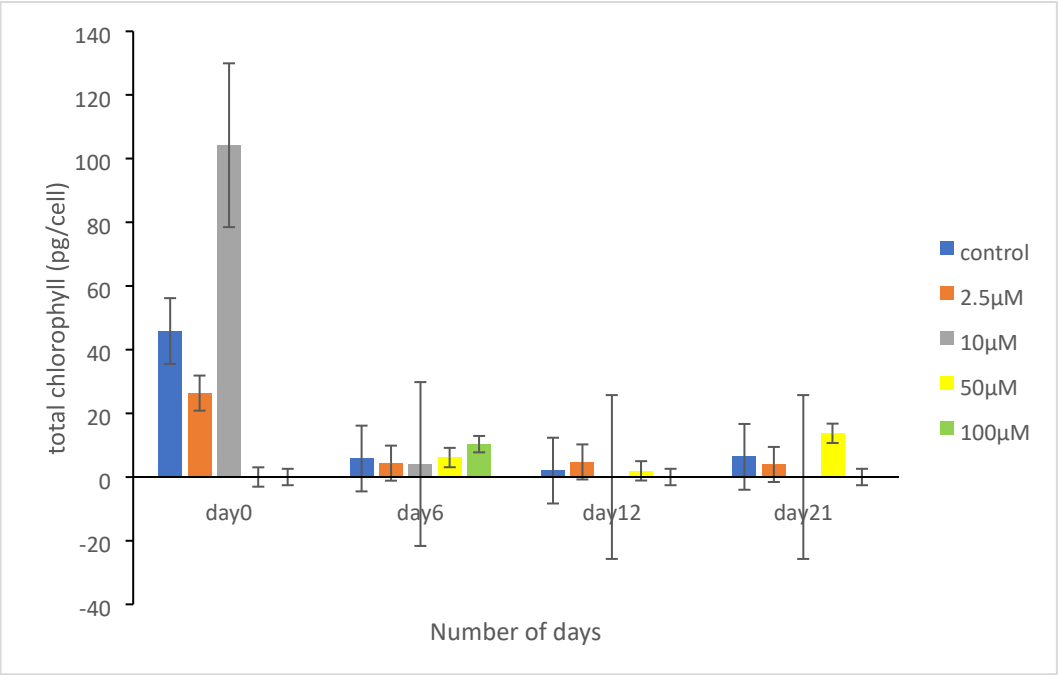

Figure 18: Effect of Cu on total chlorophyll of *Tetraselmis indica*

From figure 13- 16 it was noted that the chlorophyll *a*, chlorophyll *b*, chlorophyll *c*, and total chlorophyll content in *T. indica* decreased with increase in exposure time and with an increase in Cu concentrations. In the control flask also, a decrease in pigment was seen from day 0 to day 21. This could be because the nutrient was getting depleted as the days increased.

If chlorophyll *a*, *b* and *c* are less in phytoplankton exposed to heavy metals, it suggests that the photosynthetic system of the phytoplankton is being negatively impacted by the metal exposure (Gao et al 2008). In another study, it was known that Cu could inhibit photosynthetic activity by affecting the electron transport chain and replacing Mg in the center of chlorophyll molecules**,** which damages photosystems and the antenna complex (Silva et al, 2018).

Several studies have investigated the effect of Cu (Cu) on the chlorophyll content of *T. indica*. One such study by Chen et al. (2017) examined the effect of Cu on the chlorophyll

a, *b*, *c* and total chlorophyll content of *T. indica* in a batch culture system. The study found that *T. indica* significantly reduced chlorophyll content when exposed to Cu concentrations above 0.1 mg/L. The reduction in chlorophyll content was found to be more severe for chlorophyll *a* compared to chlorophyll *b* and *c*, resulting in a significant decrease in the chlorophyll *a/b* ratio.

Similarly, another 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 concentrations above 0.2 mg/L.

The mechanism by which high Cu (Cu) concentrations affect the chlorophyll content of *T. indica* has not been fully elucidated. However, several studies have suggested possible mechanisms. One possible mechanism is that Cu exposure may lead to oxidative stress, which can damage the photosynthetic apparatus and reduce the chlorophyll content. For example, Rani et al. (2020) found that Cu exposure led to increased levels of reactive oxygen species (ROS) in *T. indica*, which can cause oxidative damage to proteins, lipids, and pigments. This damage can ultimately result in a reduction in the chlorophyll content.

Another possible mechanism is that Cu exposure may disrupt the biosynthesis of chlorophyll. Cu is an essential cofactor for several enzymes involved in chlorophyll biosynthesis, and high concentrations of Cu may interfere with this process. This interference can result in a reduction in the chlorophyll content.

4. CONCLUSION

Phytoplankton plays an important function in the marine food web and are responsible for a considerable portion of the oxygen in our atmosphere. Understanding the effects of pollution, especially heavy metals, on phytoplankton is therefore critical for protecting the health of our seas and the numerous organisms that are dependent on them. Heavy metals' effects on phytoplankton can be complicated and vary, depending on parameters such as metal type and concentration, as well as phytoplankton species and physiological state. In our present study, with increasing exposure time, total carbohydrate production increased. This increasing biomolecule levels also helped *T. indica* respond to defense mechanisms by

producing large amounts of total carbohydrate. Heavy metal exposure can have a variety of detrimental effects on phytoplankton, as evidenced by a decrease in the growth rate and lower chlorophyll content. Overall, the precise mechanism by which elevated Cu concentrations alter *T. indica* chlorophyll content is unknown and deserves further exploration.

5. FUTURE 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 looking into the interplay between metals and important 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 have an impact on 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 for minimizing 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.

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ANNEXURE

Figure 2:

https://www.google.com/search?q=cyanobacteria&sxsrf=APwXEddPCOSXydzItyx1BYjR0NrwmvWatA:1682734545168&source=lnms&tbm=isch&sa=X&ved=2ahUKEwj8mcPWgs7-AhWmVmwGHX0BCFkQ_AUoAXoECAEQAw&biw=1707&bih=793&dpr=1.13#imgrc=jVdU7by4sQ3VDM&imgdii=OhsyGiT0W4d2cM

Figure 3:

https://www.google.com/search?q=diatoms&tbm=isch&tbs=il:cl&hl=en&sa=X&ved=0CAAQ1vwEahcKEwjg_c_Ug87-AhUAAAAAHQAAAAAQAw&biw=1688&bih=793#imgrc=eCPY0hgvOtGDDM

Figure 4:

https://www.google.com/search?q=dinoflagellates&tbm=isch&hl=en&tbs=il:cl&sa=X&ved=0CAAQ1vwEahcKEwjgm8j9g87-AhUAAAAAHQAAAAAQAw&biw=1688&bih=793#imgrc=wY9uFHm4rgZ5JM .

Figure 5:

https://www.google.com/search?q=coccolithophores&tbm=isch&hl=en&tbs=il:cl&sa=X&ved=0CAAQ1vwEahcKEwjgkLyehM7-AhUAAAAAHQAAAAAQAw&biw=1688&bih=793#imgrc=rpmarxZTyR_qeM

Figure 6:

https://www.google.com/search?q=tetraselmis&tbm=isch&ved=2ahUKEwj9rp7HhM7AhVlDLcAHdT_CM8Q2cCegQIABAA&oq=tetra&gs_lcp=CgNpbWcQARgBMgQIIxAnMgQIIxAnMgcIABCKB RBDMgoIABCKBRCxAxBDMgcIABCKBRBDMgcIABCKBRBDMgcIABCKBRBDMgcIABCKBRBDMgoIABCKBRCxAxBDMgcIABCKBRBDOgcIIxDqAhAnOggIABCABBCxAzoFCAAQgAQ6DQgAEIoFELEDEIMBEENQjgdYgBhg-

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