Repercussions of salinity variation on the growth of *Tetraselmis indica* (Chlorodendrophyceae, Chlorophyta)

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by

RUTA TUSHAR SAWANT TALAULIKAR

21P039015

Under the supervision of

DR. SANGEETA M. NAIK

School of Earth, Ocean and Atmospheric Sciences

Masters in Science

Goa University

Date: **2nd MAY 2023**

Examined by: Seal of the School

DECLARATION BY THE STUDENT

I hereby declare that the data presented in this Dissertation report entitled, **"Repercussions of salinity variation on the growth of *Tetraselmis indica* (Chlorodendrophyceae, Chlorophyta)"** is based on the results of investigations carried out by me in the M.Sc. Marine Microbiology at the School of Earth, Ocean and 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 in the dissertation.

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Ruta Tushar Sawant Talaulikar

Roll Number: 21P039015

M.Sc. Marine Microbiology

School of Earth, Ocean and Atmospheric Sciences

Date: 2nd May 2023

Place: Goa University

COMPLETION CERTIFICATE

This is to certify that the dissertation **"Repercussions of salinity variation on the growth of *Tetraselmis indica* (Chlorodendrophyceae, Chlorophyta)"** is a bonafide work carried out by **Ms. Ruta Tushar Sawant Talaulikar** under my supervision in partial fulfillment of the requirements for the award of the degree of **Masters in Science** in the Discipline **Marine Microbiology** at the School of Earth, Ocean and Atmospheric Sciences, Goa University.

Date: 2nd May 2023

Dr. Sangeeta M. Naik
M.Sc. Marine Microbiology

Sr. Prof. C. U. Rivonker
M.Sc. Marine Microbiology
School of Earth, Ocean and
Atmospheric Sciences

SCHOOL OF EARTH, OCEAN & ATMOSPHERIC SCIENCES
GOA UNIVERSITY

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Date:

Place: Goa University

Dr. C. U. Rivonker
Senior Professor and Dean,
School of Earth, Ocean
& Atmospheric Sciences,
Goa University
Goa - 403 206

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Sr. Prof. C. U. Rivonker

M.Sc. Marine Microbiology

School of Earth, Ocean and Atmospheric Sciences

M.Sc. Marine Microbiology

Date:

Place: Goa University

Dr. Sangeeta M. Naik

School Stamp

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1. INTRODUCTION

1.1. Phytoplankton in marine environment

Plankton was defined by Hensen in 1887 as any organism, plant or animal, that is passively 'drifting' along with the movement of water. Phytoplankton are autotrophic organisms that can fix inorganic carbon by using solar energy, nutrients and trace metals via photosynthesis. Phytoplankton has a broad range of taxonomic groupings that include organisms of a variety of dimensions. Currently, 4000 species of marine phytoplankton have been identified (Simon et al., 2009). Based on linear metrics, phytoplankton are classified into the following size classes: nanophytoplankton (2-20 μm), microphytoplankton (20-200 μm) and macrophytoplankton (> 200 μm). However, they can be categorised as nanoplankton ($10–10^3$ μm^3), microplankton ($10^3–10^6$ μm^3), and macroplankton ($10^6–10^9$ μm^3) on the basis of cell volume measurements (in μm^3) (Ignatiades, 2016; Sieburth et al., 1978). The main phytoplankton groups in the marine environment are coccolithophorids, cyanobacteria, diatoms and dinoflagellates.

Coccolithophores are calcifying protists that are characterized by exoskeletal coccosphere formed due to the production of calcite platelets (coccoliths) that cover the cell surface (De Vargas et al., 2007). The variations in the shape, size and arrangement of coccoliths and, in turn, coccospheres, give rise to a remarkable morphological diversity within the group. They inhabit both benthic and open-water habitats (Monteiro et al., 2016). *Emiliania huxleyi* is the most dominant species among the coccolithophorids. Other examples are, *Calcidiscus*, *Gephyrocapsa*, *Syracosphaera*, and *Umbellosphaera* (Baumann et al., 1999).

Cyanobacteria are widely distributed Gram-negative bacteria that can perform photosynthesis and are commonly referred to as blue-green algae (Lau et al., 2015). Cyanobacteria have a long evolutionary history as they are known to be the oldest photosynthetic prokaryotes on earth that originated approximately 2.6 to

3.5 billion years ago (Hedges et al., 2001). Cyanobacteria exist in different forms, such as unicellular, filamentous, planktonic or benthic, and colonial forms (Burja et al., 2001). *Anabaena*, *Microcystis*, *Nostoc*, *Oscillatoria*, *Prochlorococcus*, *Spirulina*, and *Synechococcus* are some examples of Cyanobacteria (Zahra et al., 2020).

Diatoms are functionally single-celled microscopic organisms ranging in size from 5 to 200 μm in diameter or length (Cameron, 2013). They can also take the form of filaments, chains, as well as colonies and can be found in freshwater or marine environments adhering to any particular substratum as benthos or floating in the column of water as phytoplankton. They have characteristic silica cell walls composed of two valves separated by girdle bands (Sabater, 2009). *Amphipleura*, *Asterionella*, *Chaetoceros*, *Cymbella*, *Fragilaria*, *Navicula*, *Nitzschia*, *Synedra*, *Tabellaria*, etc., are some examples of diatoms (Sabater, 2009).

Dinoflagellates are photosynthetic, unicellular, microscopic organisms ranging in size from 15 to 40 microns (Steidinger & Jangen, 1997). The characteristic feature of dinoflagellates is the presence of two flagella: the longer, posterior, longitudinal, whiplash flagellum extending out from the sulcal groove of the hypotheca and the shorter, anterior, flattened, transverse, tinsel flagellum which lies in the cingulum. Cingulum is a transverse groove that extends around the equator of the cell and divides the cell into epitheca and hypotheca (Carty, 2003). These may be found in fresh as well as marine water habitats. Some examples of dinoflagellates include *Ceratium*, *Dinophysis*, *Gonyualux*, *Gymnodinium*, *Karenia*, *Noctiluca* and so on (Soyer-Gobillard, 2009).

1.2. Significance of phytoplankton

1.2.1. Origin of Ocean and Atmosphere

Various minerals in rocks were analyzed by geologists and they discovered that, the Earth's atmosphere contained no free oxygen for the first half of its

4.6-billion-year history. Oxygen started accumulating only 2.4 billion years ago. Rocks containing fossilized cyanobacteria were found, whose present-day forms perform photosynthesis that uses the sun's energy to split water into hydrogen and oxygen, two billion years ago, before the atmospheric level of oxygen rose, no land plants produced oxygen. Thus, it is one of the theories that these photosynthetic microorganisms' oxygen created our oxygen-rich atmosphere (Davankov, 2021; Falkowski, 2012).

Phytoplankton is a critical component of the marine ecosystem, responsible for approximately half of the global (terrestrial and marine) net primary production (Field et al., 1998). Not only the chemical composition of the deep ocean was reflected by phytoplankton now, they are also considered to have created it (Falkowski, 2012). Alfred Redfield of Harvard University in Massachusetts realised that the proportions of the elements needed to construct essential cellular molecules (carbon, nitrogen and phosphorus), in the ocean were not random. Samples from every region of the ocean showed that the ratio of nitrogen atoms to phosphorus atoms in the deep ocean was 16:1, which is the same ratio as in the phytoplankton. This could be because the phytoplankton and the animals that consumed them died and sank to the bottom, along with those animals' feces and then the microorganisms in the deep sea broke this material down further into its chemical constituents, thus creating seawater which has the same proportions of nitrogen and phosphorus (Redfield, 1958).

1.2.2. The food web

In aquatic settings, phytoplankton serves as the primary part of the food chain because they serve as energy converters, transforming solar energy into the chemical energy of food. Zooplankton passes this food energy to the higher trophic levels and thus provides a link between energy producers and consumers (Falkowski, 2012). The blue-green and green algae which are very abundant in freshwater were initially considered of lesser significance in marine habitats (Zeitzschel, 1978) but this view has changed with advancement in research in recent

times. In coastal areas, microphytobenthos, macroalgae and halophytes contribute in carbon fixation (Connell & Russell, 2010; Kromkamp et al., 2006) and in the open ocean, phytoplankton constitute the only source of primary production to sustain pelagic food webs (Chavez et al., 2011; Falkowsky & Oliver, 2007). Figure 1 by Kuiper et al. (2015) shows a schematic illustration of the feeding relations in the aquatic food web. The pelagic and benthic food chains in the food web are interwoven by a common predator. The following information is provided in the order of biomass (g m^{-2}), specific death rate (per year), assimilation efficiency, and production efficiency (in square brackets) to compute feeding rates (parentheses). Feeding rates (g m^{-2} per year) are given near their respective arrows. The information relates to a clear-water state that receives 2.6 mg of phosphorus per m^{-2} per day.

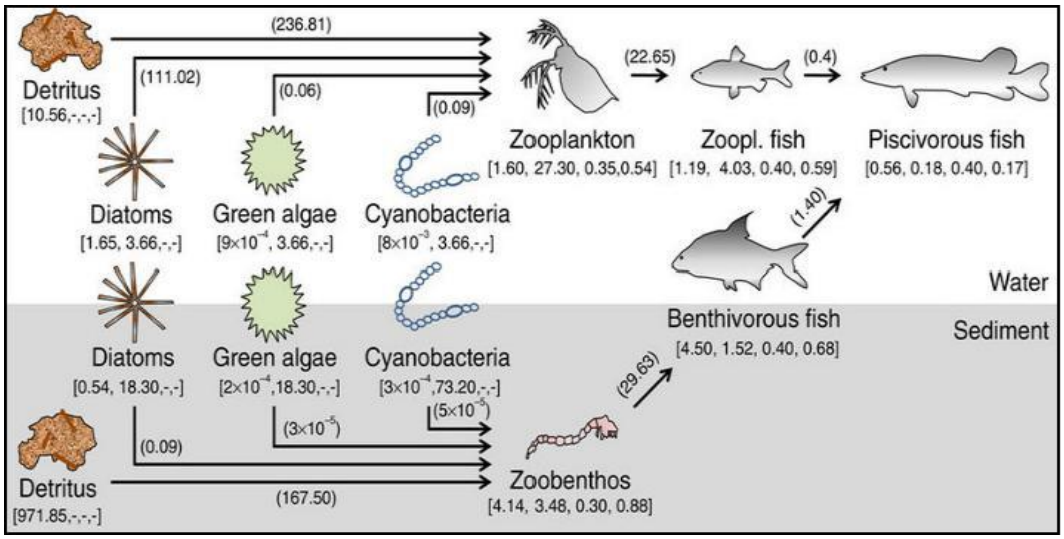


Figure 1. Aquatic food web and feeding relations depicted schematically (Kuiper et al., 2015).

1.2.3. Biogeochemical cycles

The primary contributors to the global primary productivity and the principal stakeholders in the global carbon cycle are phytoplankton. Functional groups within the phytoplankton, which are identified by distinctive features, have an impact on other significant biogeochemical cycles, including the cycles of nitrogen, phosphorus, and silica (Figure 2). Due to these groups' particular environmental sensitivity, alterations to phytoplankton community structure might have a large

impact on elemental cycling on a range of scales, from local to global (Arrigo, 2004; Litchman et al., 2015).

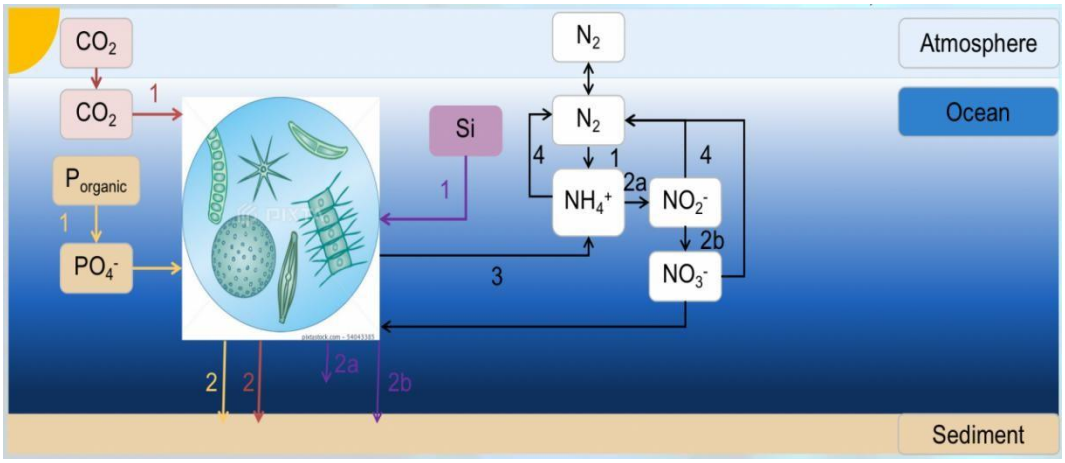


Figure 2. Role of phytoplankton in different biochemical cycles.

Marine phytoplankton fuels the global ocean biological carbon pump, whose intensity is correlated with the composition of the plankton community and is controlled by the relative rates of primary production and carbon remineralization (Field et al., 1998). The ocean's biological pump is a key piece of the Earth's carbon cycle. It is basically the process by which inorganic carbon dioxide is transformed to organic carbon via photosynthesis carried out by marine phytoplankton or by the formation of carbonate shells by some other planktonic organisms in the ocean, exported through sinking particles (Dissolved Organic

Matter, DOM and Particulate Organic Matter, POM) when these organism die, or are consumed, and then the carbon is finally sequestered into the deep ocean sediments (Figure 3) (Guidi et al., 2016).

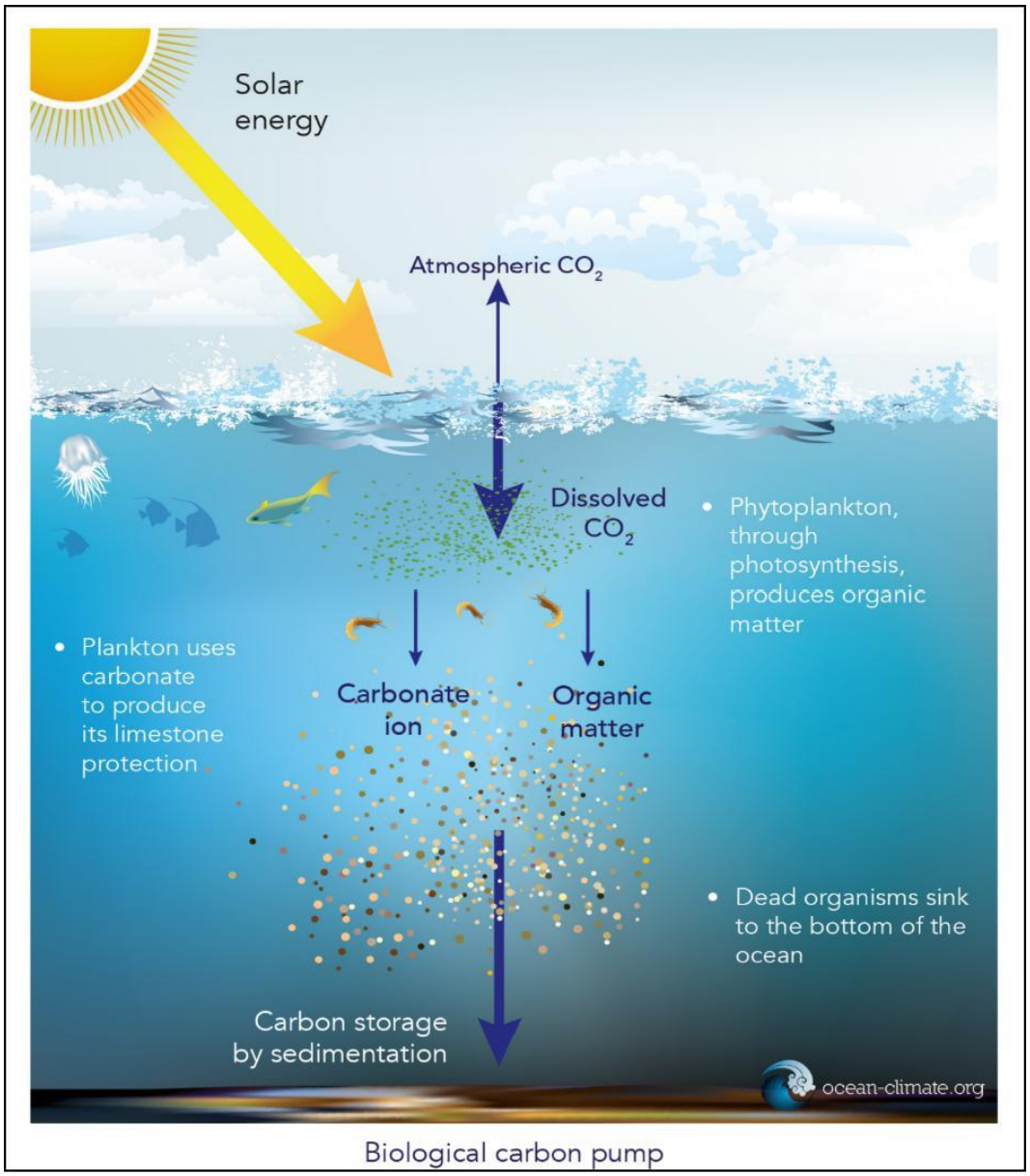


Figure 3. Schematic representation of the role of phytoplankton in the carbon cycle (Eloyan, 2020).

The most prevalent gas in the atmosphere, nitrogen, dissolves into the ocean's surface layers where it is then transformed into other nitrogenous compounds by marine microbes. Nitrogen fixation is a process by which some microorganisms, including cyanobacteria, turn N_2 into ammonium (NH_4^+), which can subsequently be directly ingested by phytoplankton. Contrarily, the majority of nitrogen is converted by bacteria into nitrite (NO_2^{2-}) or nitrate (NO_3^-), a process called as nitrification. NO_3^- is the main nitrogenous compound is a major nutrient

required for photosynthesis that is utilized by primary producers in the ocean, phytoplankton. In order to support photosynthesis and synthesis of large molecules like proteins, nucleic acids, and chlorophyll, phytoplankton utilises and incorporates nitrogen (Gao et al., 2018). The subsequent trophic level receives the nitrogen that the phytoplankton has taken up. Then, when these species pass away and sink to deeper water, it is returned to the marine environment via the decomposition of waste products and organic materials. Denitrification is another process that can be used to turn ammonium, nitrate, and nitrite back into nitrogen, which can then be recycled into the cycle or released back into the earth's atmosphere (Figure 4) (Webb, 2021; Cai et al., 2019).

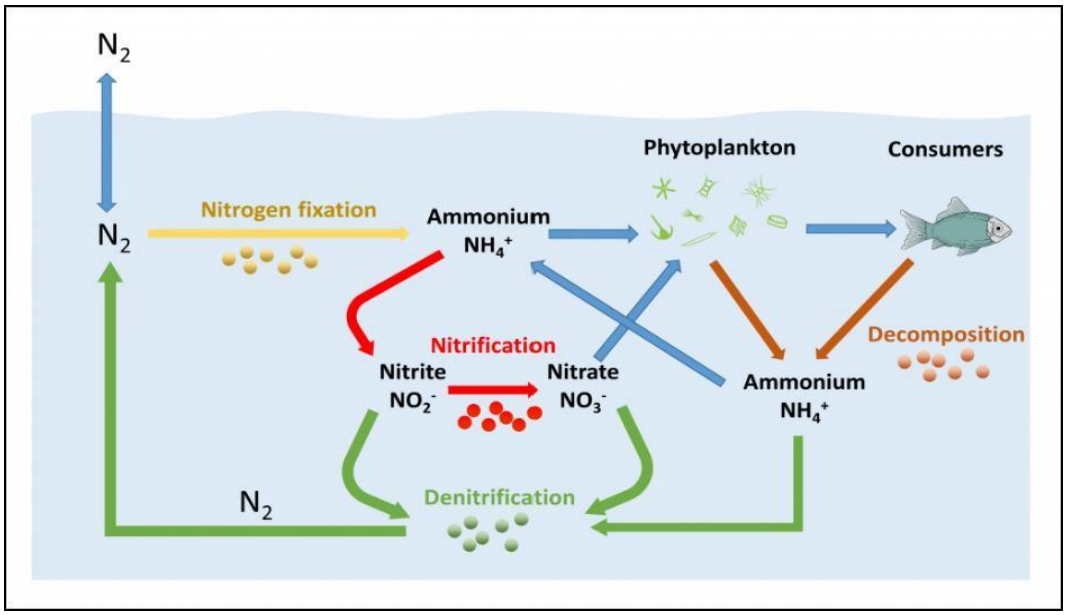


Figure 4. Schematic representation of role of phytoplankton in nitrogen cycle (Webb, 2021).

All living organisms require phosphorous to make up their genetic material (such as Deoxyribonucleic acid, DNA and Ribonucleic acid, RNA), energy molecules (Adenosine Tri-Phophate, ATP), and other essential organic compounds. Phosphate (PO_4^{3-}) ion happens to be the most abundant form of phosphorus which can be utilized by phytoplankton for growth under high phosphate conditions in the oceans. A fraction of the phosphorus is released back into the ocean pool, after assimilation, as dissolved organic phosphorus. This is, in turn, converted back to

phosphate by bacteria. Under low phosphate conditions, however, dissolved organic phosphorus will be utilized by phytoplankton and other marine organisms. They may also scavenge for phosphate by releasing phosphate from organic molecules using alkaline phosphatase (Figure 5) (Kamerlin et al., 2013; Yong et al., 2014).

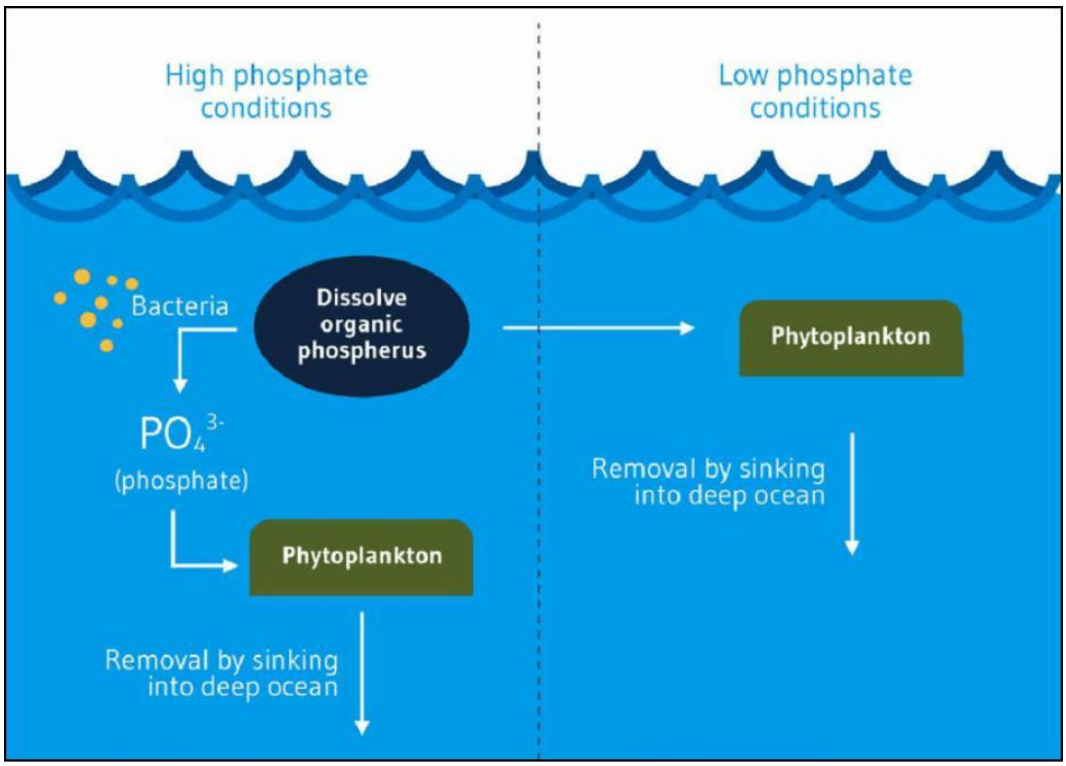


Figure 5. Schematic representation of role of phytoplankton in phosphorus cycle (Kwong, 2011).

Diatoms are a form of phytoplankton that account for 40% of marine primary production. They need silicate to develop and construct their opal shells, known as frustules, which are responsible for their attractive colour (Amo & Brzezinski, 1999; Tréguer et al., 1995). Compared to the nitrogen and carbon in sinking particles, the remineralization depth profile of the silicon in diatom shells is deeper. As these animals perish, their shells will sink and accumulate silica in the ocean's bottom (Figure 6).

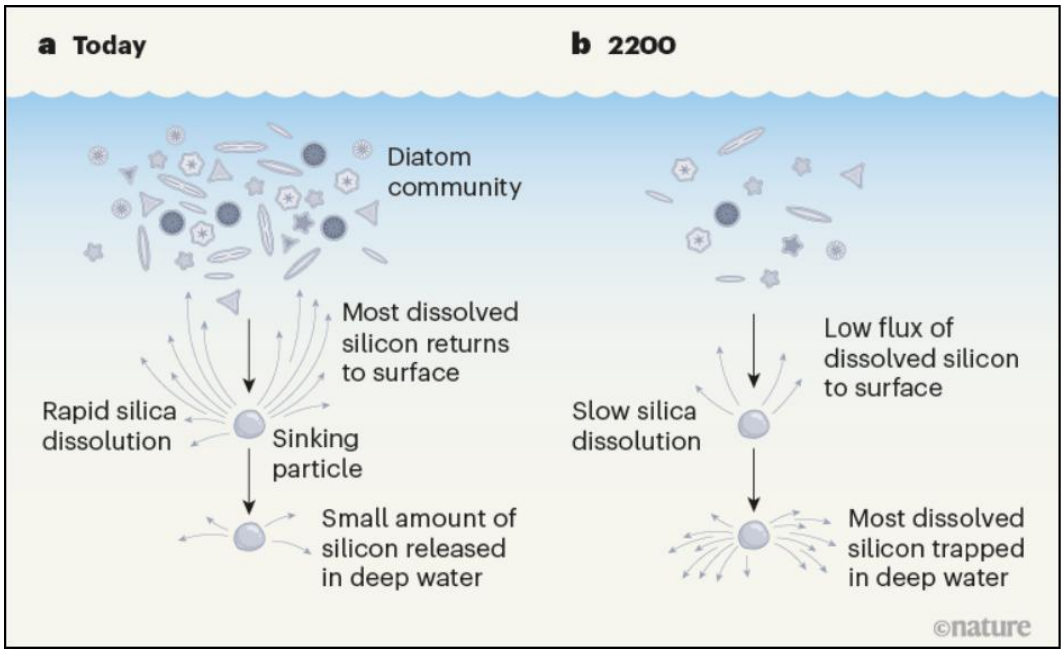


Figure 6. Schematic representation of role of phytoplankton in silica cycle (Taucher et al., 2022).

1.3. Factors affecting phytoplankton growth and community

The primary factors that influence microalgal biomass development and its composition include pH, temperature, salinity, duration of dark and light exposure, and carbon dioxide (Patrinou et al., 2022). Phytoplankton can experience elevated growth rates and attain high cell densities under certain environmental conditions. The interaction between variables such as higher carbon dioxide, higher temperature, and lower salinity may alter the composition of the phytoplankton community, according to a study on the effects of temperature, carbon dioxide, and also salinity on the phytoplankton community structure in the Western Arctic Ocean (Sugie et al., 2020). Phytoplankton blooms are essential for fisheries and coastal systems' benthic-pelagic coupling (Legendre, 1990). These increasing biomass episodes primarily result from variations in nutrients and light imposed by seasonal variations in radiation, temperature, and also water column stability. At the same time, the end phase has been ascribed to nutrient depletion and zooplankton grazing pressure (Sommer et al., 2012).

1.3.1. Temperature

The temperature has a well-known effect on phytoplankton's physiology and metabolic processes. Higher temperature increases the specific phytoplankton productivity under light-saturated conditions, by acting on photosynthetic carbon assimilation (Lewandowska et al., 2012). Under non-limiting nutrient conditions, the phytoplankton nutrient uptake increases with water temperature (Gillooly et al., 2001). Also, with the increase in temperature, the growth rates of phytoplankton also increase, almost increasing by two times with each 10 ℃ increase in temperature (Q_{10} temperature coefficient) (Rose & Caron, 2007). Furthermore, because phytoplankton develops faster at low temperatures than herbivorous grazers do, a rise in water temperature may benefit them more than their grazers, especially at relatively low in situ temperatures. This can, in turn, trigger the initiation of phytoplankton community blooms (Hunter-Cevera et al., 2016; Huntley, 1992). According to research by Trombetta et al. (2019), no specific water temperature can cause phytoplankton blooms. Instead, a wide range of water temperatures can initiate blooms. Blooms are initiated by the increase in water temperature. Hence, temperature is an important physical factor influencing the growth of planktonic plants.

1.3.2. pH

The pH of marine surface waters can influence several biological and physical processes. Inorganic carbon uptake (Carbon dioxide, CO_2) by phytoplankton during photosynthesis increases pH, while releasing CO_2 via respiration processes decreases the pH (Hansen, 2002). Variations in pH can change the availability of carbon, trace metals and essential nutrients and extreme pH levels may cause direct physiological effects on various phytoplankton species (Chen & Durbin, 1994). Although the optimum pH range for the growth of phytoplankton under laboratory conditions is pH 6.3-10, some species can grow well at a wide range of pH. In contrast, others have growth rates that may vary greatly even over a 0.5 to 1 pH unit change (Hinga, 2002).

1.3.3. Salinity

Some marine microalgae are capable of developing in an extensive range of salinities, however, they are often segregated from brackish or marine environments. Microalga requires optimal salinity conditions that are essential for the growth and accumulation of primary metabolites. Extreme salinity occasionally affects cellular metabolism by causing osmotic distress, ion stress, as well as shifts in cellular ionic ratios (Zhang et al., 2018). *Amphidinium carterae*, *Nephroselmis* sp., *Tetraselmis* sp. (var. Red Pappas), *Asteromonas gracilis*, and *Dunaliella* sp. were batch cultured at salinities 20 (low), 40 (sea), and 50/60/100 (high). Through this experiment, it was discovered that each of the five species under examination had varying responses to the various salinities that were employed. In 20, 40, and 50, *Amphidinium* sp. performed best. The growth of *Nephroselmis* sp. and *Tetraselmis* sp. was comparable at salinities of 20 and 40 but less successful above 60. While it could grow fairly well at 40 and 60, *Asteromonas* sp. fared best in 100. According to Hotos and Avramidou (2002), it was also found that *Dunaliella* sp. thrived successfully at all salinity levels (20, 40, and 60). To maximize microalgal development, a different study was conducted in which the effects of salinity and photoperiod on the proliferation of *Nannochloropsis* sp. along with *Tetraselmis* sp. were investigated (Fakhri et al., 2015). When the salinity had been high and nitrogen was limiting, it was discovered that nanophytoplankton and tiny microphytoplankton that have lower part-saturation constants and greater surface-to-volume ratios predominated (Cari et al., 2011).

1.3.4. Light and dark cycle

Alternating light and dark periods have been shown to synchronize the cell populations of numerous phytoplankton species. This entrainment by light and dark cycles regulates the cell division timing and also other cellular processes, including photosynthetic capacity and enzyme activity (Vaulot et al., 1986). In a study by Falkowski & Owens in 1978, a hysteresis was observed concerning increasing and decreasing light in all the species they examined. According to their research,

species-dependent respiration in the dark accounted for around 25% of total photosynthesis. A study on the effects of prolonged darkness on temperate and tropical marine phytoplankton showed that the organisms from both tropical and temperate locations were able to survive the prolonged dark period and were in sufficient health to reproduce and photosynthesize when returned back to a light regime (Carney et al., 2011).

1.3.5. Nutrients

The growth and stoichiometry of phytoplankton depend on the availability of multiple nutrients in their ambient surrounding (Klausmeier et al., 2004). The nutrient concentration also has an impact on the abundance and composition of phytoplankton communities (Barcelos & Ramos et al., 2017). These nutrients include the macro-nutrients nitrate and phosphate in case of most phytoplankton species. Diatoms, in addition, also require the macro-nutrient silicate inorder to construct their frustules. Trace metals such as Iron (Fe), Cobalt (Co), Copper (Cu), Molybdenum (Mo), Zinc (Zn) and Manganese (Mn) are required for the biochemical function of various enzymes and they regulate the productivity and species composition of marine phytoplankton (Barcelos & Ramos et al., 2017; DiTullio et al., 1993).

1.3.6. Climate change

The findings on climate-related modifications to marine ecosystems have increased significantly during the previous fifty years (Hays et al., 2005). Climate changes, such as the rise in atmospheric CO_2 and temperature, affect the marine biosphere by modifying the pH, carbonate availability, water column stability, nutrient and light regimes, which in turn directly impact the phytoplankton, whose short-term life cycles make them amenable to respond to subtle environmental variations quickly. Guinder & Molinero (2013) depict (Figure 7) how modifications at the bottom of the food web can permeate the trophic network because of trophic amplification and the subsequent cascading effects. Hydroclimatic variability from the large-scale and long-term to the local and short-term scales affects water

properties an,d consequently, the ecology of microalgae that follow the biophysical rules. The makeup of the phytoplankton community may change as a result of changes in the species physiological processes and behavioral patterns (such as ecological features and trade-offs). Interannual and/or interdecadal biomass patterns may eventually change as a result of these changes, which may also influence the timing of seasonal blooms.

Every level of ecological structure, from organisms to ecosystems, is impacted by climate-driven hydrological changes in marine phytoplankton. The increasing CO_2 and temperature can directly affect the phytoplankton's cell physiology (e.g., photosynthesis, growth rates and range shifts). The indirect consequences of changes in the pelagic environment (such as pH, light, nutrients, and grazing activity) include changes in phytoplankton dimensions-structure, stoichiometry, sedimentation rates, interactions between species, and bloom phenology. All these changes result in substantial alterations in the structure of pelagic food webs and ecosystem functioning (Guinder & Molinero, 2013; Hays et al., 2005).

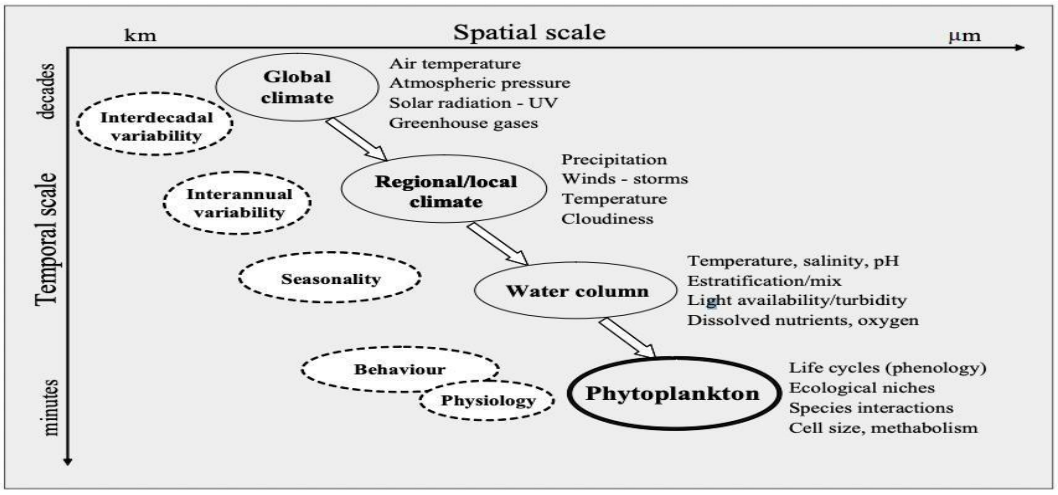


Figure 7. Schematic depiction of cascading climate-related alterations in the pelagic environment, the atmosphere-sea interfaces, and phytoplankton (Guinder & Molinero, 2013).

1.4 Tetraselmis genus

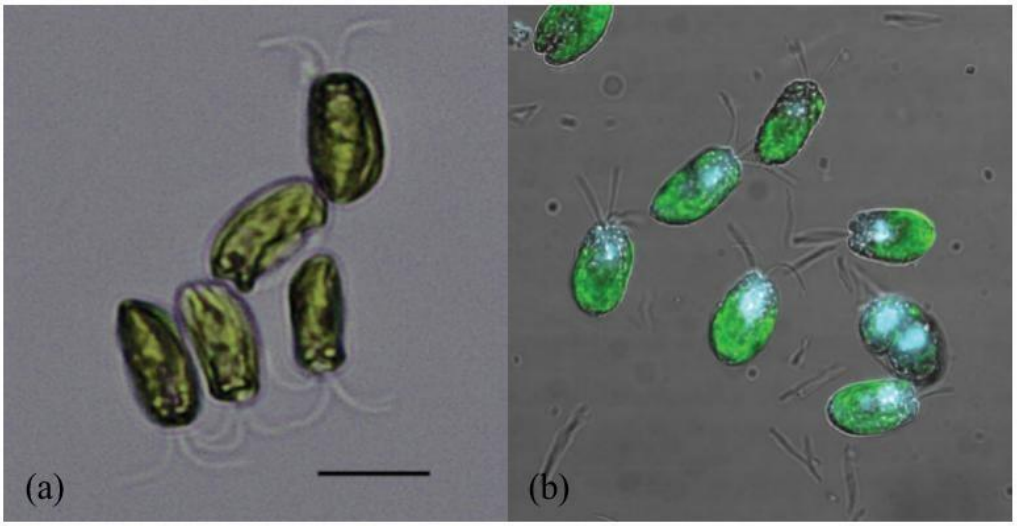


Figure 8. (a) *Tetraselmis sp.* from Rottnest Island, Western Australia's salt lake. The four flagella are clearly discernible. Scale line = 10 μm (Borowitzka, 2018). (b) *Tetraselmis suecica*, a type of microalga, under a confocal microscope (Greenwell et al., 2009).

1.4.1. Classification

Kingdom: Plantae

Subkingdom: Viridiplantae

Division: Chlorophyta

Subdivision: Chlorophytina

Class: Chlorodendrophyceae

Order: Chlorodendrales

Family: Chlorodendraceae

Genus: *Tetraselmis*

(Borowitzka, 2018; WoRMS Editorial Board, 2023).

1.4.2. Morphology

The single-celled flagellates *Tetraselmis spp.,* formerly known as *Platymonas,* have elliptical or nearly spherical and somewhat flattened cells. They possess an invagination at the anterior end, from which arise four equal flagella in two opposite pairs. A close-fitting theca of fused organic scales surround the cells. The Golgi apparatus produces the stellate scales which constitute the theca, which are subsequently released to the cell's surface (Domozych et al., 1981). The flagella are covered by square or diamond-shaped scales in twenty-four rows, which are overlaid by another twenty-four double rows of scales. From opposite sides of the flagella two rows of hair-shaped scales project.

1.4.3. Reproduction

The non-motile stage of *Tetraselmis* divide asexually within the parental periplast. Sexual reproduction in *Tetraselmis* has not been observed. Vegetative thick-walled cysts are also known in several species and germinate by dividing into four cells (Borowitzka, 2018).

1.4.4. Habitat

Marine/euryhaline and freshwater species are known. *Tetraselmis* species can be found in a variety of forms, including planktonic, benthic, sand-colonizing, and as endosymbionts in metazoans such as the acoelomate turbellarian flatworm *Symsagittifera* (Serôdio et al., 2011).

1.4.5. Significance

Tetraselmis spp. is easily culturable. Several species of *Tetraselmis*, such as *T. chui*, *T. suecica*, and *T. tetrahele*, have been known to be widely used as a feed in aquaculture (De Pauw & Persoone, 1988). The relevance of euryhaline strains of Tetraselmis, which are able to thrive over a very wide salinity range, as promising and long-lasting sources of lipids for biofuels, has increased recently (Fon-Sing & Borowitzka, 2016).

1.5. Objective of work

A few years ago, *Tetraselmis indica*, a new species of *Tetraselmis*, was discovered in salt pan nanoplankton in Goa, India. Its description was developed based upon morphological, ultrastructural, 18S rRNA gene sequence, and genome size data. (Arora et al., 2013). The characteristic features of *T. indica* have been depicted in Figure 9. which has been adopted from Arora & Anil, 2013.

Figure 9. Light microscope images of *T. indica*: 2 - Cell with emerging flagella. 3 - The locations of the eyespot (red-brown granules), pyrenoid (lower arrow), and nucleus (upper arrow). 4 - Resting cell. 5 & 6 - Narrow lateral view. 7 & 8 - Narrow lateral view of a cell attaching to the glass slide via the flagella. 9 & 10 - Posterior view. 11 - A cell's flagellar opening (left arrow) as well as its unique crease (right arrow) are visible in a confocal micrograph of the cell. Scale bars = 10 μm (Arora & Anil, 2013).

The salt pan from where *T. indica* was isolated had salinity and temperature ranging from 35 to 350 and 48.2 °C and 28.5 °C, respectively (Arora & Anil, 2013). However, no research has been done to pinpoint the physiological mechanisms of *T. indica* that might control its capacity to adapt to such a wide range of salinities. *Tetraselmis indica* has demonstrated that it can withstand extreme environmental conditions (e.g. prolonged darkness for ten months) by changing its morphology, biochemical composition and forming resting cells (Naik & Anil, 2018). These

factors have thus been beneficial biomarkers to decipher the physiological state of the cells and their adaptive response to changing environmental conditions. This study is aimed to understand the influence of a wide range of salinities on *T. indica*.

2. MATERIALS AND METHODOLOGY

2.1. Collection and treatment of seawater

2.1.1. Material required

Glassware - Measuring cylinder, Conical flasks

Chemicals - Soap solution, 10% Dilute Hydrochloric acid (HCl), Sodium Chloride (NaCl)

Other equipments - Plastic cans, Marker, 0.22 µm pore size filter paper

Instruments - Refractometer, Thermometer, Filtration unit, Autoclave

2.1.2. Prerequisites

The glasswares used were thoroughly washed with soap, soaked in 10% HCl and rinsed with distilled water before using.

2.1.3. Method

Seawater was collected from Cacra beach, the date and temperature of the water were noted down. After bringing the water to the lab it was first filtered through 0.22 µm pore size filter paper and then autoclaved for 20 minutes at 121 ℃. The water was cooled under running tap water to avoid crystal formation. Salinity was measured using a refractometer (adjusted to 35 with NaCl solution) and the water was stored for further use in a clean, cool and dark place after labeling it appropriately.

2.2. Preparation of standard f/2 media

2.2.1. Material required

Glassware - Beakers, Conical flasks, Measuring cylinders, Volumetric flasks, Glass rod

Chemicals - Sodium nitrate ($NaNO_3$), Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$), Sodium metasilicate nonahydrate ($Na_2SiO_3.9H_2O$), Copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$), Sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$), Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$), Cobalt (II) chloride hexahydrate ($CoCl_2.6H_2O$), Manganese (II) chloride tetrahydrate ($MnCl_2.4H_2O$), Iron (III) chloride hexahydrate ($FeCl_3.6H_2O$), Ethylenediaminetetraacetic acid disodium salt dihydrate ($Na_2EDTA.2H_2O$), Biotin (Vitamin H), Cyanocobalamin (Vitamin B_{12}), Thiamine.HCl (Vitamin B_1), Sterile distilled water, Sterile sea water

Other equipments - Spatula, Butter paper, Droppers, Micropipette, Pipette tips, Filter paper

Instruments - Weighing balance, Autoclave, Filtration unit, Laminar air flow

2.2.2. Method f/2 media was prepared as per the standard compositions given by Guillard, 1975. To prepare 1 L of f/2 media, trace metal solution and vitamin solution were prepared as shown in Table 1 a and Table 1 b respectively and then phosphate, nitrate, silicate, trace metal and vitamin solutions were added to sterile (filtered and autoclaved) sea water in quantities as given in Table 1 c.

Table 1 a. Preparation of trace metal solution

Trace metal (stock solution)	**Amount**	**Molar concentration in final medium**
Copper (II) sulfate pentahydrate		

($CuSO_4.5H_2O$)	1 mL	3.93×10^{-8} M
Sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$)	1 mL	2.60×10^{-8} M
Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$)	1 mL	7.65×10^{-8} M
Cobalt (II) chloride hexahydrate ($CoCl_2.6H_2O$)	1 mL	4.20×10^{-8} M
Manganese (II) chloride tetrahydrate ($MnCl_2.4H_2O$)	1 mL	9.10×10^{-7} M
Iron (III) chloride hexahydrate ($FeCl_3.6H_2O$)	3.14 g	1.17×10^{-5} M
Ethylenediaminetetraacetic acid disodium salt dihydrate ($Na_2EDTA.2H_2O$)	4.36 g	1.17×10^{-5} M

Table 1 b. Preparation of vitamin solution.

Vitamin (Stock solution)	**Amount**	**Molar concentration in final medium**
Biotin (Vitamin H)	10 mL	2.09×10^{-9} M
Cyanocobalamin (Vitamin B_{12})	1 mL	3.69×10^{-10} M

Thiamine.HCl (Vitamin B_1)	200 mg	2.96 x 10^{-7} M

Table 1 c. Preparation of 1 L of f/2 medium

Component	**Amount (mL)**	**Molar concentration in final medium**
Sodium nitrate ($NaNO_3$)	1	8.83 x10^{-4} M
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$)	1	3.63 x10^{-5} M
Sodium metasilicate nonahydrate ($Na_2SiO_3.9H_2O$)	1	1.07 x10^{-4} M
Trace metal solutio	1	-
Vitamin solution	0.5	-

2.3. Preparation of Lugol's Iodine solution

2.3.1. Material required

Glassware - Beakers, Measuring cylinder, Volumetric flask, Glass rod, Reagent bottle

Chemicals - Potassium Iodide (KI), Crystalline iodine (I_2), Glacial acetic acid, Sterile distilled water

Other equipments - Spatula, Butter paper

Instruments - Weighing scale

2.3.2. Method

Lugol's iodine solution was prepared by dissolving 100 g Potassium Iodide (KI) in 1 L of distilled water followed by dissolving 50 g crystalline iodine (I_2) in this solution and then adding 100 mL glacial acetic acid. This was then stored in an airtight reagent bottle in cool and dark place.

2.3.3. Note

Lugol's iodine must be stored in the dark because iodine is light sensitive and will degrade in the presence of light. It must be ensured that it is stored in a bottle with a tight-fitting lid and kept away from the general culture environment.

2.4. Preparation of 0.25% glutaraldehyde from 50% glutaraldehyde

2.4.1. Material required

Glassware - 50 mL amber stoppered bottle, Measuring cylinder

Chemicals - 50% glutaraldehyde, Distilled water

Other equipments - Micropipette, Pipette tips

2.4.2. Method

20 mL of glutaraldehyde was prepared by adding 4.9 mL of 50% glutaraldehyde to 20 mL of distilled water and this was used as a cell fixative.

2.5. Measurement of salinity

2.5.1. Material required

Glassware - Beakers

Chemicals - Distilled water

Other equipments - Dropper, Tissue paper

Instruments - Refractometer

2.5.2. Method

Calibration of refractometer - The refractometer platform was cleaned with distilled water and a drop of distilled water was placed to check if the salinity is 0. If not, the final salinity was corrected by either adding (if salinity of distilled water shows greater than 0) or subtracting (if salinity of distilled water shows less than 0) the value obtained.

Once calibrated a drop of sample is placed on the clean slide of the refractometer and the salinity reading is noted down. If there are more than one samples whose readings must be taken, the slide of the refractometer must be cleaned with distilled water after each reading.

2.6. Measurement of temperature

2.6.1. Material required

Glassware - Beakers

Instrument - Thermometer

2.6.2. Method

The thermometer was shaken well before use. The thermometer was dipped in the container containing the sample such that it is in the center of the container and waited for at least a minute before noting down the temperature.

2.7. Enumeration of phytoplankton

2.7.1. Material required

Glassware - Beakers

Chemicals - Lugol's iodine or 0.25% glutaraldehyde, distilled water, seawater

Other requirements - 1000 µL and 20 µL Micropipettes, 1000 µL and 100 µL Pipette tips, Eppendorf tubes

Instruments - Sedgewick rafter, Haemocytometer, Microscope

Sample - *Tetraselmis indica* culture

2.7.2. Method

Sample preparation

980 µL of culture was taken in an Eppendorf tube. The samples were fixed with 20 µL of Lugol's iodine or 0.25% glutaraldehyde.

Counting using Sedgewick rafter

Figure 10. Sedgewick rafter (Image courtesy: Graticules Optics).

A clean coverslip was placed diagonally on the Sedgwick rafter slide and using a micropipette 1 mL of sample was transferred onto the Sedgewick rafter slowly from the corner. Once the chamber was filled , the coverslip was slowly moved to cover the slide, avoiding forming any bubbles completely. Before beginning to count, the slide was placed on the microscope stage and the cells were allowed to settle. The chamber was checked at a low magnification for an even spread of settled cells and it was confirmed that no cells have floated to the top of the chamber. It is customary to only count the cells that are in contact with the top and left rulings of each square when counting cells that are close to grid lines. Under 40 X, two transects were counted each with 50 squares and the cell concentration (number of cells mL^{-1}) was calculated using the following formula:

$$C = \frac{N \times 1000\ mm^3 \times DF}{L \times D \times W \times S}$$

Where,

N = number of cells/colonies counted

DF = dilution factor

L = length of transect strip (mm)

W = width of transect strip (mm)

D = chamber depth (mm)

S = number of transects counted

Counting using a haemocytometer

To fill haemocytometer chambers, the thick cover glass was placed over both grids. The pipette was subsequently grasped at a 45-degree angle. Depending on the desired flow rate, the angle can be changed to a greater or lower angle. The sample was permitted to enter swiftly and uniformly into the chamber by placing the tip at the leading end of the coverslip while applying very light pressure. After the cells had settled for about a minute, the grid was examined under a microscope (20X objective) to ensure that the cells had been distributed satisfactorily, and evenly. Triple lines split the haemocytometer's grid into nine sizable squares, each measuring 1 mm by 1 mm. Each big square is split into 25 intermediate-sized squares with a side measurement of 0.23 mm. Additionally, 16 smaller squares split the intermediate-sized ones, each measuring 0.05 mm on one side. All nine squares in this experiment were counted to get the cell count. The average cell count was multiplied by the conversion factor ($x10^4$ for Neubauer) to determine the cell density.

2.7.3. Note

Sedgwick rafter

In case the cell density is more than 10,000 cells/mL then the sample was diluted and then counted.

In case of a large number of treatments and/or replicates wherein there may be several days (or weeks) between the collection of the sample and counting, it is

usually recommended to store preserved count tubes away from the sun and at cool temperatures (preferably in a fridge).

The Eppendorf tubes containing the sample must be shaken before taking the sample to homogenize the sample as the cells in the sample may stick to the tube bottom and sides.

The time taken to fill the chambers should be short to minimize the settling of cells in the pipette.

If flooding or bubbles occur, the chamber and coverslip must be rinsed with deionised water, dried with lint-free absorbent paper and the procedure must be repeated.

Haemocytometer

In the Neubauer brand the chamber surface is a flat mirror-like rectangle and it must be ensured that the sample must cover this rectangle but not flow over its edges. It is useful to rest the hand on a bench and steady the pipette tip.

If flooding occurs, haemocytometer and coverslip should be rinsed with distilled water, and the procedure must be repeated.

2.8. Experimental set-up

2.8.1. Material required

Glassware - Conical flasks, Measuring cylinders, Beakers

Chemicals - Filtered and autoclaved seawater, Sterile concentrated Sodium Chloride solution, Sterile distilled water, f/2 media components

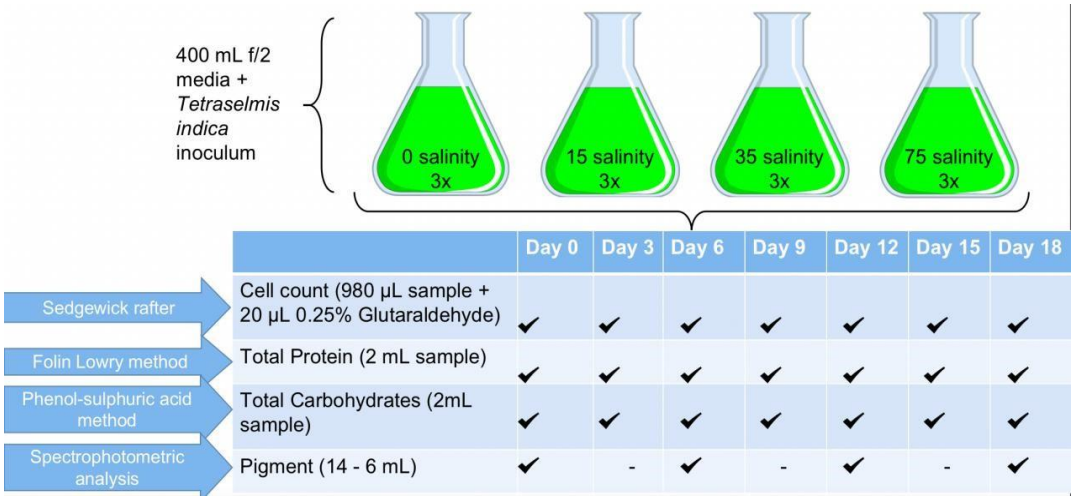
Other equipment - Micropipette, Pipette tips

Instruments - Refractometer

Culture - Pure subculture of *T. indica* subcultures that were maintained in different salinity conditions (0, 15, 25, 35, 50 and 75) were used as inoculum for experimental analysis.

2.8.2. Method

400 mL media was prepared in sterile conditions with salinities of 0, 15, 35 and 75 in triplicates and the flasks were labelled appropriately. These were then inoculated with *T. indica* culture from subcultures of respective salinity and incubated at room temperature with a 16:8 hour light:dark period. Required samples were taken on Day 0 and after every three days up till Day 18 for different analysis as shown in Figure 11.



	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18
Cell count (980 µL sample + 20 µL 0.25% Glutaraldehyde)	✔	✔	✔	✔	✔	✔	✔
Total Protein (2 mL sample)	✔	✔	✔	✔	✔	✔	✔
Total Carbohydrates (2mL sample)	✔	✔	✔	✔	✔	✔	✔
Pigment (14 - 6 mL)	✔	-	✔	-	✔	-	✔

Figure 11. Experimental set-up

2.9. Growth of T. indica under different salinity conditions

2.9.1 Material required

Glassware - Conical flasks, Beakers

Chemicals - f/2 media, NaCl solution, Sterile distilled water

Other equipment - Spatula, Micropipette, Pipette tips

Instruments - Autoclave, Laminar air flow, Refractometer, Thermometer

Culture - Pure subculture of *T. indica* that was maintained in f/2 media at 35 salinity and 16:8 light:dark conditions was used as inoculum.

2.9.2 Method

Preliminary experiment

The flasks with different salinities (0, 15, 25, 35, 50, 75 and 100) were inoculated with the culture inoculum at a final concentration of 2000 cells per mL. 980 µL of sample from each flask in duplicates in 1.5 mL Eppendorf tubes was then taken and fixed with 20 µL of Lugol's iodine starting from Day 0 and then every alternate 3 days up until Day 30. The cells were counted using Haemocytometer or Sedgewick rafter (Refer section 2.7) and a growth curve was plotted. This growth curve was then used to determine which salinities to be used for the main experiment.

Analysis of sample

980 µL samples were taken in Eppendorf tubes from the triplicate flasks of 0, 15, 35 and 75 salinity on Day 0, 3 6, 9, 12, 15 and 18 and fixed with 20 µL of 0.25% glutaraldehyde. 1 mL of each sample was loaded onto the Sedgewick rafter and cell concentration was estimated using the Sedgewick rafter method as mentioned in Section 2.7. Highly concentrated samples were diluted with filtered seawater. The obtained values were plotted to derive results and conclusion.

2.10. Total protein

Total protein estimation was done by using the Folin Lowry method given by Lowry et al. (1951) and modified by Price (1965) (Slocombe et al., 2013).

2.10.1. Material required

Glassware - Beakers, Stoppered test tubes, Glass pipettes, Measuring cylinders, 1 cm quartz cuvettes

Chemicals - Sodium Carbonate (Na_2CO_3), Sodium Hydroxide (NaOH), Copper Sulphate ($CuSO_4$), Sodium Potassium Tartrate, Folin reagent, Bovine Serum Albumin (BSA), Sterile distilled water

Other equipment - Spatula, Micropipette, Pipette tips, Testube stand

Instruments - Weighing balance, Spectrophotometer

Samples - 2 mL samples were taken in vials from the triplicate flasks of 0, 15, 35 and 75 salinity on Day 0, 3 6, 9, 12, 15 and 18 and stored at 4 ℃ until further analysis.

2.10.2. Method

Preparation of reagents

Reagent A - Equal volume of 2% Na_2CO_3 in 0.1N NaOH was mixed.

Reagent B - Equal volume of 0.5% $CuSO_4$ with 1% Sodium Potassium Tartrate was mixed.

Reagent C - Reagent A and Reagent B in the ratio 50:1 respectively were mixed (must be used within 24 hours).

Reagent D - Folin reagent.

Preparation of standards and blank

A standard stock solution of Bovine Serum Albumin (BSA) was prepared by adding 25 grams BSA in 25 mL distilled water to make a final concentration of 1000 µg/mL and this was used for the preparation of standards. 2 mL standard solutions of 20 µg/mL, 40 µg/mL, 80 µg/mL, 150 µg/mL, 250 µg/mL and 500 µg/mL concentrations were prepared in duplicates in stoppered tubes labeled accordingly. 2 mL distilled water was used as blank.

Spectrophotometric analysis of protein standards

To the stoppered tube containing 2 mL of standard solution/blank, 2 mL Reagent C was added. After waiting for 10 minutes, 1 mL of Folin reagent was added to the mixture and it was kept aside for 30 minutes. After the waiting period, the

absorbance was measured at 750 nm wavelength. Average was taken and a standard curve of concentration v/s absorbance at 750 nm was plotted to obtain an equation which would be used to determine the unknown concentration.

Spectrophotometric analysis of samples

2 mL of sample was taken in appropriately labeled stoppered tubes and 2 mL Reagent C was added. After waiting for 10 minutes, 1 mL of Folin reagent was added to the mixture and it was kept aside for 30 minutes. After the waiting period, the absorbance was measured at 750 nm wavelength. The absorbance values and the equation obtained from the standard plot was used to determine the unknown concentration. The obtained values were averaged and plotted to derive results and conclusion.

2.11. Total carbohydrates

Carbohydrate estimation was done by using the phenol-sulphuric acid method which was described by Dubois et al., 1956.

2.11.1 Material required

Glassware - Beakers, Stoppered test tubes, Glass pipettes, Measuring cylinders, 1 cm quartz cuvettes

Chemicals - 5% Phenol, Concentrated Sulphuric acid (H_2SO_4), Glucose, Sterile distilled water

Other equipment - Spatula, Micropipette, Pipette tips, Testube stand

Instruments - Weighing balance, Spectrophotometer

Samples - 2 mL samples were taken in vials from the triplicate flasks of 0, 15, 35 and 75 salinity on Day 0, 3 6, 9, 12, 15 and 18 and stored at 4 ^{o}C until further analysis.

2.11.2. Method

Preparation of standards and blank

A Glucose standard stock solution was prepared by adding 25 grams of Glucose in 25 mL distilled water to make a final concentration of 1000 µg/mL and this was used for the preparation of standards. 2 mL standard solutions of 20 µg/mL, 40 µg/mL, 80 µg/mL, 150 µg/mL, 250 µg/mL and 500 µg/mL concentrations were prepared in duplicates in stoppered tubes labeled accordingly.
2 mL distilled water was used as blank.

Spectrophotometric analysis of carbohydrate standards

To the stoppered tube containing 2 mL of standard solution/blank, 0.5 mL 5% Phenol and 5 mL of concentrated Sulphuric acid was added. The absorbance was then measured at 490 nm wavelength. Average was taken and a standard curve of concentration v/s absorbance at 490 nm was plotted to obtain an equation which would be used to determine the unknown concentration.

Spectrophotometric analysis of samples

To the stoppered tube containing 2 mL of standard solution/blank, 0.5 mL 5% Phenol was added followed by the addition of 5 mL of concentrated Sulphuric acid. The absorbance was measured at 490 nm wavelength. The absorbance values and the equation obtained from the standard plot was used to determine the unknown concentration. The obtained values were averaged and plotted to derive results and conclusion.

2.12. Pigments

Method for chlorophyll pigment estimation was adapted from Lesley Clementson CSIRO (April, 2002).

2.12.1. Material required

Glassware - Beakers, Conical flask, 1 cm quartz cuvettes

Chemicals - 90% acetone, Distilled water

Other equipment - 22 mm GF/F filter paper, Forceps, Small filtration unit, Syringe, 1.5 mL Eppendorf tubes, Centrifuge tubes, Micropipette, Pipette tips, Testube stand, Aluminium foil, Parafilm

Instruments - Vortex, Centrifuging machine, Spectrophotometer

Samples - Appropriate amount of samples were taken from the triplicate flasks of 0, 15, 35 and 75 salinity on Day 0, 6, 12 and 18, filtered through GF/F filter paper in dim light condition at low pressure and stored at -20 ^{o}C in appropriately labeled Eppendorf tubes until further analysis.

2.12.2 Method

The filter paper with the filtered sample was taken in a centrifuge tube and 5 mL of 90% acetone was added to it. It was then vortexed for 30 seconds, covered with foil, sealed with parafilm and stored in the refrigerator at 4 ℃ overnight. Next day, the tubes containing the sample with acetone were centrifuged at 5000 rpm for 5 minutes. Absorbance was then taken at wavelengths 630 nm, 647 nm, 664 nm and 750 nm. The absorbance at 750 nm was subtracted from the absorbance at the wavelengths 664 nm, 647 nm and 630 nm to obtain the corrected wavelengths and these were then substituted into the following equations:

$$[\text{Chl. a}]\ \text{extract} = \frac{11.85}{A_{664} - 1.54\, A_{647} - 0.08\, A_{630}}\, l$$

$$[\text{Chl. b}]\ \text{extract} = 21.03 \frac{}{}\, A_{647} - 5.43\, A_{664} - 2.66\, A_{630}$$

$$l$$

$$[\text{Chl. c}]\ \text{extract} = \frac{24.52\, A_{630} - 1.67\, A_{664} - 7.60\, A_{647}}{l}$$

Where,

A = Corrected absorbance. l

= Path length in cm.

The concentration of each chlorophyll in the sample in µg/L was then obtained by the following equation:

$$[\text{chl. x}]\ \text{sample} = [\text{chl. x}]\ \text{extract} \times \frac{v}{V}$$

Where, v = Volume of extract

in mL.

V = Volume of seawater filtered in L.

The total concentration of chlorophyll in the sample in µg/L was obtained by the following equation:

$$[\text{chl. }]\ \text{total} = [\text{chl. a}]\ \text{sample} + [\text{chl. b}]\ \text{sample} + [\text{chl. c}]\ \text{sample}$$

Further chlorophyll content per cell was calculated by dividing the amount of chlorophyll obtained in µg/L by the cell abundance. The obtained values were averaged and plotted to derive results and conclusion.

3. RESULTS AND DISCUSSION

3.1. Growth of T. indica in different salinities

The data obtained in the preliminary experiment is depicted in Figure 12 and Table 2.

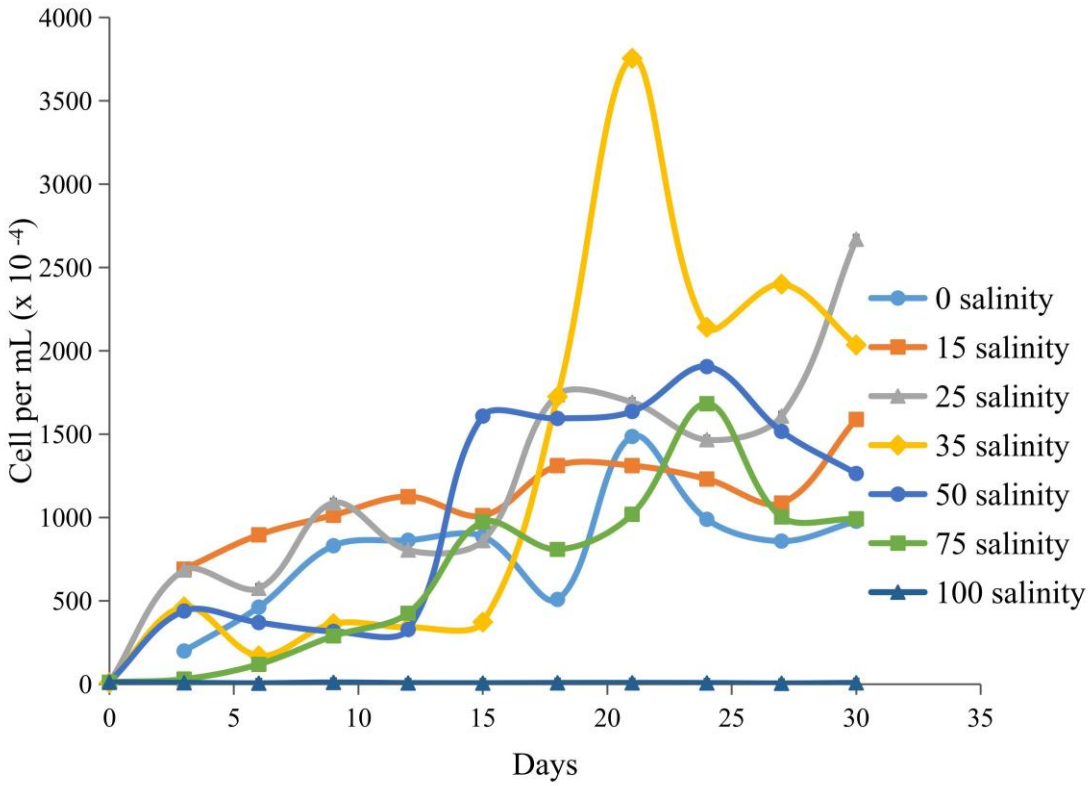


Figure 12. Growth plot obtained from preliminary experiment.

Table 2. Growth rate, doubling time and generation time obtained from preliminary experiment.

Salinity	Growth rate (d^{-1})	Doubling time	Generation time
0	0.1496	4.6129	0.2168
15	0.1658	4.1626	0.2402
25	0.1831	3.7693	0.2653
35	0.1831	3.7695	0.2653
50	0.1519	4.5436	0.2201

75	0.1475	4.6793	0.2137
100	0	0	0

The data obtained in the main experiment is depicted in Figure 13 and Table 3.

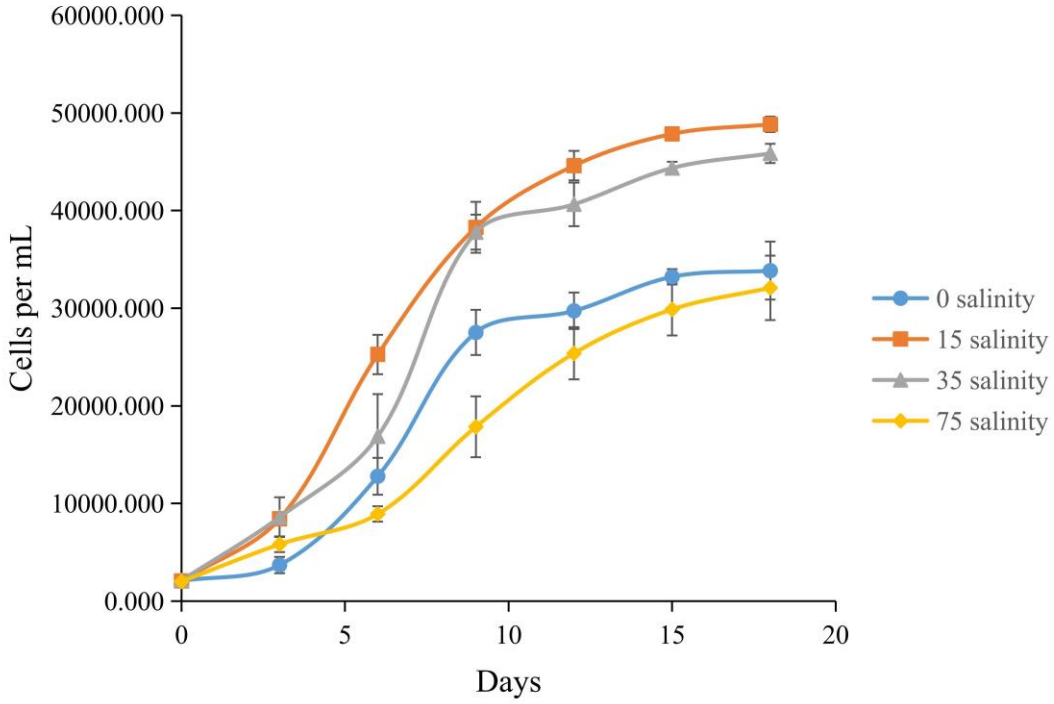


Figure 13. Growth plot obtained from main experiment.

Table 3. Growth rate, doubling time and generation time obtained from main experiment.

Salinity	Growth rate (d^{-1})	Doubling time	Generation time
0	0.155	4.444	0.225
15	0.176	3.929	0.254
35	0.171	4.044	0.247
75	0.154	4.487	0.223

Tetraselmis indica tolerated salinity upto 100, i.e. at salinity 100 and beyond *T. indica* showed no growth as per the preliminary experiments. *Tetraselmis indica* showed higher cell abundance at salinities 15 and 35 as compared to 0 and 75 in the main experiment. This result coincides with the results of a similar study that showed that *Tetraselmis* sp. cultured at different salinities of 20, 30 and 40, had significantly higher cell density under control condition at 30 ppt (Khatoon et al., 2014). It is clear from both the preliminary and main studies that the cells had a longer lag phase before the exponential phase at higher salinities (50 and 75) and salinity 0 (freshwater condition). This demonstrated that they were not growing as quickly as they would have under ideal conditions, which include salinities of 15, 25, and 35. It can be seen that *T. indica* achieved highest growth rate at 0.176 per day and fastest doubling time of 3.929 at salinity 15. Similar findings were found in a 2015 research by Fakhri et al. *Tetraselmis* sp. had a maximum growth rate of 0.63 per day (p 0.05) and the fastest time to double was 1.09 days at a salinity of 15. Additionally, they found that *Tetraselmis* sp. phytoplankton exhibits strong salinity dependence in terms of cell concentration and also growth rate.

3.2. Total protein content variation of T. indica at different salinities

The total protein content was estimated by first obtaining an equation from the standard plot of the standardizing agent for proteins i.e. Bovine Serum Albumin (BSA). The concentrations used were 20 µg/mL, 40 µg/mL, 80 µg/mL, 150 µg/mL, 250 µg/mL and 500 µg/mL and the standard curve and equation were obtained as shown in Figure 14.

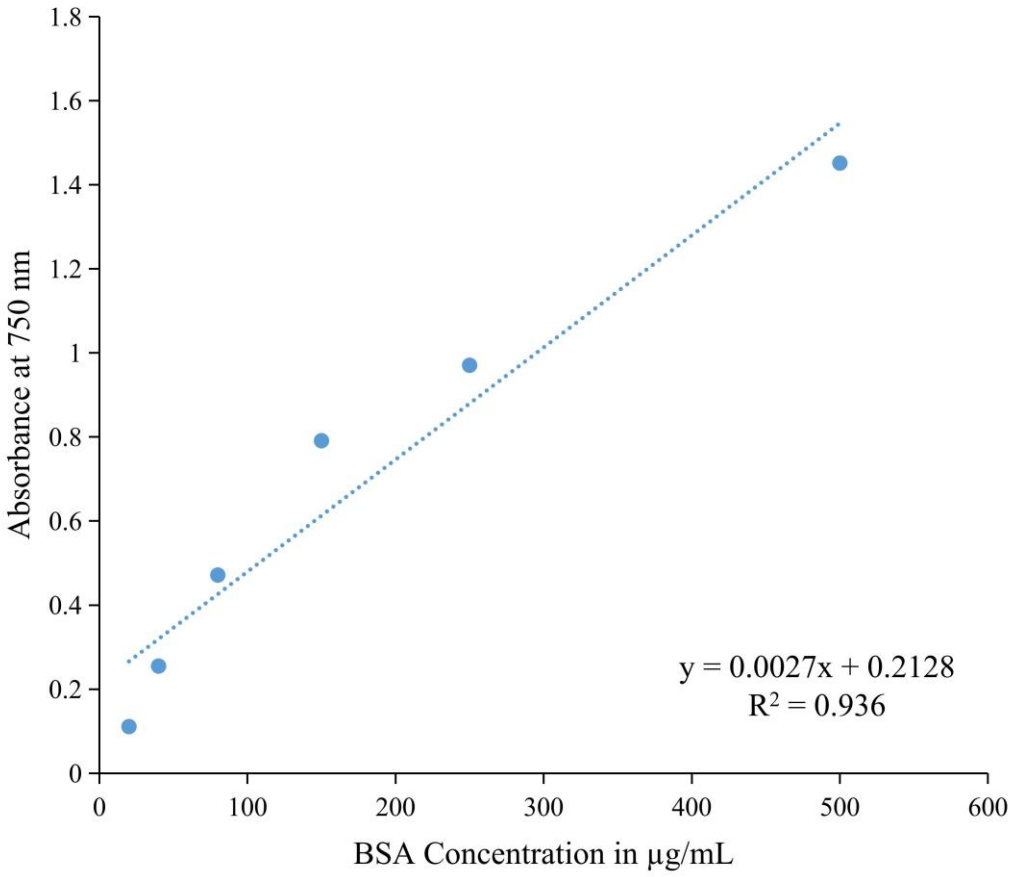


Figure 14. Standard plot for protein.

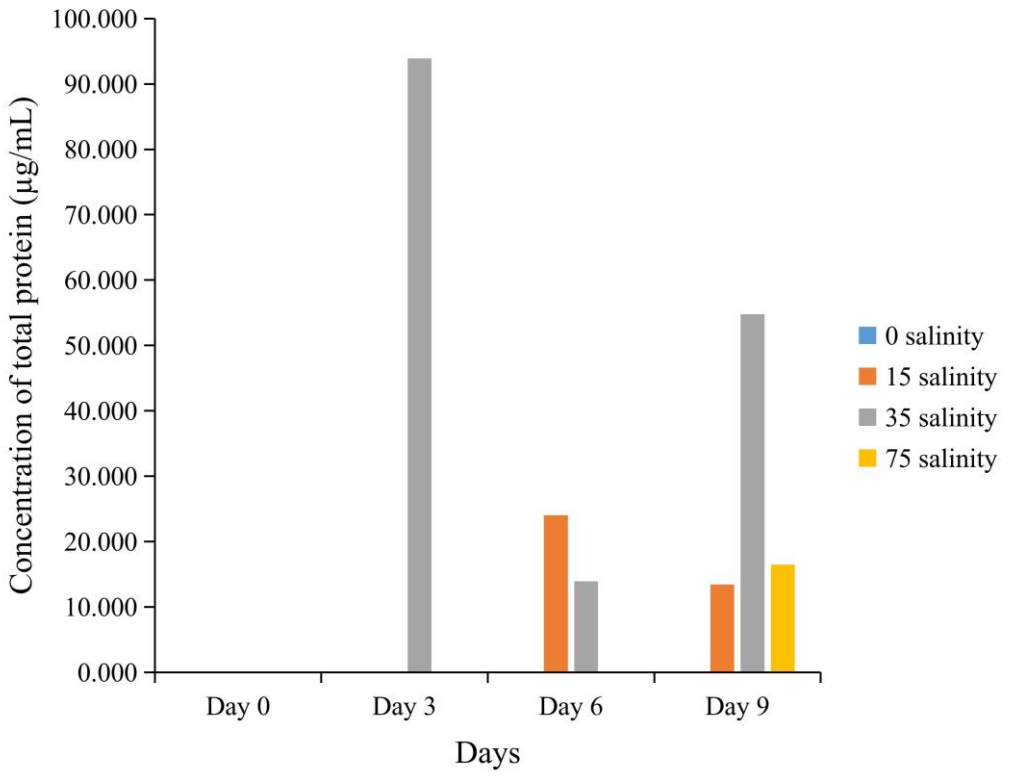


Figure 15. Total protein concentration of *T. indica* at different salinities.

The protein analysis could not be done upto Day 18 due to some issues and the results obtained were not satisfactory. Hence, nothing can be said conclusively upon the protein content.

3.3. Total carbohydrate content variation of T. indica at different salinities

The carbohydrate content was estimated by first obtaining an equation from the standard plot of the readily available standardizing agent for carbohydrate i.e. Glucose. The concentrations used were 20 µg/mL, 40 µg/mL, 80 µg/mL, 150 µg/mL, 250 µg/mL and 500 µg/mL and the standard curve and equation were obtained as shown in Figure 16.

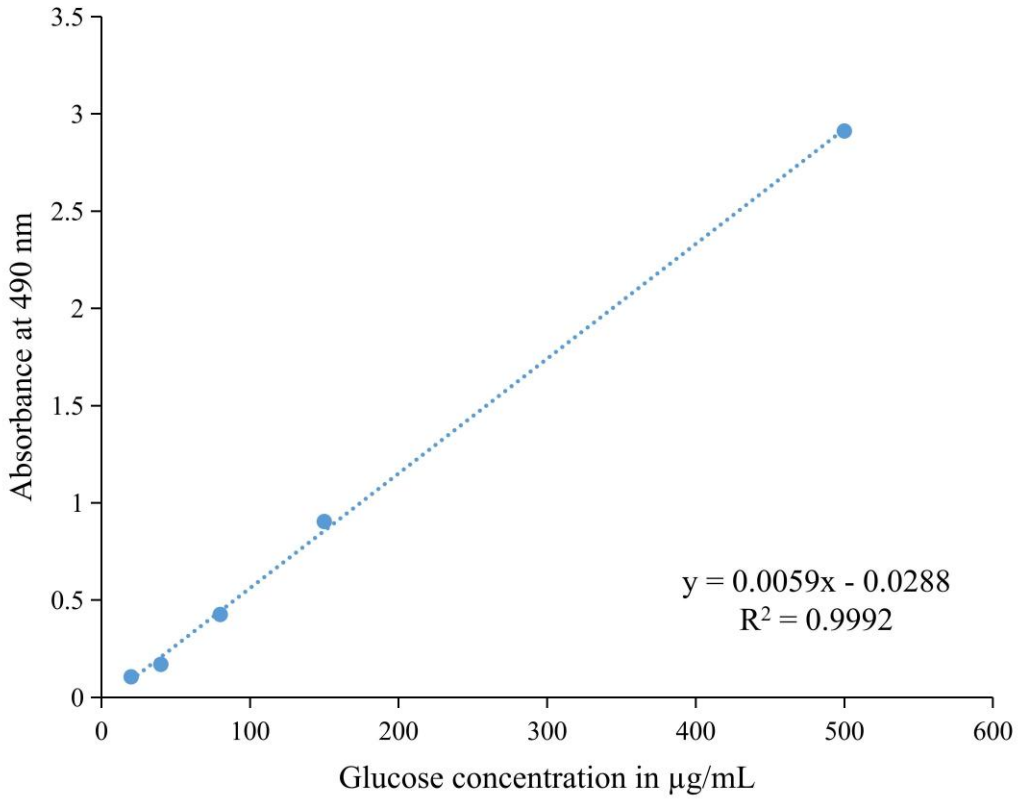


Figure 16. Standard plot for carbohydrates.

The carbohydrate content was estimated for salinities 0, 15, 35 and 75 for Day 0, 3, 6, 9, 12, 15 and 18. After plotting the curve, it was shown that *T. indica* originally had a low total carbohydrate content, however, it started declining on Days 6 and 9. Continuing on Day 12, the amount of total carbohydrate per cell subsequently began to gradually grow, as seen in Figure 17.

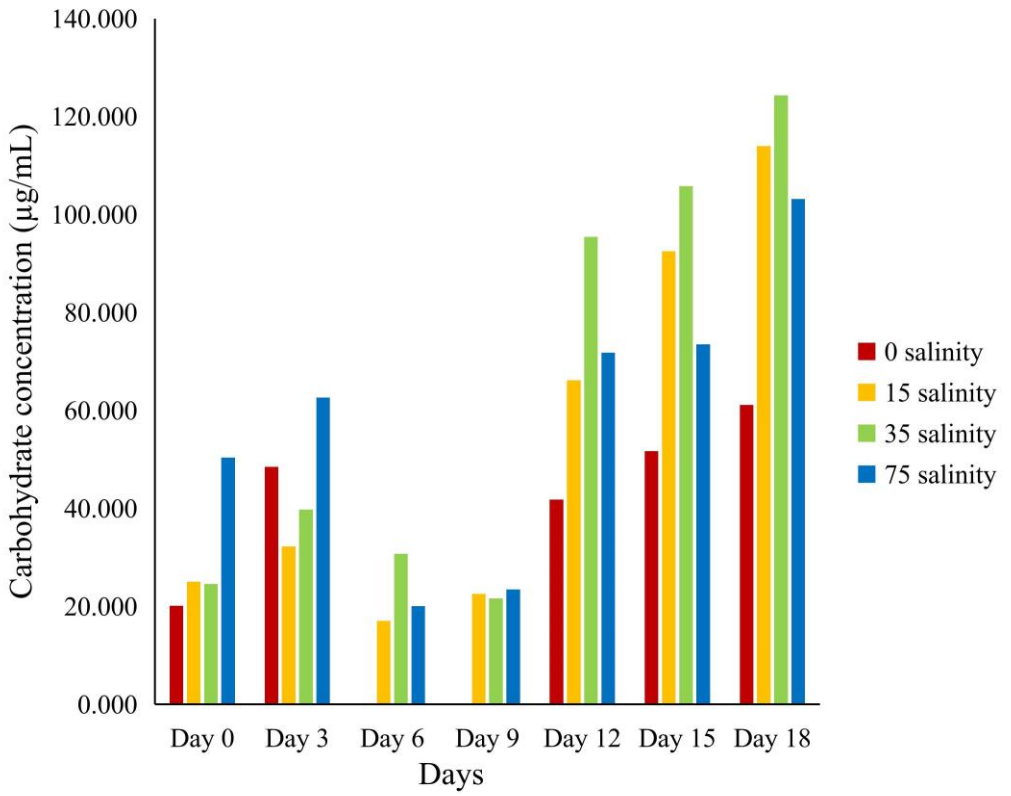


Figure 17. Total carbohydrate concentration of *T. indica* at different salinities.

3.4. Production of pigment by T. indica at different salinities

The makeup of the pigment can be used as a biomarker to determine a cell's physiological status and its ability to respond to changing circumstances in the environment (Naik & Anil, 2018). The production of pigments (Chlorophyll *a*, *b* and *c*) by *T. indica* was estimated for salinities 0, 15, 35 and 75 for Day 0, 6, 12, and 18. Figure 18 a. to 18 d. depict the total concentration of chlorophyll and the concentration of different chlorophyll pigments (*a*,*b* and *c*) produced by *T. indica*.

On Days 0, 6, and 12, the total chlorophyll and chlorophyll *c* concentration per cell for 0 salinity was almost the same, but it was lower on Day 18. On Day 0 for 0 salinity, the chlorophyll *a* level was high, but by Day 6, it had slightly reduced and remained almost unchanged for the following days. On Day 0, there was less chlorophyll *b* in each cell at 0 salinity. But on Day 6, it marginally grew, and on Days 12 and 18, it gradually declined.

On Day 0, the total chlorophyll content as well as chlorophyll *a*, *b*, and *c* content per cell was quite high for 15 salinity; however, following that, it proceeded to decline quite rapidly.

On Day 0, there was a high concentration of total chlorophyll, chlorophyll *a*, chlorophyll *b*, and chlorophyll *c* in each cell at a salinity of 35. Chlorophyll *a*, *b*, and *c* content per cell decreased on Day 6 but increased on Day 12 and Day 18. However, total chlorophyll content per cell decreased on Days 6 and 12 but increased slightly on Day 18.

At 75 salinity, the quantity of total chlorophyll, chlorophyll *a*, chlorophyll *b*, and chlorophyll *c* per cell was lower on Day 0 but increased significantly by Day 6. It subsequently declined once again on Day 12 and was somewhat higher on Day 18.

Studies on pigment can reveal a cell's physiological status and its environment-adaptive behaviour. Any shift in pigment content is essentially a mechanism for cells to make up for changes in their environment, such as variations in light intensity and nutrition concentration, so they can adapt to unfavorable circumstances (Naik & Anil, 2018). Even though there was no fluctuation in the light in this experiment, the amount of chlorophyll pigment displayed variations because the light may be necessary for the constancy of chlorophyll but not for its production. The depletion of the resources available for the formation of these pigments may be the cause of the declining chlorophyll concentration. Because *T. indica* grew faster in salinities 15 and 35 than in salinities 0 and 75, nutrients were used more quickly in cases of those salinities than in cases of 0 and 75. Also, since it’s not an axenic culture the dissolved organic matter released by phytoplankton can be converted back to inorganic nutrients by the microorganisms (Traving et al., 2017). This regeneration of small amount of nutrients would explain the slight increase in chlorophyll again. Although more studies need to be done in retrospective of the correlation between nutrient regeneration and increasing trend of chlorophyll.

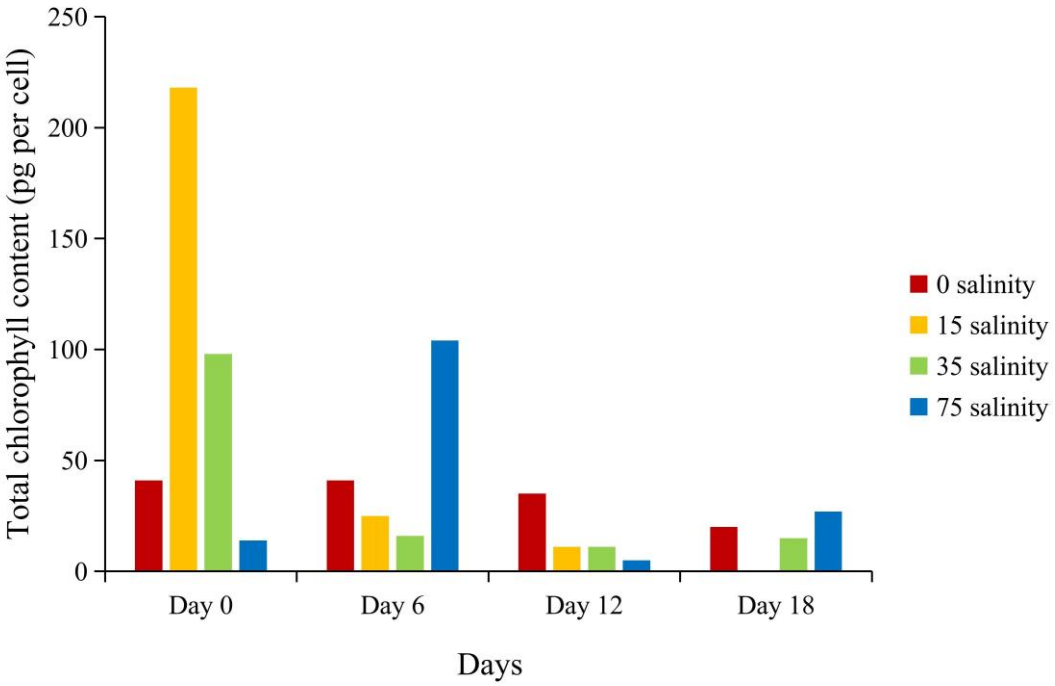


Figure 18 a. Concentration of total chlorophyll of *T. indica* at different salinities.

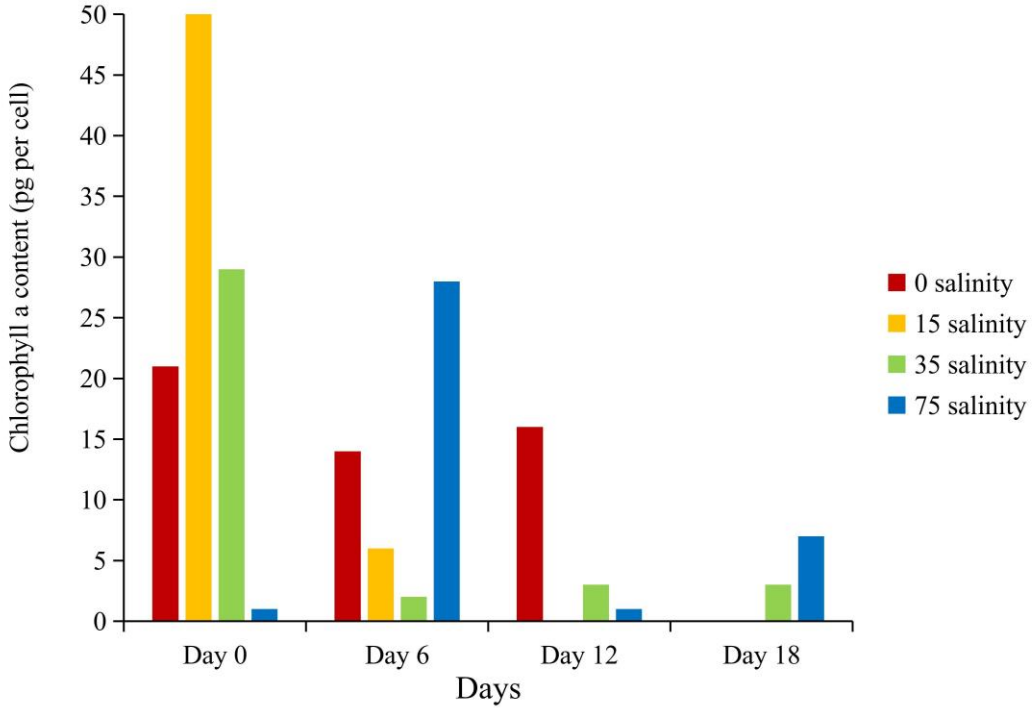


Figure 18 b. Concentration of chlorophyll *a* of *T. indica* at different salinities.

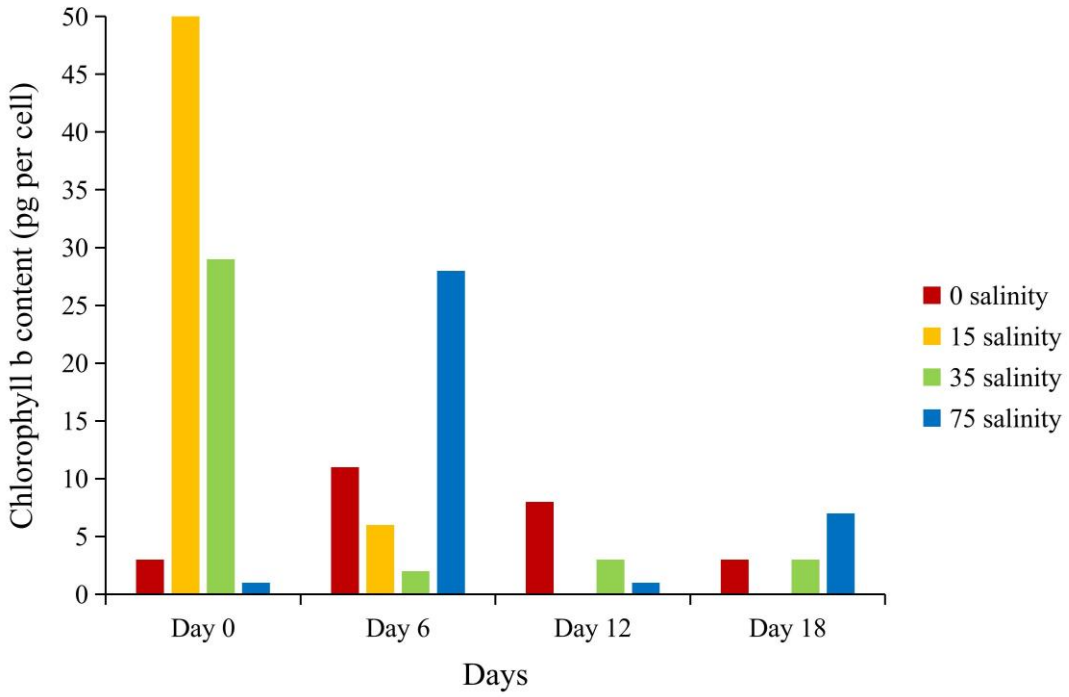


Figure 18 c. Concentration of chlorophyll *b* of T.indicaat different salinities.

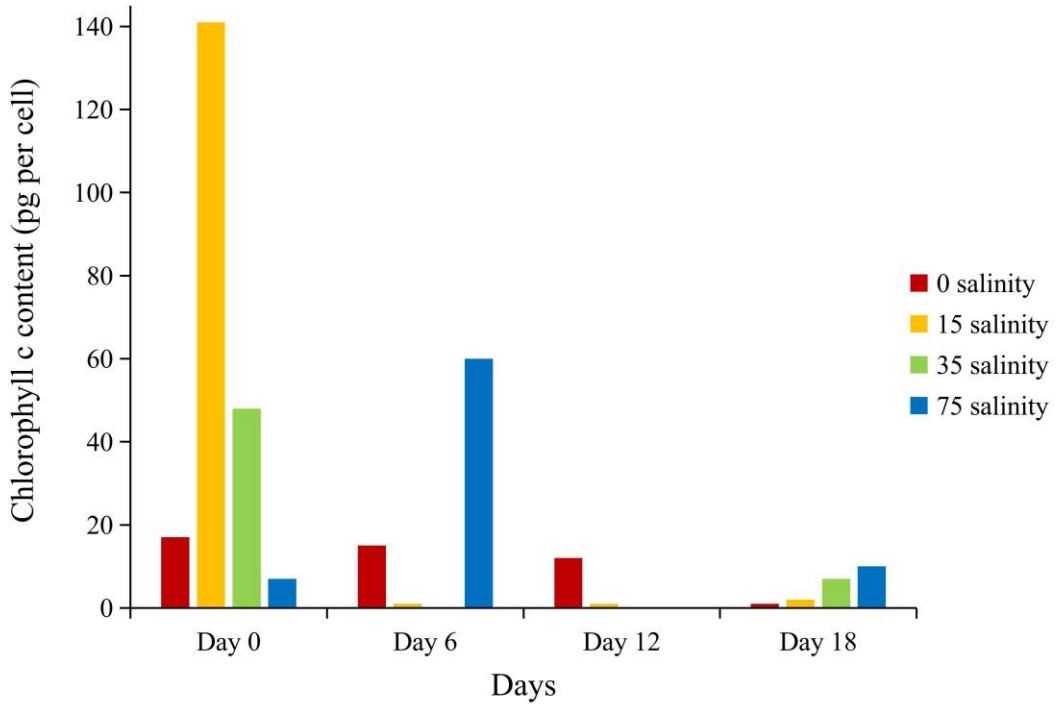


Figure 18 d. Concentration of chlorophyll *c* of T.indicaat different salinities.

4. CONCLUSION

Marine microalgae primarily consist of pigments, proteins, lipids, and carbohydrates whose composition varies in various microalgal species. Even rare,

species-exclusive metabolites can be produced by some marine microalgae. This multi-compound co-production capability is species-specific (Ma et al., 2020). In this study, cell abundance and growth rate, the total carbohydrate, protein and pigment (Total chlorophyll and chlorophyll *a*, *b* and *c*) content were analysed to study how *T. indica* may be influenced by wide range of salinities that included salinity 0 (freshwater), 15 (brackish water), 35 (sea water) and 75 (hyper-saline waters such as salt pans). It was confirmed that a wide range of salinity levels permitted the growth of the halotolerant *T. indica* as it endured salinities close to the maximal levels in marine water, although it proliferated best at brackish-water salinity. In conclusion, this work suggested that *T. indica* are euryhaline in nature thus confirming its flexible physiology inorder to adapt to adverse and widely varying conditions pointing towards its ecological role in nature.

Numerous microalgae species have the ability to create high-value bio-active substances such as long-chain polyunsaturated fatty acids, pigments, and vitamins (Pal et al., 2011). Only profitable phytoplankton have been used in the aquaculture industry, and the biomass' entire economic worth is determined by the proportion of lipids, carbohydrates, and protein (Williams & Laurens, 2010). Since salt levels in cultures grown in open systems might vary owing to evaporation, air dilutions, various water sources, etc., an understanding of strain versatility may be useful for the economic exploitation of *T. indica* on marine water resources. Experiments have been carried out with *T. indica* and studies have indicated the phycoremediation potential of *T. indica* for removal of pollutants from secondary treated domestic sewage water and also its capability of biodiesel production (Amit et al., 2017; Mittal & Ghosh, 2022). By studying more about *T. indica* and how it can survive extreme conditions such as high salinity or increased amount or dark exposure may help in understanding and improving its potential in bio-remediation and other biotechnological applications.

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