Studies on phytoplankton isolated from Mangrove ecosystem

A Dissertation for
Course code and Course Title: GBT 651 & Biotechnology
Credits: 16
Submitted in partial fulfilment of Masters Degree in Biotechnology
by

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I hereby declare that the data presented in this Dissertation report entitled, "Studies on phytoplankton isolated from mangrove ecosystem" is based on the results of investigations carried out by me in the Biotechnology Discipline at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Ms. Snesha Bhomkar 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 / College will be not be responsible for the correctness of observations / experimental or other findings given in the dissertation.

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PREFACE

Despite the wealth of studies on mangrove ecosystems, there remains a gap in understanding the biotechnological potential of phytoplankton specifically within the Chorao mangrove ecosystem. The Chorao mangrove ecosystem is renowned for its rich biodiversity and is home several birds to the famous Dr. Salim Ali Bird Sanctuary, along with other popular tourist destinations. Phytoplankton are known for their adaptability to challenging environmental conditions. In this context, the current study delves into the intricate ecosystem of Chorao to explore the diversity of phytoplankton and exploring its potential for various biotechnological applications. Isolating phytoplankton can be a challenging process due to several factors and requires lots of practice and patience.

Additionally, study was conducted to see the variation in the nutrient concentration and dissolved oxygen levels of water samples from Chorao, shedding light on the ecosystem's health. The pigments produced by these phytoplankton was investigated with a keen interest in their antioxidant properties. Antioxidants, known for their health benefits, are increasingly sought after in various industries, including pharmaceuticals and cosmetics. The study employs extraction solvents and mechanical disruption to access these valuable compounds, assessing their potential for commercial use. Furthermore, the antimicrobial properties of the isolated phytoplankton were explore, aiming to contribute to the development of new antimicrobial agents a pressing need in the face of increasing antimicrobial resistance. The extraction protocols were evaluate to check the efficacy of these organisms against pathogenic bacteria, offering a glimpse into their potential as natural sources of antimicrobial compounds.

On the frontier of sustainability, phytoplankton can be used as a potential solution to the world's energy challenges. Rich in lipids, these organisms hold promise for biofuel production a renewable

and eco-friendly alternative to traditional fuels. Through a preliminary screening of lipid bodies, we can lay the groundwork for future advancements in biofuel technology.

ACKNOWLEGEMENT

Firstly I would like to express my deepest gratitude towards my Kuldevi Shree Mahalsa Narayani for all her blessings that has guided me throughout the journey of my research. Her wisdom and grace have been my strength in my bad days.

I extend my sincere gratitude to Prof. Bernard F. Rodrigues, Senior Professor of Botany, Dean of School of Biological Sciences and Biotechnology, Goa University & Prof. Savita Kerkar, Senior Professor of Biotechnology, former Dean of School of Biological Sciences and Biotechnology, Prof. Sanjeev Ghadi, Vice Dean academics, Prof. S. Krishnan, Vice Dean research & Dr. Meghanath Prabhu programme director of Biotechnology for their support and providing the necessary facilities for the completion of this thesis. I am grateful for the opportunities provided by Goa University to pursue my research goals.

A special sincere gratitude to Ms. Snesha Bhomkar, my guide, for her continuous support and guidance throughout this research project. She provided invaluable feedback and encouragement that helped shape this dissertation. I am equally thankful towards the faculty members of Biotechnology discipline Prof. Savita Kerkar, Prof. Sanjeev Ghadi, Dr. Saanika Samant, Dr. Meghanath Prabhu, Ms. Dviti Mapari, Dr. Samantha Fernanades D'Mello & Ms. Snigdha Mayenkar for imparting their knowledge and expertise during my M.Sc. course.

Special thanks to Dr.Nitin S. Sawant program director, Zoology discipline, SBSB for granting me permission to use their inverted and epifluorescence microscope facilities & research scholars Sasha and Ankit for their assistance during the operation of the instrument for my research work.

I would also thank Dr. Savita Kerkar and research scholar Preeti for granting me permission to use the pathogenic strains and assisting me during the use of the Biosafety cabinet.

I would also thank the other research scholars of the Biotechnology disciple Hetika, Diksha, Deepti, Elaine, Veda and Devika for guiding me in operation of instruments. A special thanks to Serrao sir for all his services to repair the inverted microscope. I am also thankful to the other non-teaching staff including Sandhya ma'am, Jaya ma'am, Sameer sir, Ashish sir and Sanjana ma'am for their support and fulfilling the basic needs required for my project work.

Lastly I would thank my friends Hari, Vedha, Sushant, Varisha, Samradni, Tanmay, Kaushiki & Anurag for their constant encouragement throughout this journey and my family – my father Mr. Sachin Pai, my mother Mrs. Sonia Pai, my uncles Mr. Shashikant Pai and Mr. Devidas Pai and my aunts Mrs. Milan Redkar and Mrs. Sujata Patil for their unwavering support and belief in me.

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ABBREVIATIONS USED

Entity	Abbreviation
%	Percentage
µM	Micromolar
Abs	Absorbance
℃	Degree Celsius
Chl a	Chlorophyll *a*
Chl c	Chlorophyll *c*
Ddx	Diadinoxanthin
DO	Dissolved oxygen
DPPH	2,2-diphenyl-1-picrylhydrazy
Dtx	Diatoxanthin
Fx	Fucoxanthin
g	Gram
hr	Hour
Hz	Hertz
L	Litre
LMWA	Low molecular weight antioixidants
mg	Milli gram
MHB	Mueller hinton broth
mins.	Minutes
ml	Milli litre
ppm	Parts per million
ROS	Reactive oxygen species
rpm	Revolutions per minute

ZMB	Zobell marine broth
β – car	Beta carotenoid

ABSTRACT

Phytoplankton are a diverse group of organisms that are found in various ecosystems including freshwater, estuarine water bodies, marine waters, mangroves, saltpans etc. In this study a total of 10 phytoplankton from a mangrove ecosystem located in Chorao, Goa were isolated. The water samples were analysed for salinity, dissolved oxygen (DO), silicate, phosphates, and nitrites, showing significant variation over the sampling period. The isolates were identified as *Dunaliella*, *Amphiphora*, *Navicula distans*, *Amphora ovalis*, *Navicula transitions var. derasa f. delicatula*, *Amphora*, *Navicula directa*, *Cylindrotheca closterium*, *Cymbella* and *Pleurosigma*.

The isolates were screened for pigment concentration and antioxidant production using extraction solvents. *Amphora ovalis* showed the highest content of chlorophyll *a*, while *Navicula transitions var. derasa f. delicatula* showed the highest content of carotenoids using 90% acetone. Chlorophyll *a* in *Navicula transitions var. derasa f. delicatula* was highest using a chloroform:methanol extract, while *Amphiphora* showed the highest concentration of carotenoids. *Cylindrotheca closterium* exhibited the highest scavenging activity (83.40%), followed by *Dunaliella* indicating its potential as an antioxidant supplement.

The isolates were also tested against three pathogenic bacterial strains (*E. coli*, *Pseudomonas aeruginosa*, and *Shigella*) to assess their antimicrobial activity. Additionally, the isolates underwent preliminary screening for lipid content to explore their potential for use in biofuel production. Red fluorescence was observed when the cells were stained with Nile red and viewed under an epifluorescence microscope. Overall, the isolates obtained show potential as sources of natural antioxidants and lipids that can be further studied to test their ability to be used to combat human diseases, in laboratory experiments, and in promoting green technology.

Keywords: Phytoplanktons, biodiversity, mangrove ecosystem, pigments, antioxidants, , antimicrobial activity, lipid content.

CHAPTER 1:
INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1.BACKGROUND

1.1.1. PHYTOPLANKTON

Phytoplankton are tiny creatures that live in aquatic environment, both salty and fresh, and are named after the Greek terms phyto meaning plant and plankton coined by Victor Hensen in 1887 meaning made to wander or drift (Verlecar et al., 2004; Gopinathan et al., 2007; Lindsey et al.,2010; Santhanam et al., 2019). Common types include cyanobacteria, silica-encased diatoms, dinoflagellates, green algae, and chalk-coated coccolithophores (Lindsey et al., 2010; Gopinanthan et al., 2007; Gireesh et al., 2015; Santhanam et al., 2019). Phytoplankton, like terrestrial plants, have chlorophyll that absorbs sunlight and converts it into chemical energy through photosynthesis accounting <1 % photosynthetic biomass on earth but are still the main source of energy for aquatic ecosystems and account for about 50% of worldwide net primary production. They need different amounts of nutrients like calcium, phosphate, silicate, and nitrate, depending on the species. The temperature and salinity of the water, the depth of the water, the wind, and the kinds of predators that graze on phytoplankton are other elements that affect their growth and development rates. (Lindsey et al., 2010; National Ocean Service). They absorb CO_2 and release O_2 (Lindsey et al., 2010; Winder et al., 2012; Gireesh et al., 2015). The size of phytoplankton cells can vary from approximately 1 µm to 1 mm (Verlecar et al., 2004; Gopinanthan et al., 2007; Gireesh et al., 2015). Through a variety of food chains, phytoplankton serves as the main link in the energy flow leading to higher trophic levels. A wide range of species, including larvae, young fish and invertebrates, and zooplankters, rely heavily on phytoplankters as a food supply (Gireesh et al., 2015; National Ocean Service). They are divided into groups based on their size under: more than 1mm: Macroplankton, less than 1mm: Microplankton, organisms of 5-50µ: Nannoplankton, less than 5µ:

Ultraplankton, less than 1μ: Picoplankton (Gopinathan et al., 2007; Gireesh et al., 2015). Various ways of collecting concentrated samples of phytoplankton are known. Surface samples are typically taken in coastal waters, estuaries, or lagoons using a clean bucket with a defined volume. Water samplers like Van Dorn samplers, Niskin bottles, Meyer's water sampler, or Friedinger water sampler are used to gather subsurface samples at various depths (Gopinanthan et al., 2007; Gireesh et al., 2015). The cells must be kept intact as soon as possible in order to count the phytoplankton. A common fixative and preservative for phytoplankton cells is formalin but not satisfactory for delicate organisms. Lugol's iodine solution is another useful preservative, especially for diatoms and nanoplanktons (Gopinanthan et al., 2007; Gireesh et al., 2015). The diatoms *Fragilaria oceanica* and *Hemidiscus hardmanianus* are indicators of clupeid fish and oil sardine abundance in Indian waters. Certain phytoplankters, such *Phaeodactylum tricornutum* and *Dunaliella tertiolecta,* function as markers of metal contamination, particularly copper pollution. In addition to serving as effective filters for fruit juice, syrup, and varnish, diatoms can also be used as insulators in freezers, electric ovens, and boilers (Santhanam et al., 2019). Phytoplankton can yield a variety of beneficial chemicals, according to research findings. It is believed that phytoplankton is a rich source of biofuel, human nourishment, animal feed, and bio fertilizers (Ibanez et al., 2011). The industrial exploitation of these organisms through mass culture and the transformation of the harvested biomass into value-added products has been described in phytoplankton biotechnology, and it has experienced unanticipated global expansion (Ibanez et al., 2011; Santhanam et al., 2019). Manufacturers of nutraceuticals, cosmeceuticals, feed for aquaculture, fertiliser for agriculture, fine chemicals, natural products, biodiesel, municipal wastewater remedy corporations, and meal processors using nutrient-enriched wastewaters are among the industries engaged in the biotechnological applications of phytoplankton (Santhanam

et al., 2019). There are many different sizes, shapes, and forms of marine phytoplankton, some of which are quite exquisite. They seem to have a major role in regulating atmospheric carbon dioxide (CO2), a greenhouse gas that can affect how much heat the Earth's atmosphere retains. The marine food chain is built on the foundation of bacteria and phytoplankton. For instance, the food source for bivalve shellfish, such as clams, mussels, oysters, and scallops, is virtually entirely phytoplankton. Finally, the fact that marine algae may produce a wide range of extremely harmful substances, or marine bio toxins, makes them significant (Verlecar et al., 2004; Gopinanthan et al., 2007; Gireesh et al., 2015).

1.1.2. MANGROVE ECOSYSTEM

One of the world's most prolific ecosystems is thought to be the mangrove ecosystem (Kathiresan, K. 2000; Bouillon et al., 2008; Alongi 2009;Shoaib et al., 2017,). Only tropical and subtropical regions are home to these mangroves, which are intertidal woodlands (Tomlinson, P. B, 2016; Shoaib et al., 2017). They have been treasured for their contributions to the ecosystem, such as constructing soil, preventing flooding and hurricanes, and serving as a home and breeding ground for numerous fish and shellfish that are significant to the economy (Odum, W. E., & Heald, E. J. 1972; Kathiresan. K, 2000). However, mangroves are seriously threatened by degradation; in the last century, India has lost almost 40% of its mangrove cover (Krishnamurthy et al., 1987). Mangrove sediments get major inputs of organic carbon via subsurface root growth and litter from mangroves, including leaves, twigs, and paragules (Alongi 1989). Bouillon and Dehairs (2000) state that a variety of additional sources, such as allochthonous riverine or marine material, autochthonous production by benthic or epiphytic micro or macro algae, and local water column production by phytoplankton, may also supply significant organic carbon imports. Within the estuarine ecology, mangroves are essential for biodiversity, energy exchange, and the upkeep of

healthy food chains. Phytoplankton is one of the principal producers in these mangroves. Studies have shown that in tropical environments, the distribution and abundance of phytoplankton are positively correlated with nitrates, phosphates, salinity, and turbidity (Saifullah et al., 2016). The branches of mangroves serve as nesting sites for coastal wading birds such as egrets, herons, cormorants, and roseate spoonbills (Why are mangroves important? 2020; The Nature Conservancy). Goa's mangroves are primarily found around muddy creeks and marshy riverbanks in coastal areas. North Goa's Chorao Island is located on the Mandovi River. Out of all the 17 islands in the state, it is the biggest the mangrove ecosystem that is home to several plant and animal species, is the reason Chorao is most well-known today. For photographers and bird enthusiasts, this is one of the largest bird sanctuaries in the nation and a must-visit location. Mangroves occupy the whole western portion of the island, and numerous canals crisscross the landscape (Forest department, Goa; Kothari et al., 1990;Goa Tourism 2023).

1.1.3. BIOACTIVE COMPOUNDS

Plant-based functional foods have gained popularity due to their physiological advantages for humans. Polyphenols, which are found in fruits, vegetables, grains, tea, and coffee, are the most prevalent and widely dispersed chemical component of these meals. The possible function of plant-derived polyphenolic compound-based functional meals involves minimising the risk of chronic conditions like cancer, diabetes, and hypertension (Zhang, L., Han, Z., & Granato, D., 2021). However, while it is known that aquatic species may constitute an ideal source of chemicals for nutraceutical purposes, only a small number of functional foods have been isolated from them thus far. Bioactive chemicals such as vitamins, pigments, polyphenolic compounds, polyunsaturated fatty acids (PUFAs), polysaccharides, critical minerals, enzymes, and peptides are produced by thousands of species found in marine settings (Kim, S. K., & Wijesekara, I. 2010;

Jaramillo et al., 2019). The extraction of high-value compounds from microalgae is now limited to a few species, including *Arthrospira spp.* (a cyanobacterium known by the trade name Spirulina), *Chlorella spp.,* and *Dunaliella spp.* However, the use of microalgae to produce economic goods is still underexplored (Nieri et al., 2023). Specifically, diatoms generate an extensive array of wound-activated compounds that have evolved for deterrent functions. These compounds may be engaged in a number of physiological and cellular processes, such as detoxification and cell death. Lastly, certain diatoms collaborate with symbiotic bacteria to create chemicals, as is typically seen in marine sponges (Welker, C., & Nichetto, P. 1996; Jüttner et al., 2010; Maibam et al., 2014). The chemical complexity and wide variety of dinoflagellates make them attractive sources of bioactive substances for the pharmaceutical industry's pursuit of novel drugs. The chemical diversity of dinoflagellate bioactive compounds has led to their screening for various biological uses in various ROS-associated illnesses (Kiuru et al., 2014).

1.1.4. ANTIOXIDANTS

An antioxidant is a chemical that shields living things from free radical damage and is added to food as a way to stop lipid oxidation (Haoujar et al., 2022). The primary components of both enzymatic and non-enzymatic antioxidant defence mechanisms in biological systems are reactions. Antioxidants such as water- and fat-soluble vitamins, carotenoids, α-tocopherol, ascorbic acid, glutathione, flavonoids, uric acid, and plasma proteins including albumin, transferrin, ceruloplasmin, metal othionein, etc. are among the non-enzymatic antioxidants (Prior, R. L. 1998, Dantas et al., 2019). Because of their potential to encourage the development of cancer and because most consumers dislike artificial food additives, the usage of synthetic antioxidants has declined (Bran, A. L. 1975; Namiki, M. 1990). Therefore, it's critical to create and employ potent natural antioxidants to fend off free radicals and slow the advancement of numerous chronic

illnesses (Hayashi et al., 1993).Because of their varied chemical structures and biological activities, as well as their diversified phyto metabolic activity, photosynthetic microorganisms, like microalgae, have garnered growing attention in recent years for their potential applications (Sun et al., 2014; Zakaria et al., 2017; Dantas et al., 2019; Haoujar et al., 2022). These important bioactive substances are produced to environmental stressors such UV radiation exposure, high oxygen tension, and free radical exposure (Singh et al., 2005). It has been reported that several microalgal species from a variety of genera, including *Chlorella*, *Dunaliella*, *Nostoc*, and *Spirulina*, have had their antioxidant capabilities evaluated (Jaime et al., 2005; Wu et al.,2005; Herrero et al., 2006;Li et al., 2007).

1.1.5. ANTIMICROBIAL AGENTS

Antimicrobials are pharmaceutical compounds that can be used to treat numerous human diseases. Antiseptics, antibiotics, antivirals, antifungals, and antiparasitics are some of the classes of antimicrobial agents (Martino, 2022). Targeting cellular metabolism, such as the production of biological macromolecules, the activity of cellular enzymes, or cellular structures like the cell wall and cell membranes can help eradicate germs or stop them from growing with the help of this agents (Purssell n.d; Romani, M., et al., 2022).Whether they are synthetic or natural, antimicrobial agents always have an ecological effect when they are present in an ecosystem (Grenni, P et al., 2018). Antimicrobials and their byproducts are released into the environment, especially the aquatic environment which makes it easier for them to disperse and accumulate in various compartments (Felis, E., et al., 2020). The study of interactions between different microorganisms, whether prokaryotes or eukaryotes, is an important source of new antimicrobial discovery (Molloy, E. M., & Hertweck, C. (2017), Pringgenies, D., & Setyati, W. A. (2021). Numerous chemical classes, such as indoles, terpenes, acetogenins, phenols, fatty acids, and volatile halogenated

hydrocarbons, have been linked to the antimicrobial activity of microalgae (Mayer, A. M., & Hamann, M. T. 2005; Cardozo, K et al., 2007). For example, the lipid composition of the microalga *Chaetoceros muelleri* was linked to the antimicrobial activity of supercritical extracts obtained from it (Mendiola, J. A., et al., 2007). Nevertheless, fatty acids alone may not fully account for the antibacterial action for example in *Dunaliella salina* other potential contributors such α- and λ-ionone, -cyclocitral, neophytadiene, and phytol were responsible for antimicrobial activity (Herrero et al., 2006). The antibacterial action of cell extracts from *Phaeodactylum tricornutum*, a marine diatom was linked to eicosapentaenoic acid (EPA), a fatty acid (Desbois et al., 2008).

1.1.6. LIPIDS

Many scientist believe that marine sources have intriguing lipid compositions, which makes them desirable as a source for lipid extraction (E.Ibanez et al., 2011). The nutritional state of phytoplankton, particularly microalgae, has been shown to have a significant impact on their lipid composition (Marchett et al., 2010;Shifrin & Chisholm 1980) & differ significantly amongst several phytoplankton species (Ben-Amotz et al. 1985; Shifrin and Chisholm 1981). In addition to fundamental research that identifies the types and amounts of lipids present, there has been a recent resurgence of interest in the lipids of microalgae due to their promising applications in biotechnology. Microalgae are appealing for their rapid growth, high lipid levels, and the absence of a cellulosic cell wall in many species, making them well-suited for the production of biofuels and various other bioproducts (DOE 2016; Gordon and Polle 2007; Greene et al. 2016; Huntley et al. 2015; Walsh et al. 2016; Williams and Laurens 2010). The FTIR findings indicated that five diatom species, including *Amphora sp*., *Nitzschia sp*., *Nitzschia alexandrina*, *Opephora sp*., and *Staurosira sp*., exhibited higher lipid rates superior to 15% DW (Cointet et al., 2019). Using sonication method & gas chromatography it was observed that *Navicula cryptocephala* contained

traces of Palmitic acid, Oleic acid, Palmitolic acid and linoleic acid as major fatty acids (Sanjay et al., 2012). Sterols were extracted and analyzed from *Dunaliella tertiolecta* and *Dunaliella salina* which mainly consisted of Polysterols which have been shown to have therapeutic applications (Francavilla et al., 2009).

1.2. AIM

To study the phytoplankton isolated from Mangrove ecosystem

1.2.2. OBJECTIVES:

1. To determine of DO and nutrient concentration of the Chorao mangrove water samples.

2. To assess the phytoplankton diversity from the Chorao mangrove water samples.

3. To isolate phytoplankton monocultures.

4. Screening for pigments and its antioxidant activity.

5. Screening for antimicrobial activity.

6. Preliminary screening for lipids.

1.3. RESEARCH HYPOTHESIS

It is hypothesized that the stressful conditions in the mangrove ecosystem have led to the development of coping mechanisms in phytoplankton to protect against reactive oxygen species (ROS). Being photosynthetic organisms, there is a known correlation between the pigments present in phytoplankton and their antioxidant activity. Additionally, the diverse ecosystem of mangroves, which includes bacteria, may influence phytoplankton, with some species exhibiting antimicrobial properties. Certain phytoplankton species are known to be lipid-rich. Conducting a preliminary screening of lipids using Nile red can be a strategic approach to avoid wasting resources and time associated with extensive extraction protocols.

1.4. SCOPE

This thesis aims to explore the potential of phytoplankton isolated from the Chorao Mangrove ecosystem to produce bioactive compounds. The research will focus on isolating phytoplankton monocultures from the Chorao mangrove ecosystem, extracting pigments and screening them for antioxidant properties & screening the isolates for antimicrobial properties. Additionally, the study will include a preliminary screening of lipids. The results of these screenings can be used to produce natural antioxidant compounds for the pharmaceutical industry or for laboratory-scale cryopreservation processes. Isolates showing promise in lipid production can undergo further screening for use in biofuel production, offering a sustainable alternative to fossil fuels and promoting green technology. Furthermore, seasonal diversity studies and nutrient concentration analyses can provide valuable insights for ecologists studying the impact of environmental parameters on these organisms.

CHAPTER 2: LITERATURE REVIEW

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2.1 PHYTOPLANKTON DIVERSITY IN MANGROVE ECOSYSTEM

Throughout 2004, apochlorotic pennate diatoms of four morphotypes (I, II, V, and VI) were isolated from the plankton and neuston of mangrove water in Florida (Blackburn et al., 2009). A study conducted identified a total of 134 phytoplankton species dominated by diatoms from Sundarbans Mangrove Forest of Bangladesh. These species were categorised into different groups, including 99 species from 41 genera of Bacillariophyta (diatoms), 18 species from 6 genera of Pyrophyta, 12 species from 9 genera of Chlorophyta, 4 species from 4 genera of Cyanobacteria, and 1 species of Ochrophyta (Rahaman et al., 2013). *Rhizosolenia styliformes, Rhizosolenia alata, Rhizosolenia curvata, Rhizosolenia polydactyla, Rhizosolenia clevei, and Rhizosolenia* imbricata were the six diatom species that predominated in Kuala Sibuti Mangrove estuary. These were followed by four species of *Coscinodiscus*, which included *Coscinodiscus sp., Coscinodiscus centralis, Coscinodiscus thorii, and Coscinodiscus granii. Coscinodiscus sp., Coscinodiscus centralis, Coscinodiscus thorii, Coscinodiscus jonesianus, and Coscinodiscus granii*, was the predominant group of phytoplankton in Kuala Mangrove estuary. In both places, the genus Ceratium dominated the dinoflagellate population (Saifullah et al.,2014). The condition factor of the majority of fish species indicates that the aquatic ecosystems of Lampung Mangrove Center are highly supportive of fish population expansion, as indicated by the species diversity index and good phytoplankton diversity, which may serve as a potential source of food for the fish. Six types of phytoplankton belonging to 14 taxa were identified: *Tetraselmis, Nannochloropsis, Isochrysis, Chaetoceros, Cyclotella, and Thalassiosira* (Tugiyono, T., & Master, J. 2017).

82 species of phytoplankton were found in a research on the tropical mangrove ecosystem in Pichavaram, southeast India. Of them, 67 species are diatoms, 12 species are dinoflagellates,

and 3 species are blue-green algae (Kathiresan, K, 2000). 94 different phytoplankton species were identified from the mangrove forest at Pichavaram, India representing a variety of taxa including the Bacillariophyceae, Dinophyceae, Cyanophyceae , Chlorophyceae , and Chrysophyceae . Diatoms were generally observed to predominate in mangrove waterways (Rajkumar et al.,2009). In the southeast coast of India, the marine phytoplankton *Picochlorum maculatum* was originally identified. The Marine phytoplankton was separated and identified by rDNA sequencing after being collected in the mangrove waters of Muthukkuda, Tamil Nadu (Kumar et al., 2017).

Regular monthly sampling from salinity gradient zones in the intertidal waters along the Goan coast was conducted from October 2007 to September 2008 as part of a yearly analysis on the distribution and composition of phytoplankton. Up to 11 of the 179 phytoplankton species were observed during this investigation and were toxic. Particularly in May 2008, the pre-monsoon month, the poisonous diatom species *Pseudo-nitzschia pungens* was highly prevalent off Chapora, a permanently low salinity area. *Dinophysis acuminata* and *Alexandrium minutum*, two of the ten hazardous dinoflagellate species identified, were found to have the highest cell counts in the research region (Alkawri et al., 2011). On being reared on f/2 media without silicate, nanoplankton harvested from salt pans in Goa gave rise to a new species of *Tetraselmis*, *T. indica,* according to Arora & Anil (Arora et al., 2013). Between June 2007 and May 2008, studies of seasonal changes in the salinity and nutrient levels of phytoplankton were conducted at three distinct locations along the Mandovi Estuary. With 71% of all identified species, Bacillariophyta constituted the predominant category of phytoplankton. Dinophyta, Chlorophyta, Cyanophyta, Haptophyta, Chromophyta, and Chrysophyta contributed 25%, 0.5%, 1%, 0.5%, 1%, and 1% of the total species, respectively (Pednekar et al., 2014). Taxonomic diversity of bacteria, high salt tolerant fungal genera have been studied from the mangrove ecosystem of Goa particularly from regions

of Divar ad Chorao (Dastager et al., 2012;Nayak et al., 2012; Fernandes et al., 2014; Haldar, S., & Nazareth, S. W. ,2018; Lotlikar, G., & Naik-Samant, S. 2020). But not much studies have been conducted on isolation of phytoplankton from particularly the Mangrove ecosystem of Goa. Therefore this research has been carried out to check the diversity of phytoplankton from the mangrove ecosystem of Chorao Island and to isolate them in monoculture form and identify them using authentic key resources.

2.2. BIOACTIVE COMPOUNDS EXTRACTED FROM PHYTOPLANKTONS

2.2.1. ANTIOXIDANTS

Due to their ability to produce bioactive compounds in culture, which allows for the synthesis of structurally complex molecules, microalgae are particularly appealing as natural sources of these compounds. Microalgae have a wide range of physiological, biochemical, and molecular stress-reduction mechanisms that enable them to synthesise a wide range of bioactive compounds. Microalgae are photosynthetic organisms that can be used for food and cosmetics because they contain chlorophyll. Because certain species of microalgae produce bioactive substances like antioxidants, antitoxins, anticancer, anti-inflammatory, and antiviral agents, they can also be used in the pharmaceutical industry. Furthermore, because microalgae have high protein, vitamin, and polysaccharide contents, they are used as nutrient supplements for human consumption. Bioactive compounds have been separated using extraction techniques that have been developed to produce highly pure products with a variety of uses. These technologies may offer a novel way to boost the desired compound production, such as the extraction of bioactive compounds from microalgae. To extract the bioactive components, numerous extraction techniques have been developed. The majority of today's methods, including the soxhlet, microwave, ultrasonic, and supercritical extraction techniques, entail heating and solvents (

Carballo-Cárdenas et al., 2003; Jaime et al., 2005; Mendiola et al. 2005; Herrero et al., 2006; Spolaore et al., 2006; Rodriguez et al., 2008; Garcia Casal et al., 2009; Plaza et al., 2009; Zakaria, et al 2017).

There are numerous uses for antioxidants in the chemical and food industries. They are used in the chemical industry to stop rubber and plastic from oxidising and to stabilise fuels and lubricants. Antioxidants are crucial additives in the food industry that extend food's shelf life by primarily preventing lipid oxidation. Whereas industry usually relies on synthetic antioxidants, there is a potential to replace synthetic antioxidants with natural antioxidants. Because synthetic antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have the potential to cause cancer, natural antioxidants are becoming more and more popular, especially in food applications. A growing number of natural antioxidants are being utilised as nutraceuticals because they are said to have positive health effects. Nowadays, plants are the primary source of natural antioxidants. Microalgae are thought to offer a different source of naturally occurring antioxidants. Microalgae are phototrophic, which means that during photosynthesis, reactive oxygen species (ROS) are produced, causing severe oxidative stress. Microalgae have antioxidant defence mechanisms, such as antioxidant enzymes (like catalase or superoxide dismutase) and low molecular weight antioxidants (LMWA), to stop cellular damage from these ROS.

Because they can produce and accumulate valuable pigments, microalgae are appealing for industrial production because they have advantages over other vegetable sources, such as their wide natural distribution and diversity and the fact that they don't require arable land to grow. Furthermore, because some species of microalgae can fix nitrogen, eliminating the need for nitrogen supplements, and because they do not require carbon supplementation like heterotrophic organisms do, their photosynthetic nature may also lower production costs.The most widely used

microalgae are *Arthrospira platensis* for C-phycocyanin, *Haematococcus pluvialis* for astaxanthin, and *Dunaliella salina* for β-carotene. Additionally, there are pilot-scale lutein production projects being conducted utilising *Scenedesmus almeriensis* and *Muriellopsis sp* (Jin, E. S., & Melis, A. 2003; Del Campo et al., 2007; Ceron et al., 2008; Santhanam et al.,2019). Carotenoids, glutathione, vitamins (ascorbate and tocopherols), and phenolics are some of these LMWA. Microalgae have been the subject of extensive research on carotenoids, and these algae are currently utilised as a commercial source of carotenoid antioxidants (astaxanthin from *Haematococcus* and β-carotene from *Dunaliella*) (Namiki et al., 1990; Pokorny, J 1991; Pulz et al., 2004; Spolaore et al., 2006; Natrah et al., 2007; Rodriguez-Garcia et al., 2008; Hajimahmoodi et al., 2010; Goiris et al., 2012). Another study conducted to screen Microalgae for Antioxidant Activities found that the extracts with the highest antioxidant activity were the ethanolic extract of *Oscillatoria sp.* (69.1%), followed by the extracts of *S. obliquus* (64.3%) and *Nostoc sp.* (chloroform and ethyl acetate) (Ali, et al.,2014). A study demonstrated that extracting *Chlorella sp*. using subcritical water results in extracts that are rich in polysaccharides. For subcritical water extraction, 150°C is the ideal operating temperature to achieve the maximum polysaccharide concentration and yield of 23.6%. *Chlorella sp*. yielded an effective antioxidant extraction using environmentally safe subcritical water and showed strong free radical scavenging activities and are free of organic solvent residue (Zakaria, et al 2017). Using the DPPH free radical scavenging assay, the antioxidant activity of the following four microalgae was examined: *Phaedactylium trichodronum*, *Nannochloropsis gaditana*, *Nannochloris* sp., and *Tetraselmis suecica*. Three solvents with varying polarities (ethyl acetate, hexane, and water extract) were used to measure the content of natural antioxidants, such as carotenoids and phenolics. *Phaedactylium tricornutum* and *Nannochloropsis gaditana* were found to contain the highest and lowest concentrations of these compounds, respectively. The

findings demonstrated a robust relationship between the microalgae's total antioxidant activity, carotenoid content, and total polyphenol (Haoujar et al., 2022). The first method used to analyse phytoplankton pigments in oceanography was spectrophotometry. This method allowed for the analysis of three chlorophylls (*a*, *b* and *c*) from samples of seawater and demonstrated the selectivity of chlorophyll *a* as a phytoplankton biomarker in the presence of bacteria, zooplankton, and detritus (Richards, F. A., & Thompson, T. G., 1952). More precise spectrophotometric equations for chlorophylls *a*, *b*, and *c1* and *c2* could later be obtained from crystallisation of chlorophyll *c1* and *c2* and the reanalysis of pure chlorophylls *a* and *b* (Jeffrey, S. W. 1969, 1972). Sensitive fluorometric techniques were developed in the 1960s to measure chlorophyll *a* and pheopigments- in vivo as well as in vitro. Due to the in vivo method's success, in situ phytoplankton profiling was introduced and subsequently became a standard component of sea-going oceanography (Yentsch, C. S., & Menzel, D. W. 1963; Holm-Hansen et al., 1965; Lorenzen, C. J., 1966).

Although studies using thin-layer chromatography (TLC) of phytoplankton pigments in the 1970s and 1980s demonstrated that it was possible to separate phytoplankton marker pigments with ease, oceanographers viewed these techniques as specialised rather than routine methods, and field oceanography did not immediately adopt them. Nevertheless, it has been demonstrated that the analysis of phytoplankton populations benefits from the use of separation techniques for chlorophylls, carotenoids, and the breakdown products of these compounds. In fact, TLC discovered two pigments in the subtropical Atlantic in 1978 that were subsequently identified as divinyl chlorophylls a and b. It was discovered that TLC pigment signatures were useful indicators of phytoplankton processes and taxa. With the advent of automated HPLC techniques for pigment separations in the 1980s, which were helpful for routine shipboard or shore-based determinations,

the field of pigment analysis saw a rapid expansion in scope. With the aid of these HPLC techniques, it was possible to separate up to 20 taxonomically significant carotenoids from mixed phytoplankton populations, with over 40 pigments (chlorophylls, carotenoids, and derivatives) being separated in a single run. Accurate quantitative analyses of chlorophylls were also made possible (Jeffrey, S. W. 1968; 1974; 1976; Mantoura, R. F. C., & Llewellyn, C. A.1983, Gieskes, W. W. C., & Kraay, G. W. 1983; Wright, S. W., & Shearer, J. D. 1984; Jeffrey et al., 1999,). In the study of in vitro Antioxidant Activity of Marine Diatoms, *Pleurosigma angulatum* showed the highest overall antioxidant activity of 45.03 ± 2.1 ascorbic acid equivalent/g, followed by the centric diatom *Chaetoceros* simplex. The extracts of isolates were prepared using acetone (Karthikeyan et al., 2013). Using methanol suspension and ultra-sonication the phycobiliproteins were released from cyanobacteria. The DPPH assay was used to screen antioxidant activity and *Spirulina platensis* (40.28) showed the highest percentage of radical scavenged, followed by *Synechocystis aquatilis* (36.32) and *Phormidium laminosum* (34.80), in that order respectively(Deshmukh et al., 2014). Three strains of cyanobacteria that were obtained from the Cochin Estuary namely *Oscillatoria limosa, Synechococcus elongatus,* and *Synechocystis aquatilis* that exhibited strong antioxidant activity (Rajishamol et al., 2016). At a concentration of 100µg/ml, the *Oscillatoria limosawere* methanolic extract was found to exhibit an inhibition level of 68.11±0.21, 62.11±0.11, and 69±0.31 percent for the DPPH radical scavenging activity, hydroxyl radical scavenging activity, and total antioxidant activity, respectively. The isolate was obtained from the polythene surface in domestic sewage water in Silchar, Assam (Sarmah, P., & Rout, J. (2018). A study on the distribution of phytoplankton pigments was conducted in the Nethravathi estuary near Mangalore between February 1993 and May 1994. 90% acetone was used to release the pigments, and spectrophotometric analysis was performed. The range of

concentrations for chlorophyll *a*, *b*, and *c* was 1.18 - 11.35 mg/m3, 0.12 - 14.88 mg/m3, and 0.08 - 25.82 mg/m3, respectively, with the highest values recorded in May, June - July, and November - December. The carotenoids had peaks in October and November and March and April, and they ranged from 0.01 to 16.31 mg/m3(Gowda et al., 2002). In October 2004, at the end of upwelling season phytoplankton composition and abundance were investigated along the southwest Indian coast. To ascertain the community structure, phytoplankton pigment analyses were carried out of which chlorophyll *a* was the most prevalent, followed by fucoxanthin (Roy et al., 2006).Using HPLC-CHEMTAX analytical techniques, phytoplankton marker pigments and their functional groups were discovered for the first time in the Gulf of Mannar (GoM) and Palk Bay (PB), on India's southeast coast. In contrast to the PB, which was characterized by a high concentration of zeaxanthin, showing the dominance of photosynthetic prokaryotes (cyanobacteria), the GoM had a high diversity and concentration of marker pigments(Madhu et al., 2014). A study in the Cochin Estuary was conducted to identify phytoplankton marker pigments and explain how functional groups are seasonal based on HPLC/chemotaxonomy analytical data. Regardless of the season, fucoxanthin a diatom biomarker was clearly dominant throughout the research area. Additionally pigments found were peridinin, zeaxanthin, and alloxanthin, chlorophyll *c2* and diadinoxanthin (Paul et al., 2021).

Studies have been conducted on Archaea and Bacteria for their antioxidant potential but not much has been explored on isolation of antioxidants from phytoplankton in Goa (Kamat, T., & Kerkar, S. 2011; Alvares, J. J., & Furtado, I., 2018; Lotlikar, G., & Naik-Samant, S. 2020; Pereira et al., 2021).In the Goa estuarine complex, seasonal variations in the amounts of carotenoid and chlorophyll were examined. With a peak in May, the annual mean concentration of chlorophyll *a* was 3.05, 4.42, and 4.12 mg/m3 in the Mandovi Estuary, Cumbarjua Canal, and Zuari Estuary,

respectively. The values of carotenoids were modest, ranging from 0 to 3.6 m-SPU/m3, with a peak observed in May that was closely associated with chl *a* (Bhargava, R. M. S., & Dwivedi, S. N. , 1976). The pigment studies of the seawater samples along the coast of Goa were conducted through the use of HPLC, which identified marker pigments associated with dinoflagellates, diatoms, and prasinophytes. The primary diagnostic pigments were fucoxanthin (7–15%), prasinoxanthin (11–17%), peridinin (15–30%), and chlorophyll *b* (36–56%). The remaining pigments, which comprised alloxanthin and zeaxanthin, were generated by cryptophytes and cyanobacteria (Bhaskar et al., 2011).During the spring inter-monsoon of 2014–2018, large areas of marine cyanobacteria called *Trichodesmium* blooms were seen in the coastal waters off Goa. The most common pigment in the bloom area was zeaxanthin.The experimental investigation demonstrates that, in addition to photo-degradation, the isolate was played an important role to the breakdown of organic matter (Dias, A., Kurian, S., & Thayapurath, S. (2020).Two species namely *Dunaliella* .sp. & *D. salina*, were isolated from Goa's salt pans. *D. salina* was cultivated at 0.75 M salinity and exposed to high light intensity (1000 μmol m− 2 S−1), resulted in a significant accumulation of total carotenoid (36.95 pg. cell-1). On the other hand, when *Dunaliella* sp. was cultivated with ideal salinity (0.5M) and strong light intensity, the carotenoid concentration per cell was low (3.07 pg. cell-1), suggesting photo inhibition (Joseph, S., Ramadoss, D., & Chellandi, M. (2022). Since the pigments are known to be rich in antioxidants the present study aims to screen for pigments and their antioxidant activity. The extracted pigments can indeed be further identified using chromatography techniques, such as high-performance liquid chromatography (HPLC) or thin-layer chromatography (TLC). These techniques can help identify the specific types of pigments present in the phytoplankton extracts. The antioxidant properties of these pigments can be valuable for human supplementation, as they can help protect against oxidative stress.

Additionally, these properties can be beneficial in cryopreservation processes, where antioxidants can help prevent damage to cells during freezing and thawing.

2.2.2. ANTIMICROBIAL COMPOUNDS

Finding novel bioactive chemicals has become urgently necessary on a global scale to combat the growing problem of antibiotic resistance, which is linked to an increase in the number of bacterial infections in both veterinary and human health settings (Campanile et al., 2007; Bradley et al., 2017; European Centre for Disease Prevention and Control 2020). Numerous novel bioactive substances have been identified from marine microbial sources, according to current investigations (Murray et al., 2004; Blunt et al., 2015; Karpiński, T. M. 2019). Nielsen (1955) was the first to highlight the possibility that the production of antibiotics by planktonic algae could regulate the growth of marine bacteria. A few researchers (Jeldersen 1962; Sieburth and Pratt 1962; Burkholder 1963), examined the impact of easily cultivated freshwater and marine algae species on microbes that are likely to contaminate seawater on land. Further research incorporating a broader range of algae and test bacteria is required due to the still-in-progress nature of these studies and their limitation to a few number of standard test organisms (Duff et al., 1996). In a studied conducted on cell extracts of 14 species of Marine Phytoplankters, the coccoid *Myxophyceae* exhibited no noticeable antibacterial activity while the *Bacillariophyceae, Chrysophyceae,* and *Cryptophyceae* had a significantly wider range and degree of activity than the *Chlorophyceae* (Duff et al., 1996). By using the Agar disc diffusion method, the antimicrobial potential of *Tetraselmis sp.* extracts was assessed against human pathogens of Gram positive *(Proteus vulgaris*) and Gram negative (*E.coli, Pseudomonas aeruginosa, Bacillus subtilis, Staphlococcus aureus*) bacteria as well as three fungal pathogens (*Aspergillus fumigatus, Aspergillus niger, Candida albicans*) that were tested against the common antibiotics streptomycin

and chloramphenicol. The greatest inhibitory impact was shown against *E. coli* (16mm) and *S. aureus* (15mm) (Matharasi et al., 2018). Compounds from the green microalgae *S. subspicatus* were extracted using a variety of solvents, including ethanol, methanol, butanol, acetone, dimethyl sulfoxide, and water. The compounds were then tested for phytochemical screening, antioxidant activity, and antibacterial qualities. Gram-positive bacteria like *B. subtilis* and gram-negative bacteria like *P. mirabilis, E. coli,* and *K. pneumoniae* showed inhibitions. The ability of the extracts dimethyl sulfoxide, acetone, and water to prevent *Bacillus subtilis* from growing was demonstrated. But only dimethyl sulfoxide inhibited *Escherichia coli* and *Klebsiella pneumoniae* from growing. *Amphiprora paludosa*, *Porphyridium cruentum*, *Chaetoceros muelleri*, *Synechococcus elongatus*, *Synechocystis sp.,* and *Dunaliella tertiolecta* were the six phytoplankton species whose antibiotic activity was investigated. All of the extracts prevented *B. subtilis* from growing and caused inhibitory halos to form in several bacterial species, with diameters ranging from 7.06 to 15.23 mm. Extracts from *S. elongatus, Synechocystis sp., A. paludosa, P. cruentum,* and *C. muelleri* all showed broad-spectrum antibacterial activity (Saavedra et al., 2010). In a study conducted by Hemalatha et al., *Odontella aurita* was shown to possess antibacterial properties. They concluded that the release of antibiotics is dependent on extractive solvents and the physical or chemical rupture of algal cells. Tests were conducted on the antibacterial activity of the methanol-prepared diatom extracts against Gram-positive *Bacillus subtilis*, *Staphylococcus aureus*, pneumonia caused by *Streptococcus* and Gram-negative *Aeromonas* and *Escherichia coli* bacteria using the agar well diffusion method. The outcomes of using methanolic extracts of T*halassiosira sp., Chaetoceros sp., and Skeletonema sp.* in an agar well diffusion method against *E. coli, S. Aeromonas, B. subtilis, S. pneumoniae, and aureus* all exhibited notable inhibition. *Skeletonema* sp. whole cell extracts had the highest antibacterial activity with a 20 mm and 16 mm

zone of inhibition against *Staphylococcus aureus* and *Bacillus subtilis*, respectively, but the inhibition zone for *Chaetoceros sp.* was 12 mm against the majority of the tested bacterial strains (Bhattacharjya et al., 2020). This study aims to screen the phytoplankton isolates for their antimicrobial activity which may play a crucial role in improving human health developing new treatments, and addressing challenges related to antimicrobial resistance.

2.3 SCREENING OF LIPIDS

Lipids are one of the four main biochemical components of cells, along with proteins, carbohydrates, and nucleic acids (Ben-Amotz et al. 1985; Shifrin and Chisholm 1981). Photosynthetic organisms known as phytoplankton use light, water, and carbon dioxide to convert carbon dioxide into sugars, which are then used to make biological macromolecules like lipids. The two most popular methods for directly extracting total lipids from cells (or tissue) are the revised Bligh and Dyer method (Bligh and Dyer 1959), which uses a 1:1:0.8 $CHCl_3$:MeOH:H_2O solvent mixture, or the Folch method (Folch et al. 1957), which uses an 8:4:3 $CHCl_3$:MeOH:H_2O solvent mixture. Developed lipid-specific stains provide an alternative to extraction-based techniques as they don't necessitate solvent extractions or vast amounts of material. Since Nile red's development as a modification of Nile blue, it has been utilised as an affordable way to measure the quantity and position of lipids in many cell types due to its strong fluorescence signal (Greenspan et al. 1985). The lipids from microalgae could be utilized in a variety of energy-harvesting procedures, such as straightforward combustion in a boiler or diesel engine (Li et al., 2008;Converti et al., 2009;Mata et al.,2010; Santhanam et al.,2019).

Early in the 1960s, Nihon Chlorella in Japan initiated the first large-scale microalgae culture using *Chlorella*. During the first oil crisis in the 1970s, interest in using microalgae for

renewable energy rose (Spolaore et al., (2006). Through the Aquatic Species Program (ASP), the U.S. National Renewable Energy Laboratory (NREL) created a specific research and development program devoted to alternative renewable fuels, including biodiesel from microalgae. This program ran from 1978 to 1996. Studying the biochemistry and physiology of lipid synthesis in oleaginous microalgae was one of its primary goals (Sheehan et al., 1998). A study using an ultrasonic technique to produce biodiesel from the lipid of phytoplankton called *Chaetoceros calcitrans* has been conducted. The physical characteristics of density and viscosity, as well as the chemical characteristics of FFA concentration, saponification value, and iodine value, of biodiesel, were well-characterized. With the exception of the viscosity value, which was 1.14 g·cm−3, these values meet the American Society for Testing and Materials (ASTM D6751) standard limits (Kwangdinata et al., 2014). A similar research was carried out on *Porphyridium cruentum* (Kwangdinata et al., 2014). A study was set out to evaluate the lipid content and consequent biodiesel producing capability of several microalgae found in the marine water of Mauritius. There were also differences ($p<0.005$) in the percentage of lipid in the various microalgae, with *Symbiodinium clade C* having the greatest percentage (38.39±6.58%) (Beetul et al., 2014). Seawater samples were gathered from the Lebanese coast's coastal region. Together with the dry matter yield, lipid content, fatty acid profile, and prospective biodiesel yield, the growth dynamics were ascertained. Two of the natural blooms produced biodiesel in the cylinders and raceway ponds in amounts comparable to the *Chlorella* reference isolate. The Nahr El-Kalb (NK2) bloom produced 4.3 mg/L of biodiesel per day in raceway ponds, while the Saida bloom produced 5.2 mg/L per day in cylinders (Abdelkader et al., 2014). To identify the different kinds of algae in Teluk Kupang, laboratory testing and observation were the research methods employed. According to the findings, 34 different species of microalgae were identified, including species of *Nitzschia*

and *Thallassiosira*. These species' cells were composed of cellulose, glucan, and oils (triglycerides), which could be extracted and used to produce valuable chemicals and renewable fuels (Djoru, M. R., & Gimin, R., 2020).

The microalga *Botryococcus braunii*, which produces hydrocarbons, has been isolated and characterized from Indian waters by Ravishankar and his team at the Plant Cell Biotechnology Department of the Central Food Technological Research Institute in Mysore (Dayananda et al., 2005; Dayananda et al., 2006; Sivasubramanian, V. 2009). Research has been conducted by Ramachandra et al. 2009, on the manufacture of biofuels from diatom species.Six species of freshwater microalgae from the *Chlorophyta* genus that were isolated from freshwater bodies in Assam, India, had their fatty acid compositions determined. The six freshwater microalgae under study were shown to synthesis primarily palmitic, oleic, linoleic, and linolenic acids.The fuel qualities of biodiesel, such as cetane number, oxidative stability, and cold flow, are mostly determined by the saturated (16:0) and monounsaturated (18:1) fatty acids (Kaur et al., 2012). In a study conducted the main objectives were to quantify physical and chemical characteristics and ascertain the diversity of algae in ten lakes located in the Hubli and Dharwad districts. The lipid content of about 20 distinct algae species was investigated after isolation. Four of these species demonstrated a great potential for producing biofuels namely: *Chlorella vulgaris, Chlorella pyrenoidosa, Scenedesmus dimorphus and Scenedesmus quadricauda* (Adhoni, et al., 2015). Following identification using the internal transcribed spacer 2 (ITS2) region of ribosomal DNA sequencing, 11 microalgal strains were isolated from freshwater bodies in northeastern states. Biomass was produced from the microalgal biomass, and FAs were extracted to investigate the suitability of microalgae FAMEs as biodiesel by Trans esterification to FAMEs. After that, the criteria and threshold of the Biodiesel Standard EN 14214 were taken into consideration and the

most promising strains for biodiesel production were identified using PROMETHEE and GAIA software (Sinha, S. K., Gupta, A., & Bharalee, R. (2016). In order to evaluate microalgae's potential for producing biodiesel, a study was conducted in Kerala, India, looking at their variety across the seasons. After isolated microalgal lipids were transesterified, biodiesel that complied with EN and ASTM regulations was produced.This study highlights the potential of *Nodosilinea nodulosa* microalgae for producing biodiesel sustainably, providing low-carbon energy sources and environmentally beneficial solutions (Aravindh et al., 2023).

The Energy Resources Institute (TERI) is investigating a novel method for producing algal biodiesel, or biofuel from seaweed, using algae cultivated on seaweed farms (Sequeira, N. (2022, April 24). This research aims screen the phytoplankton monocultures for lipids which can be further extracted using standardised protocols & investigate the same for production of biofuel compared with standard values of ideal biofuel.

CHAPTER 3:

METHODOLOGY

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3. 1. COLLECTION OF SAMPLE

A known volume of sample was collected and filtered through a 20 μm mesh and stored in a sterile bottle. Another set of water sample was collected in a similar manner in an amber coloured bottle and fixed with Lugols's iodine for diversity studies. Samples for estimation of Dissolved oxygen (DO) were collected in DO bottles avoiding air bubbles and were fixed using reagents mention in section 3.2.1. Water samples for the estimation of other nutrients were collected in cryovials. The weather conditions of the site were noted down (Andersen, R. A. (Ed.). 2005; Gireesh et al., 2015; Saxena et al., 2021). The collection of samples was done in the month of September, October, December, January and February.

3.2. DETERMINATION OF DO AND NUTRIENT CONCENTRATION

3.2.1. DETERMINATION OF DO

To the collected sample 1ml of winklers A and 1ml of winklers B solution were added at the sampling site and the bottle was brought to the laboratory for further analysis. To the fixed sample, 1 ml of conc. H_2SO_4 was added and the bottle was shaken thoroughly to dissolve the precipitate. 50 ml of the sample was then titrated against $Na_2S_2O_3$ using starch as an indicator (Grasshoff, Kremling, Ehrhardt, & Anderson, 1999).

3.2.2. DETERMINATION OF SILICATES

25 mL of the water sample was taken in a test tube. To it 1mL of Molybdate reagent was added and the tube was allowed to stand for 10-20 mins. 1 mL of Oxalic acid was added followed

by addition of 0.5 mL of Ascorbic acid and the tube was incubated at RT for 30 mins to allow a blue color to develop. The absorbance was measured at 810nm (Grasshoff et al., 1999).

3.2.3. DETERMINATION OF PHOSPHATES

To 25 mL of water sample, 0.5 mL of Ascorbic acid and 0.5 mL of mixed reagent were added. The tubes were mixed well and kept at RT for 10 mins to allow the development of a blue color, followed by determination of absorbance at 880nm (Grasshoff et al., 1999).

3.2.4. DETERMINATION OF NITRITES

To 25 mL water sample, 0.5mL Sulfanilamide solution was added and mixed and kept for 5 minutes incubation. 0.5mL diamine was added and shaken, incubated for 15 minutes to allow development of Azo dye. The readings were taken at 543 nm (Grasshoff et al., 1999).

3.3. PHYTOPLANKTON DIVERISTY STUDIES

The fixed samples were allowed to sediment for 48 hours post which the sample of 500 ml was reduced to 10 ml by siphoning out the rest. 1 ml of this concentrated sample was pipetted onto a Sedgwick rafter and observed under inverted microscope. Different fields were observed and the diversity of phytoplankton were recorded. Identification was done using the Tomas 1997 Key (Andersen, R. A. (Ed.). 2005; Gireesh et al., 2015).

3.4. ISOLATION OF PHYTOPLANKTON

The samples collected for isolation were brought to the laboratory and their salinity values were noted. Required volumes of f/2 stock solutions (Guillard, R. R. L., Jr., & Ryther, J. H. 1975)

were added to these bottles and were incubated. The bottles were kept under artificial light and dark conditions until visible growth in terms of turbidity was observed. The samples were pipetted out into petri plates and viewed under an Olympus CK2 model inverted microscope. Using the aspiration technique phytoplankton of different morphology were aspirated separately into 6 well plates containing f/2 media of the respective salinity. The plates were kept under light (3100 lux) and dark incubation i.e 16:8 hours. The cultures were subcultured until monocultures were obtained. The isolated monocultures were captured using MICAPS software (Andersen, R. A. (Ed.). 2005, Pachiappan et al., 2015).

3.4.1 GROWTH STUDIES

In a 30ml conical flask containing f/2 media, 5% of inoculum of respective monocultures were added separately at their respective salinity. The flasks were kept under artificial light and dark conditions. Every alternate day 0.01ml was pipetted on haemocytometer and the cells were counted under compound microscope. The average cell count was taken and multiplied by 10,000. All the phases of growth were observed by plotting a graph of cell/ml $\times 10^4$ vs. number of days for respective monocultures on y and x axis respectively (Sournia, A. 1982; Andersen, R. A. (Ed.). 2005).

3.5. ISOLATION OF BACTERIA ASSOICATED WITH PHYTOPLANKTON MONOCULTURES

1ml of respective monocultures were pipetted into eppendorf tube and centrifuged at 3,500rpm. The supernatant was gently pipetted out and serially diluted in saline. The dilutions were plated on Zobell marine agar (ZMA) and the colony characteristics were noted after 24 hrs

of incubation. The isolated colonies were streaked and the Gram character was determined (Buchan et al., 2014).

3.6. EXTRACTION OF ANTIOXIDANTS

3.6.1. HARVESTING OF PHYTOPLANKTON BIOMASS

100 mL of sterile f/2 media was prepared in conical flasks and 5% of monoculture was inoculated separately in each flask of respective salinity. The cell mass was obtained after visible turbidity was observed by centrifugation at 4000 rpm for 10 minutes. The cell pellet was washed twice with distilled water and centrifuge to remove traces of f/2 media. The pellet was stored at -80℃ overnight. The pellet was spread onto a pre-weighed butter paper and dried in a hot air oven at 40℃. The weight of the dried biomass was calculated by subtraction of pre-weighed butter paper to the weight of after spreading biomass (Huang et al., 2016, Dantas et al., 2018).

3.6.2. EXTRACTION OF PIGMENTS

The dried biomass was scraped off and butter paper was washed in 100% acetone to remove any traces of adhered biomass. The solution was added to a Falcon tube covered with aluminium foil and distilled water was added to yield a final concentration of 90% acetone. The tubes were covered with parafilm to avoid evaporation of acetone. The tubes were sonicated at 50Hz for 30 minutes and kept -20℃ overnight. The solution was centrifuged at 3500 rpm for 10 minutes to remove any traces of acetone. The supernatant was sterilised through a millipore syringe filter (0.2 micron to remove traces of acetone) and kept in dark until further analysis. The procedure was repeated with chloroform: ethanol solvent. (Karthikeyan et al., 2013; Huang et al., 2016; Dantas et al., 2018). For bacteria associated with phytoplankton methanol was used as extraction solvent

and the procedure was repeated with minor modifications (Sinha, S., et al., 2017, Smith, J. L., & Alford, J. A. (1970).

3.6.3. DETERMINATION OF CHLOROPHYLL *a* AND TOTAL CAROTENOID CONTENTS

Absorbance of supernatant was taken according to Linchtenthaler HK (1987) equations & the concentration of photosynthetic pigments were calculated (Huang et al., 2016; Dantas et al., 2018).

3.6.4. DETERMINATION OF DPPH RADICAL SCAVENGING ACTIVITY

Standards of Ascorbic acid were prepared in the range of 0.2-1 mg/mL. 330µl of DPPH was added to all the tubes and incubated under dark conditions for 30 minutes. The absorbance was taken at 520 nm (Karthikeyan et al., 2013; Huang et al., 2016; Zakaria et al., 2017).

3.7. SCREENING FOR ANTIMICROBIAL ACTIVITY

The dried biomass was dissolved in 5mL of hexane and sonicated at 50Hz for 30 minutes. The solution was centrifuged and filter sterilise. The procedure was repeated with Chloroform: methanol (Duff et al., 1966; Del et al., 2010; Matharasi et al., 2018). For bacteria associated with phytoplankton methanol was used as extraction solvent and the procedure was repeated with minor modifications (Sinha, S., et al., 2017, Smith, J. L., & Alford, J. A. (1970). The sonicated samples were stored at -20℃ until further analysis. The pathogenic cultures (*Escherichia coli*, *Pseudomonas aeruginosa* & *Shigella*) were inoculated in nutrient broth and incubated for 24 hours on a rotary shaker. Muller Hinton agar plates were prepared and wells were bored with the help of cork borer. The pathogenic cultures were spread plated with the help of cotton swab inside

biosafety cabinet taking all necessary precautions. 0.1ml of the extracts were pipetted in the respective wells. Phytoplankton monocultures without any extraction procedure were also tested. Tetracycline, chloramphenicol and streptomycin were kept as positive control whereas the extraction solvents were used to check for comparison purpose.

3.8. SCREENING OF LIPIDS

Nile red solution (0.1 mg/mL) in acetone was added to cultures for a final concentration of 1 μg Nile Red. The cultures were stained for 5 minutes and immediately observed under epifluorescence microscope (Johnson et al., 2017).

CHAPTER 4:

RESULTS AND DISCUSSION

CHAPTER 4: RESULTS AND DISCUSSION

4.1. COLLECTION OF SAMPLE

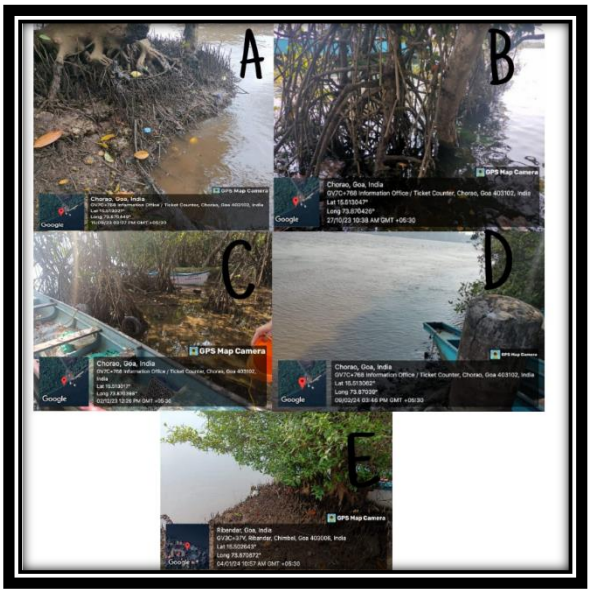


Figure 1: Sampling site (A-E: September, October, December, January & February).

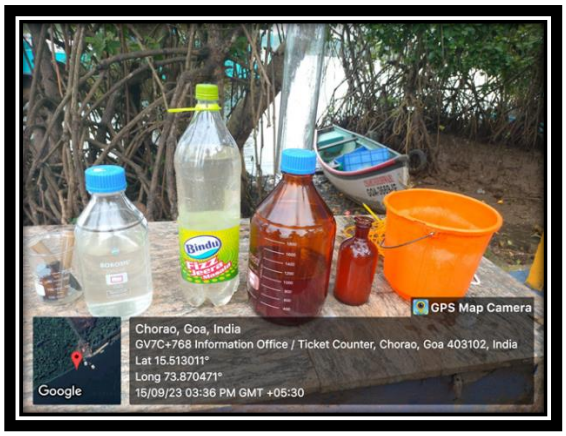


Figure 2: Sampling set up.

The efficiency of the collection technique is critical for success, as ineffective methods can result in the collection of damaged or deceased cells (Fig 1 & Fig.2). The collected samples were inoculated with f/2 media. The salinity of the water samples exhibited significant variation from September to October, with a sudden increase observed from 8ppt to 30ppt (Fig.3). The

temperature of the water samples ranged from 29-33 °C, with the highest temperature recorded in December (Fig.4).

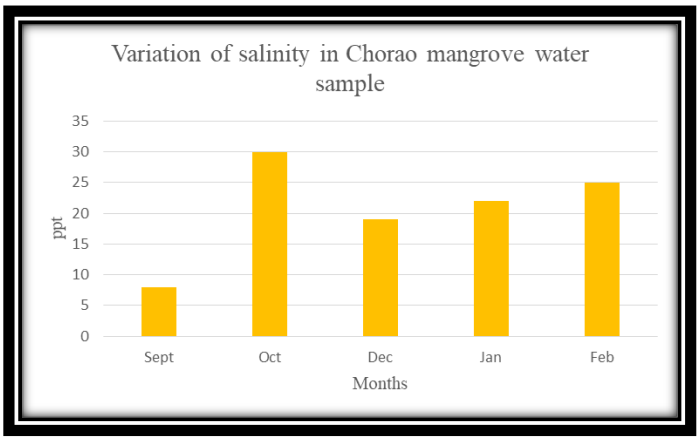


Figure 3: Variation in salinity observed over the months on the day of sampling.

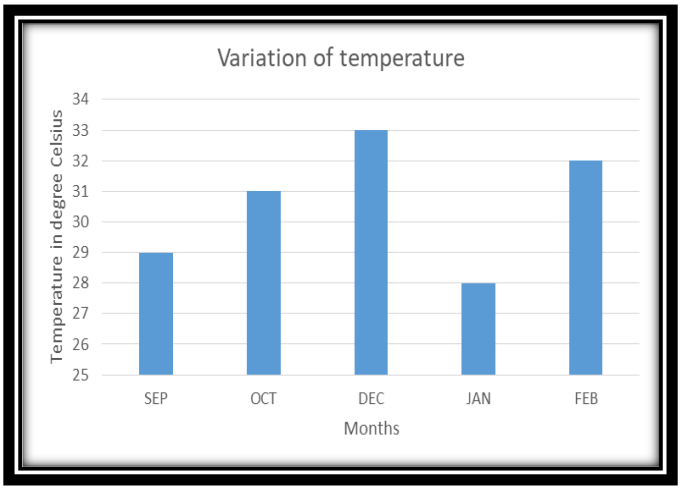


Figure 4: Variation in temperature observed over the months on the day of sampling.

Mangrove sediments have the ability to retain nutrients based on the flow patterns and sediment characteristics of the site (Saifullah et al., 2015). Several ecological factors have been identified as influencing the response of phytoplankton to shifting estuarine gradients (Underwood et al., 1998; Leland et al., 2001; Martin et al., 2007; Quinlan and Philips, 2007; Pednekar et al., 2014). Salinity is a crucial factor influencing phytoplankton growth (Qasim et al., 1972; Shetye et al., 2007; Pednekar et al., 2014). During the monsoon season, there is a decrease in salinity and an

increase in nutrients, which plays a significant role in regulating the geographic distribution, abundance, and growth of phytoplankton (Underwood et al., 1998; Leland et al., 2001; Martin et al., 2007; Quinlan and Philips, 2007; Pednekar et al., 2014). Pednekar et al.,2014 observed a sharp decrease in salinity levels during the monsoon season along the Mandovi estuary, with salinity ranging from 0 to 23 ppt in the upper section. Higher values may be related to the study area's predominance of neritic water, little rainfall, and a higher rate of evaporation (Sampathkumar and Kannan, 1998; Govindasamy et al., 2000; Gowda et al., 2001; Rajasegar, 2003).

2.2. DETERMINATION OF DO AND NUTRIENT CONCENTRATION

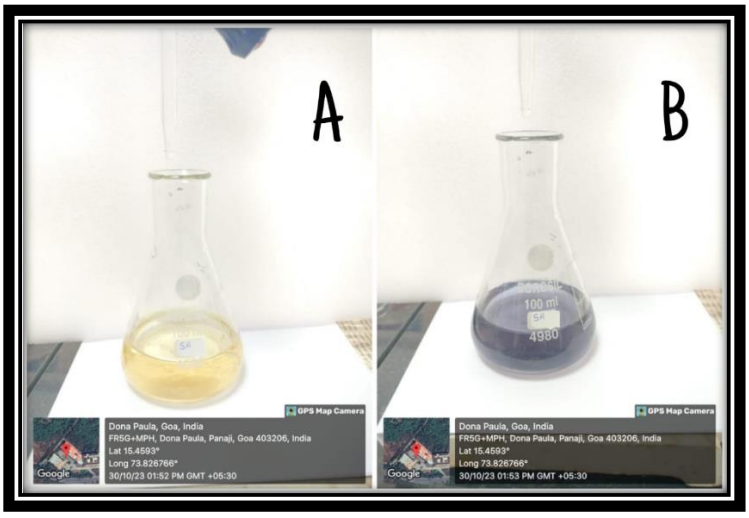


Figure 5: Titrating of DO sample against $Na_2S_2O_3$ A : Before addition of starch indicator. B: After addition of starch indicator

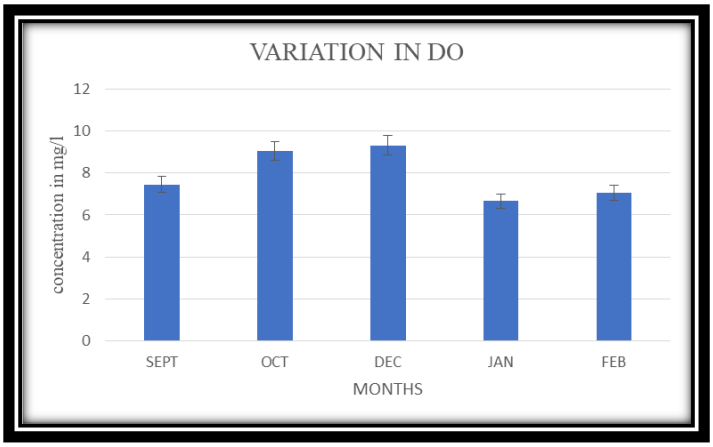


Figure 6: Variation in dissolved oxygen observed over the months on the day of sampling.

The fixed water samples after addition of concentrated H_2SO_4 were titrated against $Na_2S_2O_3$ (Fig.5).Constant burette readings of the samples were taken and the amount of DO was calculated. From the calculation it was found that the highest amount of DO on the day of sampling was in the month of December 9.33 mg/L ± 0.23 and lowest in the month of January 6.66 mg/L ± 1.15 (Fig.6). It is commonly recognized that salinity and temperature have an impact on how easily oxygen dissolves (Vijayakumar et al.,2000 ;Prabhu et al., 2007). Since oxygen dissolves more readily in cold water than in warm water, freshwater and cold water can hold more oxygen than can saltwater and warm water. Higher dissolved oxygen (DO) levels are associated with better water quality and higher biodiversity and vice versa (Gandaseca et al., 2011). In a study conducted by Gandaseca et al, the concentration of DO at Wildlife Sanctuary Sibuti Mangrove Forest, Miri Sarawak ranged from 3.2-4.7 mg L−1 & 2.76-3.74 mg L−1 from 12 sampling points and a man-made canal in the month of June, August and October. In a study conducted by Yin et al., at 2004 estuaries higher DO values > 4mg/L could be due to mixing of freshwater from rivers and saltwater from the ocean. This mixing can increase the oxygen solubility in water, leading to higher DO levels. Higher photosynthetic rate can also increase dissolve oxygen levels.

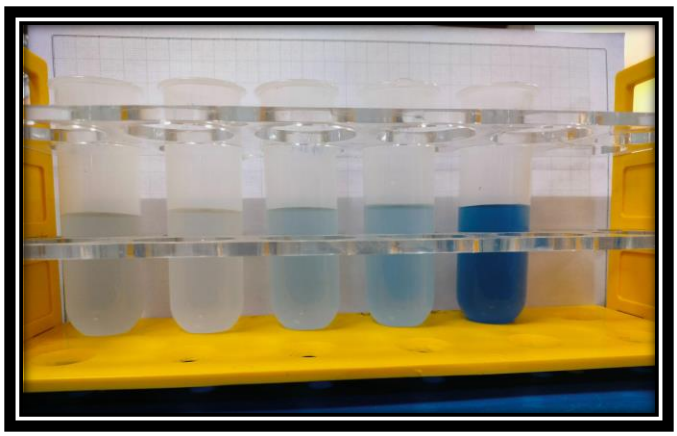


Figure 7: Standard tubes for estimation of silica

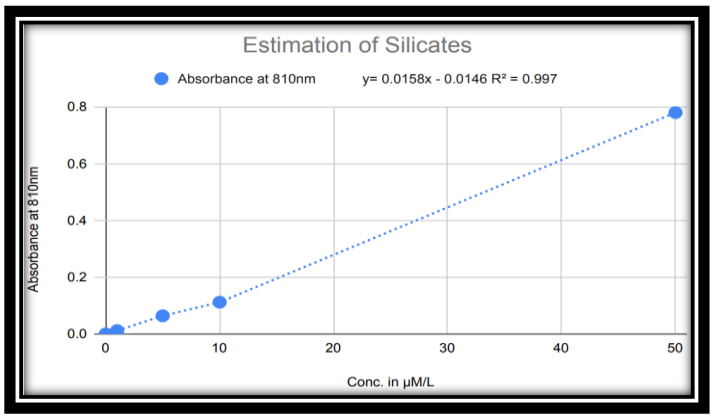


Figure 8: Standard graph for estimation of silica

Figure 9: Standard tubes for estimation of phosphates

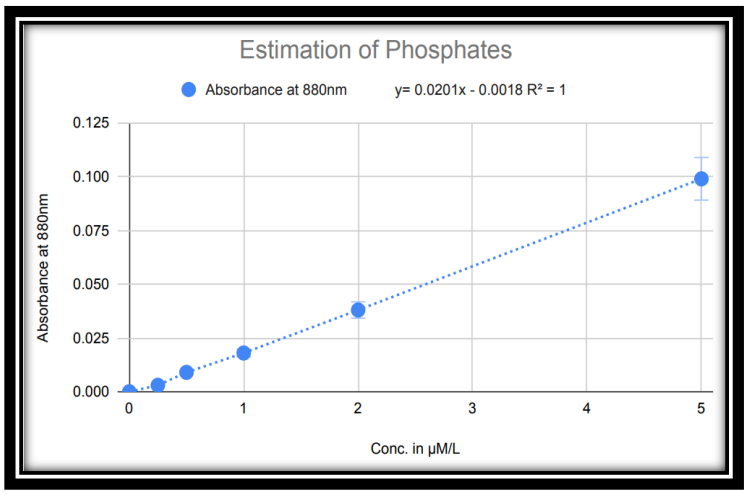


Figure 10: Standard graph for estimation of phosphates

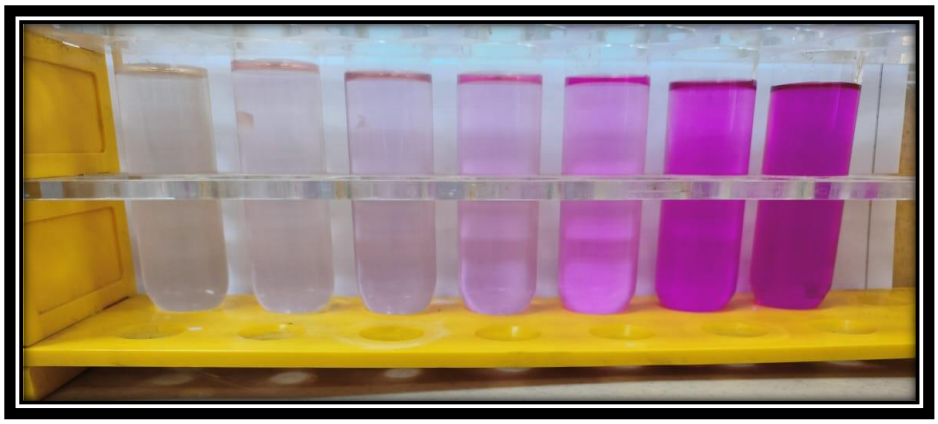


Figure 11: Standard tubes for estimation of nitrites

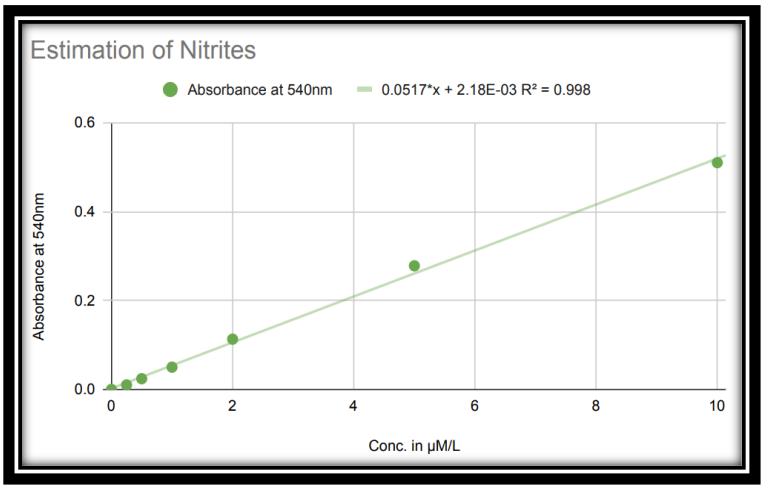


Figure 12: Standard graph for estimation of nitrites

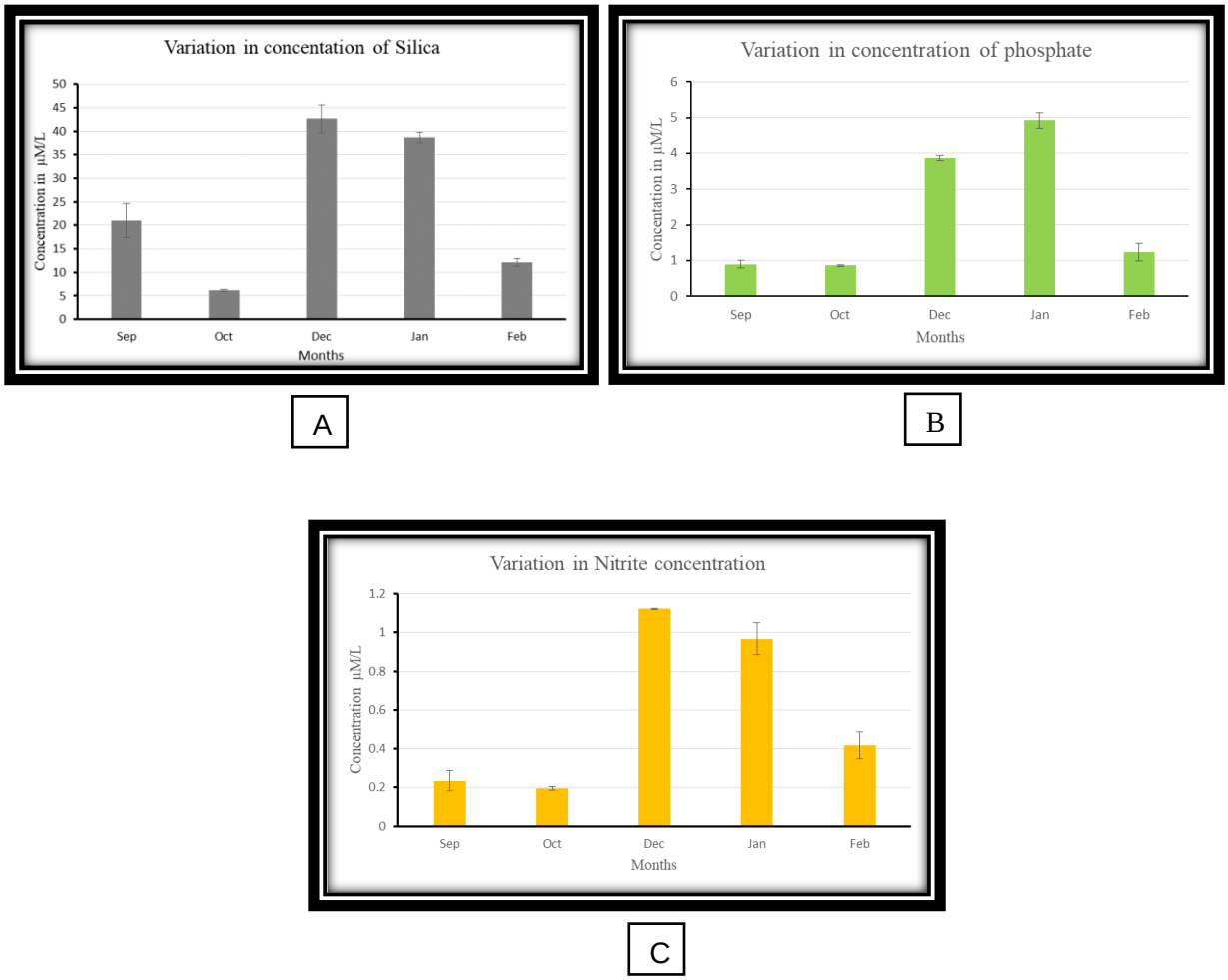


Figure 13: Variation in nutrient concentration: A: silica, B: phosphates & C: Nitrite

The highest concentration of silicates, phosphates & nitrites were observed in the month of December (42.66 µM/L ± 2.95), January (4.91µM/L ± 0.21) and December (1.12µM/L ± 0.002) respectively on the day of sampling (Fig.13). Land runoff during the monsoon season has a significant impact on the distribution of nutrients in the Mandovi estuary. This flushing process brings nutrients into the estuary, supplemented by anthropogenic activities and sediment resuspension (DeSousa, 1983; Martin et al., 2007). During the monsoon, higher concentrations of nitrite and phosphate (2.8 µM and 4.0 µM) were observed in the lower portion, whereas an extremely high concentration of silicate (129.7 µM) was reported in the top section of Mandovi

estuary (Pednekar et al., 2014). The observed elevated levels of nitrites may be attributed to amplified phytoplankton excretion, ammonia oxidation and nitrate reduction, as well as nitrogen recycling and bacterial breakdown of planktonic debris in the surrounding milieu (Swami et al., 1996;Govindasamy et al., 2000; Prabhu et al.,2007). Higher values of phosphates could be possibly due to weathering of rocks, addition of super phosphates, which are used as fertilisers in agricultural areas, and alkyl phosphates, which are used as detergents in homes. Similar reason would be for higher concentration of Silica. (Tiwari & Nair, 1993; Prabhu et al., 2007).

4.3. DIVERSITY STUDIES

Seasonal changes in the abundance of phytoplankton communities were studied, and a checklist of the encountered phytoplankton was made and is represented in Table 1. In September, Chlorophyta were the predominant group (83.4%), followed by Bacillariophyta (16.6%). In October, Bacillariophyta (92.5%) dominated, followed by Chlorophyta (3.9%) and Dinophyta (3%). In the month of December it was observed that Bacillariophyta was yet again the most dominant group (49.6%), however Dinophyta (27.9%) dominated over Chlorophyta (22.3%). Bacillariophyta were also dominant in January (93.8%) and February (97.1%). Cyanophyta accounted for 0.6% of observations only in the month of October and December (Fig. 15). It could be due to competition amongst the phytoplankton and predation rate leading to nutrient limitation to these organisms (Jouanneau et al., 1982). Overall, Bacillariophyta were the dominant group of phytoplankton in the Chorao mangrove ecosystem, with *Coscinodiscus sp*. present in all samples. *Dunaliella sp*. were dominant in September, while centric diatoms *Thalassiosira* and *Coscinodiscus* were predominant in October. *Prorocentrum caribbaeum* showed a greater number in December, and Pennales were dominant in January and February.

A similar pattern was observed by Pednekar et al. in 2014 at three different regions along the Mandovi estuary from June 2007 to May 2008. Bacillariophyta comprised 71% of the total species in this study, followed by Dinophyta, Chlorophyta, Cyanophyta, Haptophyta, Chromophyta, and Chrysophyta. It is hypothesized that the abundance of Bacillariophyta is related to increase in the concentration of silica and nitrate and more uptake of nutrients by these group (Underwood et al., 1998; Tanaka and Choo, 2000). Since Pennales are benthic the concentration of them from lower to upper sections are in decreasing order than centric diatoms (Underwood et al., 1998; Tanaka and Choo, 2000). Hence the distribution of groups can be dependent on numerous factors.

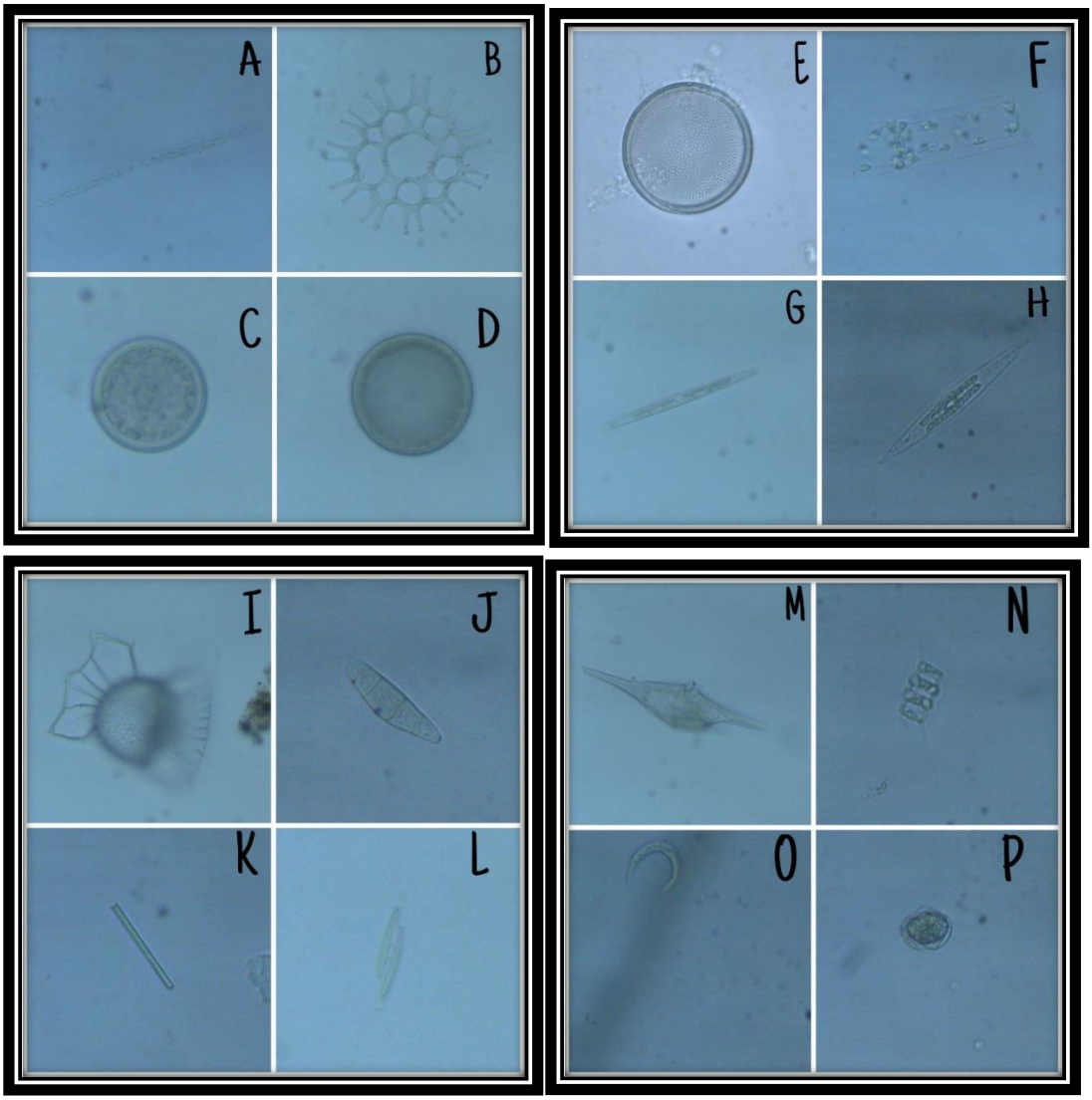
Table 1. Phytoplankton diversity

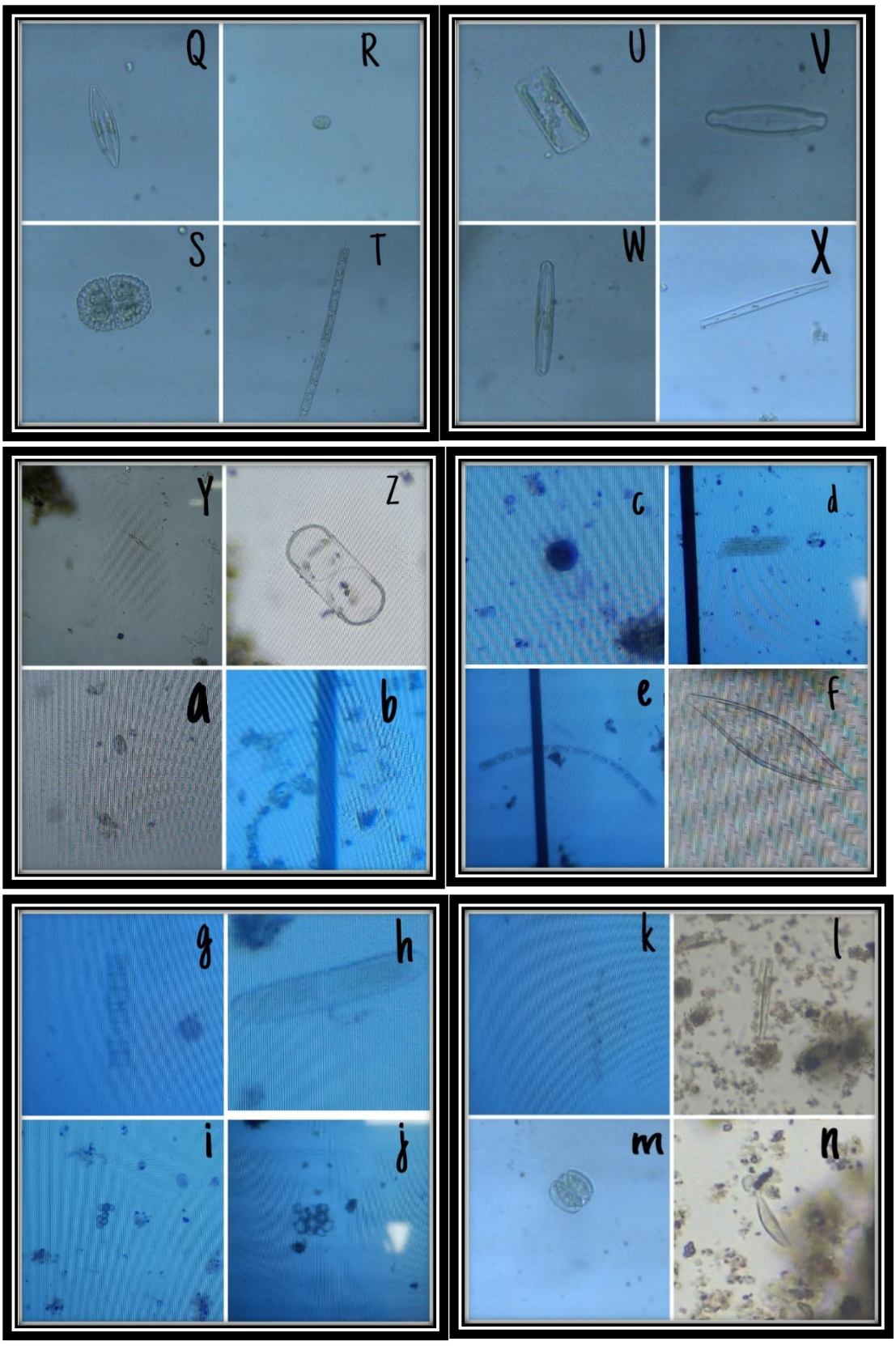
CULTURE	**SEPTEMBER**	**OCTOBER**	**DECEMBER**	**JANUARY**	**FEBRUARY**
Aulacoseira	0	0	150	0	0
Ceratium	0	2	0	0	0
Coscinodiscus	5	167	40	2	39
Cosmarium	0	0	19	0	0
Cyclindrotheca	0	22	0	0	0
Cyclotella	29	0	0	0	0
Cymbella	0	0	0	4	4
Deotonula	0	0	29	0	0

Dunaliella	350	0	0	0	0
Epithemia	0	0	1	0	0
Eucampia	0	0	0	0	3
Fragilaria	5	0	0	0	0
Golenkinia	7	0	0	0	0
Grammatophora	0	2	0	0	3
Guinardia	0	1	0	0	0
Gyrosigma	0	0	4	0	16
Haslea	0	10	0	1	0
Kirchneriella	2	0	3	0	0
Leptocylindrus	0	0	0	0	6
Manguinea	0	0	4	1	0
Mastogloia	0	0	4	0	0
Melosira	0	6	14	3	0
Minidiscus	0	0	10	0	0
Monoraphidium	0	0	4	0	0
Navicula	0	0	13	0	0
Naviculales	0	4	17	0	12

Nitzschia	7	12	0	0	17
Nitzschia palea	3	1	0	1	0
Ornithocercus	0	14	0	0	0
Pediastrum	0	18	3	0	4
Pinnularia	1	0	23	32	110
Pleurosigma	0	0	0	4	7
Prorocentrum caribbaeum	0	0	190	0	0
Radiococcus	11	3	5	6	0
Rhizosolenia	0	0	0	0	2
Scenedesmus	0	0	22	0	0
Scenedesmus dimorphus	7	0	0	0	0
Scenedesmus communiss	0	0	94	0	0
Skeletonema	4	0	2	13	7
Staurodesmus	0	0	2	0	3
Stephanopyxis	1	0	0	0	0
Synechocystis	0	3	2	0	0

Synedra	4	0	0	4	5
Thalassiosira	1	270	0	25	0
Thalassiothrix	15	0	0	0	0
Tropidoneis	0	0	27	0	0

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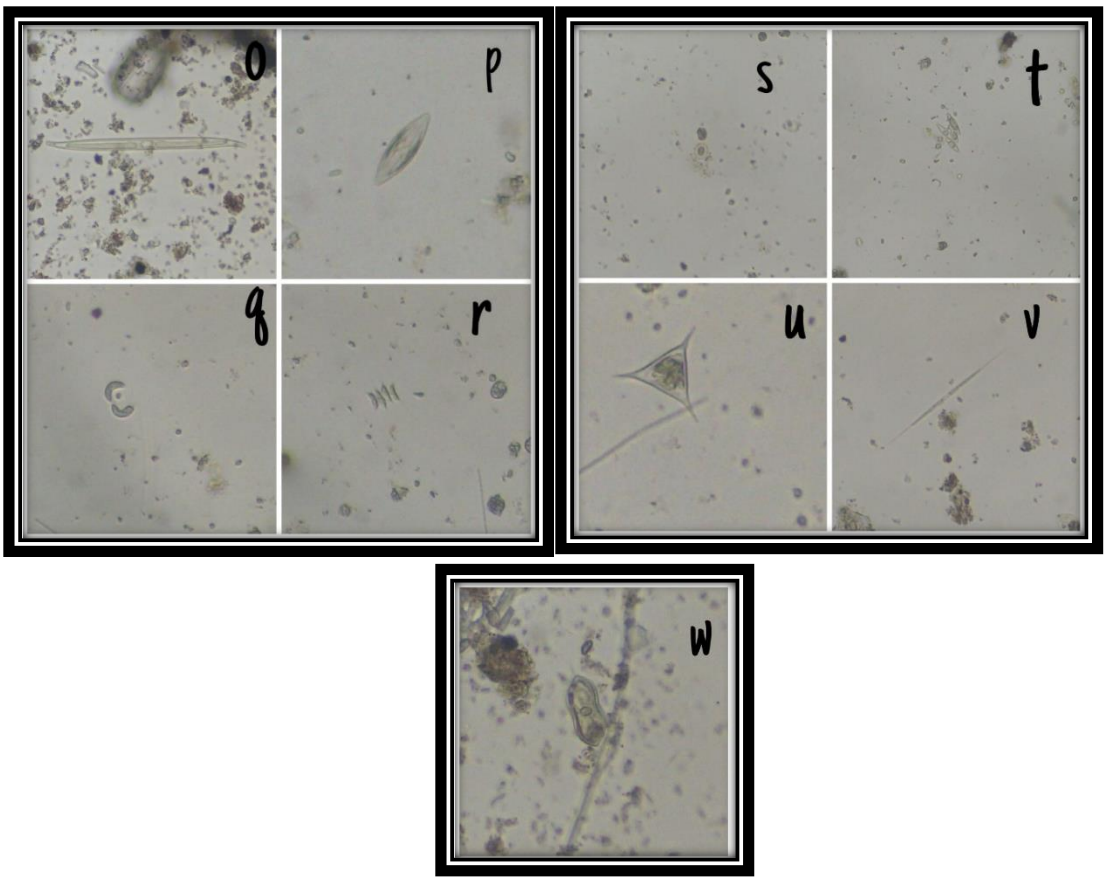

Figure 14: Diveristy study A: *Leptocylindrus, B: Pediastrum, C: Cyclotella , D: Thalassiosira ,E: Coscinodiscus ,F: Grammatophora ,G: Synedra, H: Haslea, I: Ornithocercus, J: Epithemia , K:Thalassiothrix, L: Navicula, M: Ceratium, N: Scenedesmus communis, O: Monoraphidium, P: Prorocentrum caribaeum* , Q: *Navicula,* R: *Dunaliella,* S: *Cosmarium,* T: *Aulacoseira , U: Tropidoneis,* V: *Mastogloia,* W: *Pinnularia,* X: *Nitzschia,*Y: *Cyclindrotheca,* Z: *Deotonula,* a: *Dunaliella,* b: *Eupcampia,* c: *Golenkinia,* d: *Fragilaria,* e:*Guinardia,*f: *Gyrosigma,*g:*Melosira,* h: *Nitzchia palea,*i: *Radiococcus,* j: *Synechocystis,* k: *Skeletomnema,* l: *Manguinea,* m: , n: *Cymbella,* o: *Thalassira spp, Pleurosigma,*p:*Naviculales,* q: *Kirchneriella,* r: *Scenedesmus,* s: *Minidiscus,* t: *Scenedesmus dimorphus,* u: *Staurodesmus,* v: *Rhizosolenia* , w: *Nitzschia panduriformis*

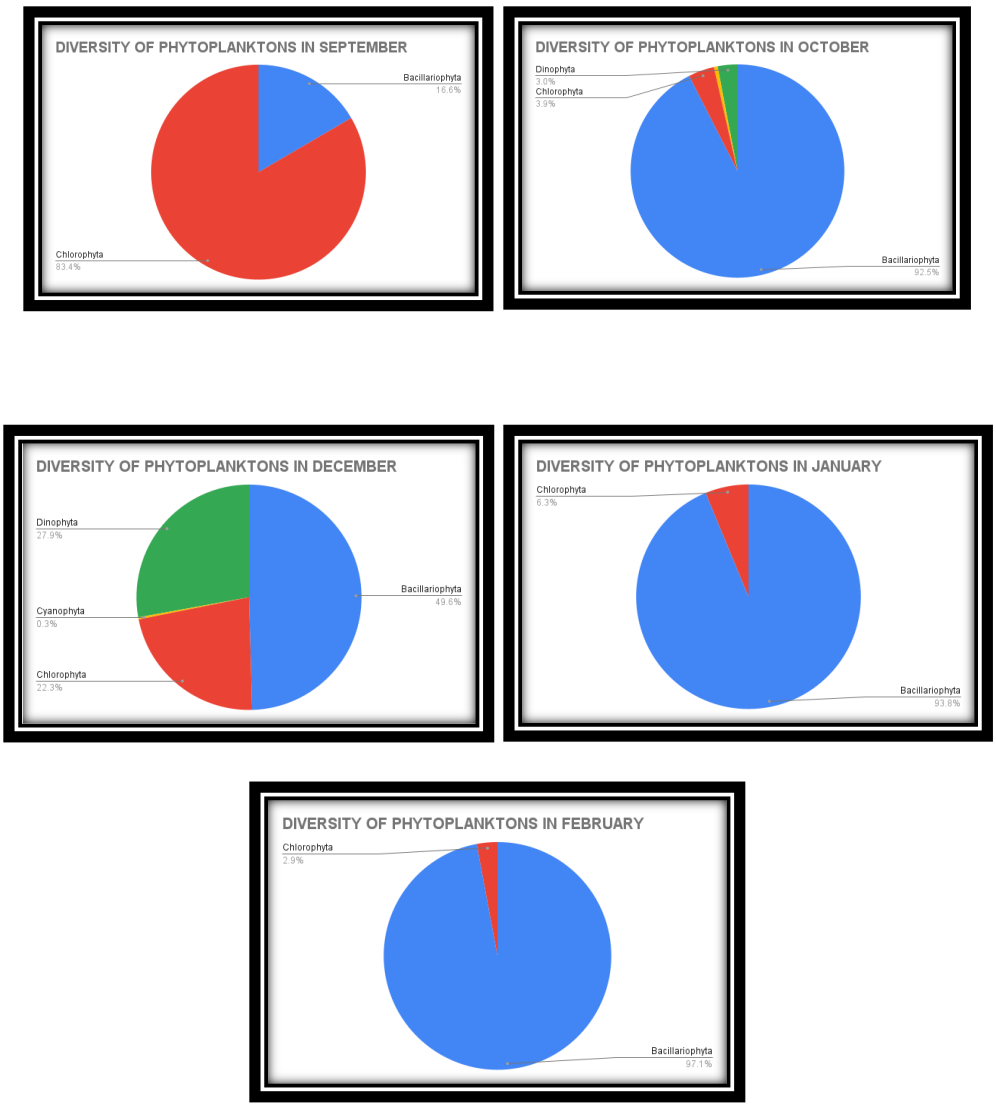

Fig.15 Distribution of phytoplankton community along Chorao mangrove ecosystem in the month of September, October, December, January & February.

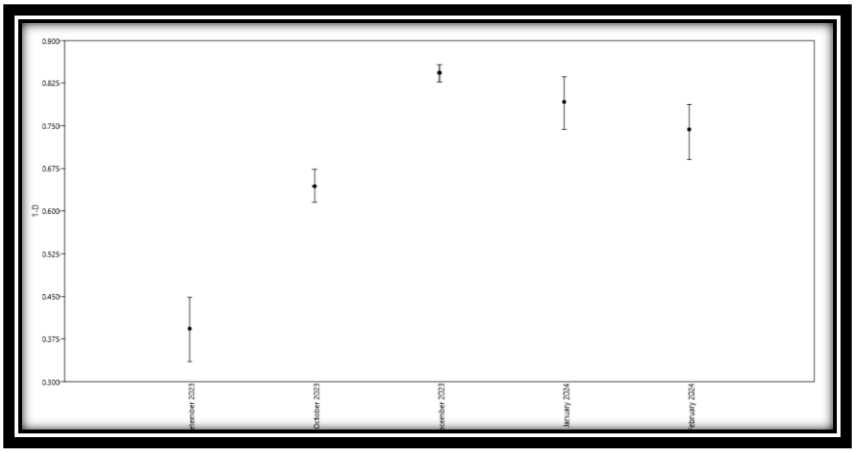

Fig.16 Simpson diversity index graph

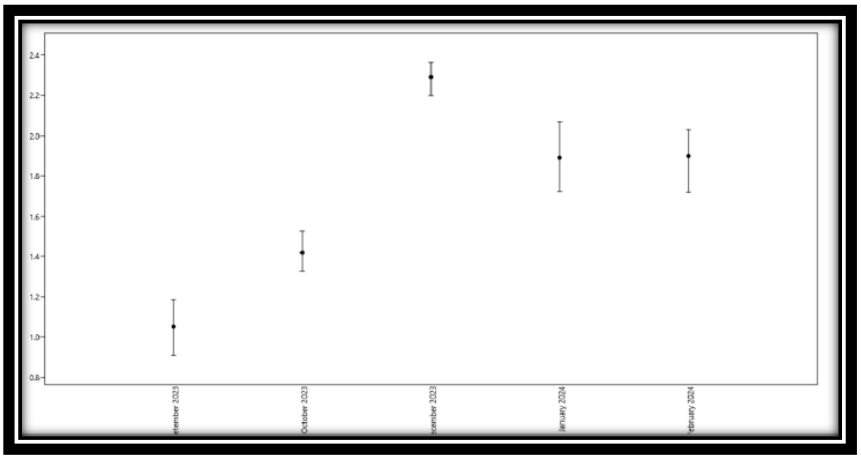

Fig.17 Shannon diversity index graph

Species count and evenness index are frequently employed to quantify diversity. However, a composite statistic that integrates these metrics can also be utilized (Stirling & Wilsey, 2001). To comprehend the seasonal change and characterize the mangrove environment, diversity indices were computed of the phytoplankton counted from the Chorao mangrove ecosystem. Variations in environmental conditions, such as nutrient levels, temperature, and salinity, may have contributed to the differences in species composition observed between the seasons (Webb 1960). The species diversity index can be used to assess the growth rate, occurrence, dispersion, and species

composition of the phytoplankton community. Certain species like *Coscinodiscus*, *Cyclotella*, *Nitzschia*, and *Biddulphia* can serve as indicators of organic pollution (Effiong et al., 2018).

The Simpson diversity index considers both the total number of species and the relative abundance of each species in a habitat. It calculates the probability that two individuals chosen at random from a sample will belong to the same species (or a different category altogether) (Effiong et al., 2018) . The Simpson index (Fig.16) for September, October, December, January, and February were 0.3934, 0.6432, 0.8434, 0.7919, and 0.7435 respectively. Low diversity index values typically indicate the dominance of one or two species, while high diversity index values suggest a more even distribution of species numbers across the habitat (Effiong et al., 2018). The Simpson index was highest in the month of December followed by January and February. Shannon's index, rooted in information theory, suggests that the information content within a system can serve as a measure of its disorderliness or randomness (Wiener, 1948). Similarly, the Shannon index (Fig.17) were calculated as 1.052, 1.419, 2.29, 1.889, and 1.898 for the respective months. High value of Shannon index was calculated in the month of December followed by January and February and least in the month of October. Bajpai 1997; Adesalu and Nwankwo 2008; Rajagopal 2010 reported that low values of Shannon index could be possibly due to heavy rains which can lead to dilution of area.

In December, the diatoms *Aulacoseira* followed by *Coscinodiscus*, were the dominant groups out of 13 diatoms identified. Among the Chlorophyta, *Scenedesmus* was the dominant species out of 8 Chlorophyta identified. The dinoflagellate *Prorocentrum caribbaeum* was the dominant Dinophyta group in December. In case of October diatoms *Cyclotella* followed by *Thalassiosira* were the dominating species among 10 species identified. *Dunaliella sp* among 5 *sp.* of phytoplankton was the dominating group of Chlorophyta whereas no Dinophyta were

observed. Liebig's law of the minimum states that the growth and development of an organism are limited by the resource that is in shortest supply relative to the organism's requirements. Similarly Sukumaran & Das in 2002 stated that low densities of phytoplankton may be due to low nutrients and nutrients play an important role in regulating the succession & growth rate. From the DO & nutrient estimation studies of Chorao mangrove water sample as stated earlier in section 4.2 it is observed that highest concentration DO, silica & nitrite were in the month of December and lowest in the month of January , September & October respectively. Hence higher diversity in the month of December can be correlated with high concentration of DO and nutrients.

4.4. ISOLATION OF PHYTPLANKTON

4 species of phytoplankton namely *Dunaliella*, *Amphiphora*, *Navicula distans*, and *Amphora ovalis*, from the Chorao mangrove ecosystem at a salinity of 30 ppt were isolated in the month of October (Fig.18). Similarly, two diatoms each, *Navicula transitions var. derasa f. delicatula*, *Amphora* & *Navicula directa*, *Cylindrotheca closterium*, were isolated at salinities of 19 (Fig.19) and 22 (Fig.20) ppt respectively in the month of December and January . Additionally, at a salinity of 25 ppt, *Cymbella* and *Pleurosigma* were also isolated in the month of February. Replicating and comprehending the natural world is often the first step toward a successful isolation. For coastal marine algae, temperature and salinity are critical, while for oceanic phytoplankton in open ocean habitats, water quality and metal toxicity are significant factors. Emulating these circumstances can improve isolation (Andersen, R. A. (Ed.). 2005). As per literature it has been observed that diatoms are known to dominate the mangrove ecosystem, as recorded in this study as well. Diatoms can thrive in estuarine environments due to their ability to survive in diverse conditions, including common features like turbidity, fluctuating salinity, and varying nutrient levels. Their capability to adjust their buoyancy allows diatoms to position

themselves in optimal light and nutrient environments, aiding their survival in estuarine ecosystems. The unique silica cell wall of diatoms enables them to efficiently absorb silicate, an essential nutrient for their growth which gives them a competitive advantage (Blackburn et al., 2009; Rahaman et al., 2013; Kathiresan, K,2000 Rajkumar et al.,2009;Pednekar et al., 2014).

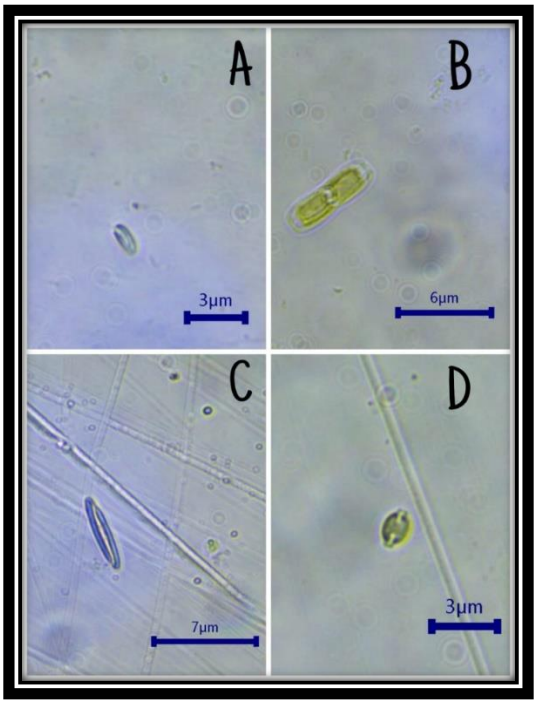

Figure 18. Phytoplankton monocultures isolated in the month of October.: A: *Dunaliella,* B: *Amphiphora,* C: *Navicula distans* & D: *Amphora ovalis*

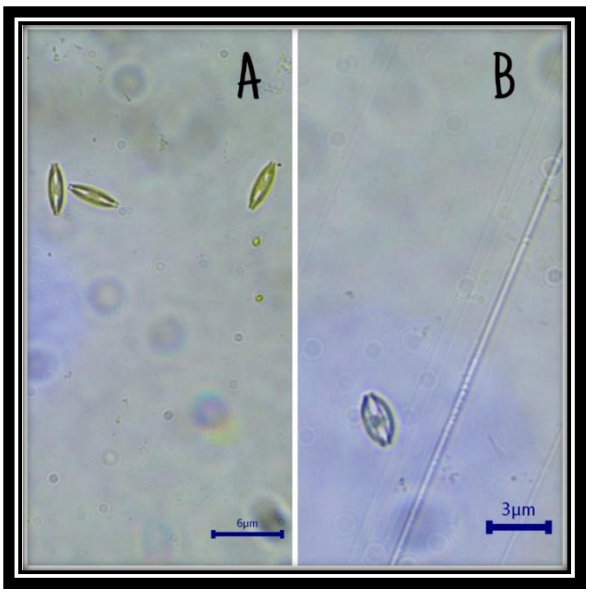

Figure 19. Phytoplankton monocultures isolated in the month of December.A: *Navicula transitions var.derasa f. delicatula* & B:*Amphora sp*

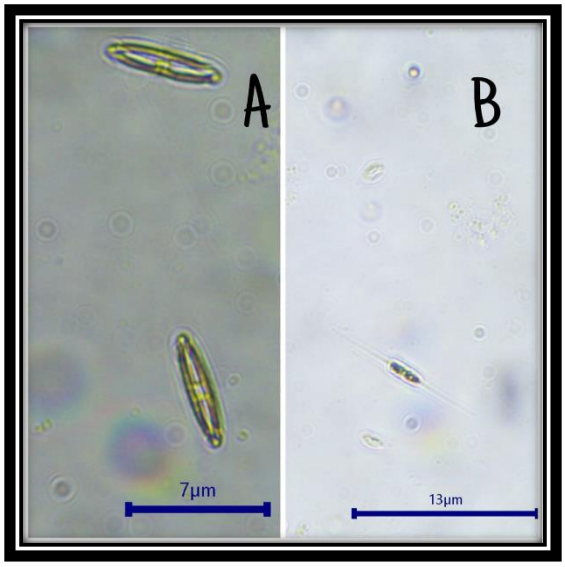

Figure 20. Phytoplankton monocultures isolated in the month of January. A*: Navicula directa* & B: *Cylindrotheca Closterium*

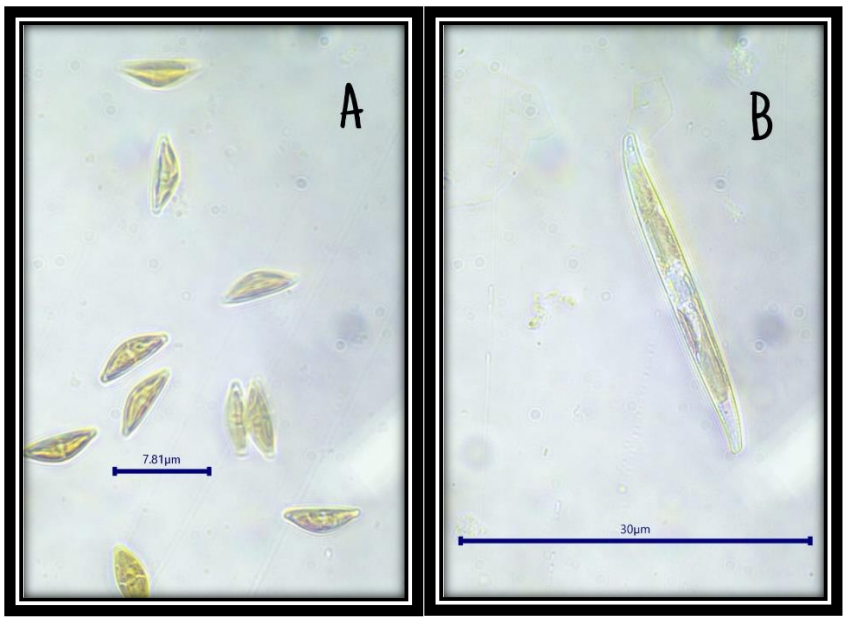

Figure 21. Phytoplankton monocultures isolated in the month of February: A: *Cymbella* & B: *Pleurosigma*

4.4.1 GROWTH STUDIES

The motile microalgae *Dunaliella* exhibited its highest cell density on day 8 (270×10^4 cells/mL), entering the stationary phase from day 10 onwards. In a study conducted by Subba Rao 1981 & Cifuentes et al., 1996 on growth of *Dunaliella sp.* the maximum cell density was observed between day 10 to day 12 for some *sp.* while some attained maximum cell density before day 10. *Amphiphora* showed a rapid increase in cell density from day 8 to day 16 followed by steady decrease from day 16. *Navicula distans* experienced a rapid increase in cell density on day 6 (244×10^4 cells/mL), followed by a sharp decrease on day 12 (56×10^4 cells/mL). Similarly, *Amphora ovalis* showed a rapid increase in cell density on day 6 (345×10^4 cells/mL), with the stationary phase observed from day 8.

Navicula transitions var. derasa f. delicatula entered the early log on day 6 (29.75×10^4 cells/mL), with a rapid exponential increase in cell density on day 8 (67.5×10^4 cells/mL), followed

by a gradual decrease from day 10. *Amphora* exhibited a gradual increase in cell density from day 6 with highest cell density observed on day 10 (29.5×10^4 cells/mL) & stationary phase on day 12 (18.25×10^4 cells/mL). In a study reported by Dias et al., 2018 a lag phase of 2 days followed by exponential phase up to day 7 was observed with maximum cell density observed on day 11 on *Amphora sp*. *Navicula directa* reached its highest cell density of 280×10^4 cells/mL on day 8, with the stationary phase observed on day 10 onwards. Vrieling et al., conducted a study on *Navicula sp.* which showed that the cell density was increased until day 10, after which a decline in cell numbers was observed. *Cylindrotheca closterium* reached its highest cell count of 58×10^4 cells/mL on day 10, entering the stationary phase on day 12. A similar growth curve was obtained on *Cylindrotheca closterium* reported by Affan et al., 2009 at 20 °C 30ppt.

Controlled variables and limited nutrient supply can significantly impact phytoplankton growth such as light, temperature, nutrient concentration and temperature (McCombie, A. M. (1953). To meet the specific nutrient requirements of different species, it is crucial to optimize the components of the growth medium. This approach ensures that species-specific needs are addressed, rather than using a uniform medium for all species in the same environment (Li et al., 2017). Light plays a crucial role in the growth of microalgae, influencing their growth rate and biomass composition. Both the quality and quantity of light are important factors in the development of these organisms (Markou et al., 2012). The light conditions provided to the cultures in the current study artificially were less (3100 lux) compared to natural light conditions (5300 lux).

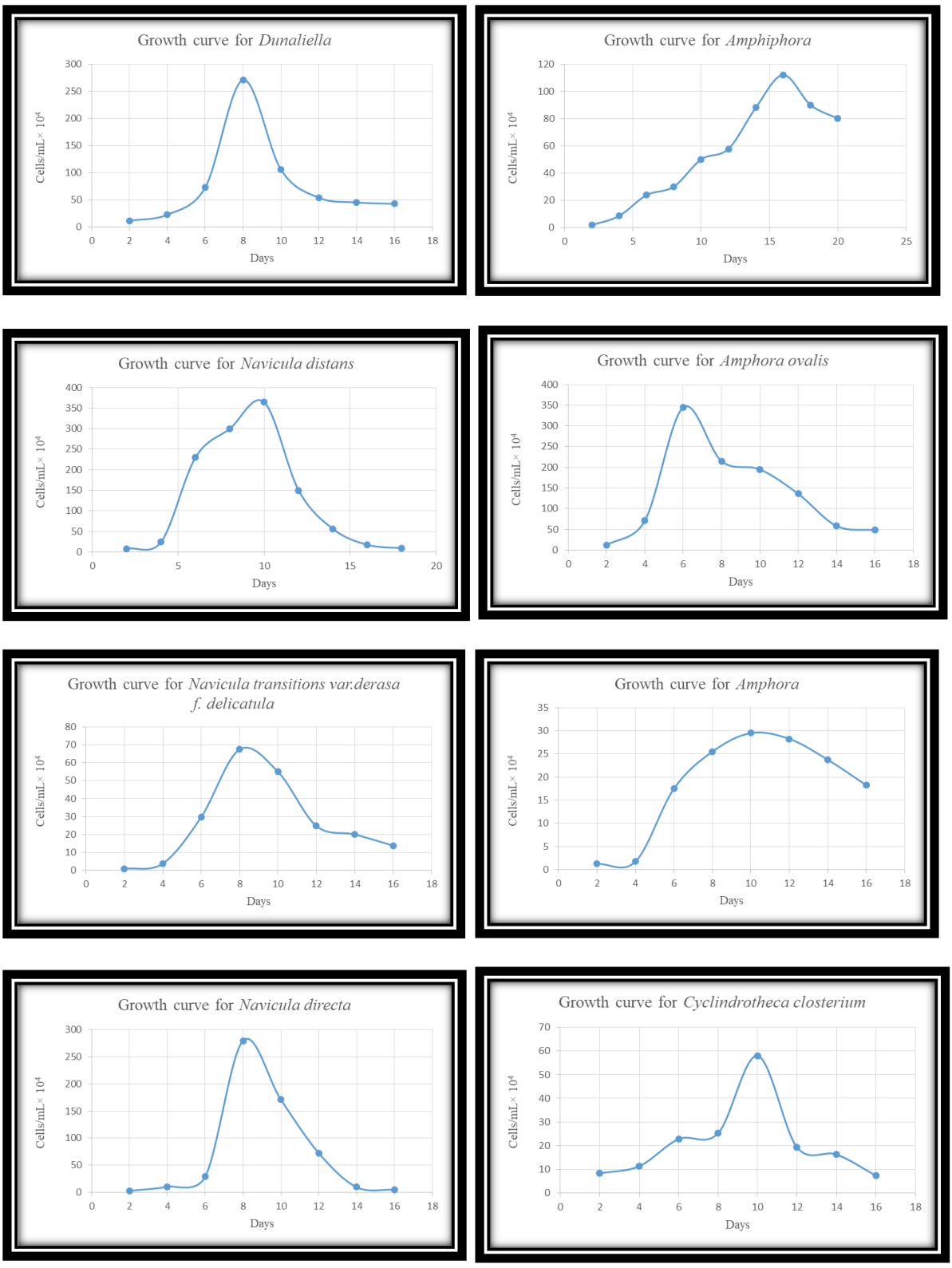

Fig 22. Growth studies of isolated phytoplankton monocultures.

4.5 ISOLATION OF BACTERIA FROM PHYTOPLANKTONS

The colony characteristic and Gram character of bacteria associated with isolated phytoplankton isolated by serial dilution and spread plating on ZMA plates are tabulated in the table.2 below. The colony morphologies were found to be somewhat similar.

Diatoms and bacteria have coexisted in shared environments in oceans for nearly 200 million years, fostering interactions between these species over evolutionary time scales. Diatom genomes have acquired hundreds of genes from bacteria, which likely played a significant role in the success and diversity of diatoms (Armbrust et al., 2004; Bowler et al., 2008). Many scientist have observed that many dinoflagellate species, including the dangerous algal bloom-causing species *Gymnodinium catenatum*, require bacteria for their development (Mayali, X. (2018). Attempts to remove bacteria from dinoflagellate laboratory cultures have been unsuccessful (Guillard and Keller, 1984). In a study by Bolch et al.,2017 laboratory batch co-cultures of *Gymnodinium catenatum* and various bacterial species were created. The researchers found that the effects of bacteria on growth could be as significant as those of light and temperature. Heterotrophic bacteria commonly scavenge and break down organic matter, utilizing the organic carbon produced by diatoms and other autotrophic organisms to convert it back into CO_2 (Cho et al., 1988). Certain microalgal strains have been observed to exhibit co-benefits with bacteria. For example, there is an exchange of metabolic components, such as vitamin B_{12}, between the two species. The bacteria provide the algae with vitamin B_{12} in exchange for the algal exudates, which serve as a carbon substrate for the algae's growth (Croft, et al., 2005). Therefore, the bacteria isolated from phytoplankton were equally screened for their antioxidant and antimicrobial activity.

Table 2: Colony characteristics, viable count and gram character

Culture from which isolated	Number of colonies	Size	Colour	Shape	Margin	Opacity	Elevation	Consistency	Cfu/mL	Gram Character
Dunaliella	4	Small	Cream	Wrinkled	Smooth	Opaque	Convex	Dry	4×10^4	Negative
Amphiphora	18	Moderate	Cream	Wrinkled	irregular	Opaque	Convex	Dry	18×10^4	Negative
Navicula distans	1	Small	Cream	Wrinkled	Smooth	Opaque	Convex	Dry	1×10^3	Negative
Amphora ovalis	2	Moderate	Cream	Wrinkled	Irregular	Opaque	Convex	Dry	2×10^4	Negative
Navicula transitions var.derasa f. delicatula	7	Moderate	Cream	Wrinkled	Irregular	Opaque	Convex	Dry	7×10^4	Negative
Amphora	3	Moderate	Cream	Wrinkled	Irregular	Opaque	Convex	Dry	3×10^5	Negative
Navicula directa	3	Moderate	Cream	Wrinkled	Irregular	Opaque	Convex	Dry	3×10^5	Negative
Cylindrotheca Closterium	6	Small	Cream	Wrinkled	Smooth	Opaque	Convex	Dry	6×10^4	Negative

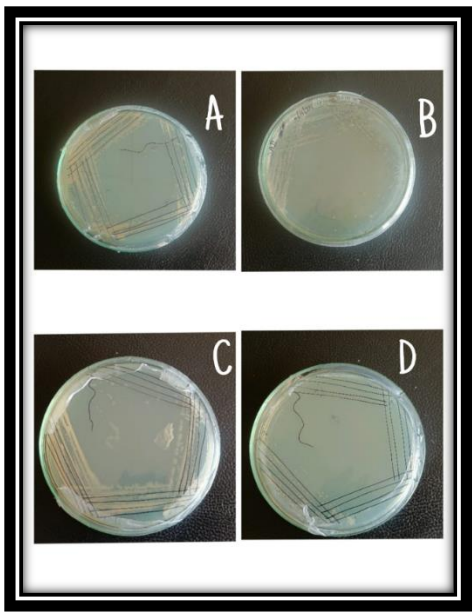

Figure 23. Quadrant streak of bacteria isolated from A: Navicula *distans*, B: *Amphiphora*, C: *Amphora ovalis* & D: *Dunaliella*

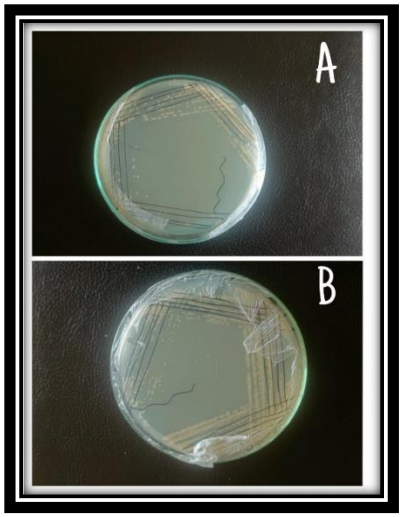

Figure 24. Quadrant streak of bacteria isolated from A: *Navicula transitions var.derasa delicatula* & B: *Amphora*

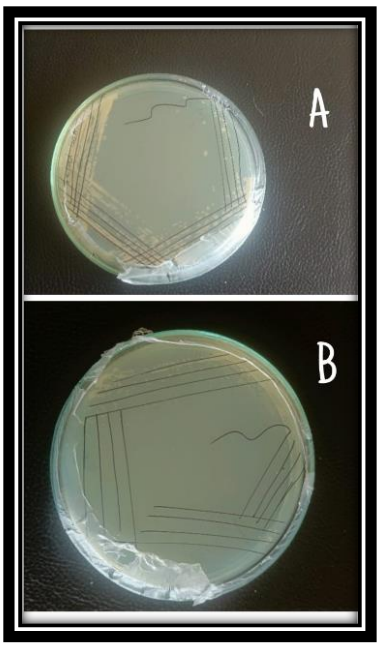

Figure 25. Quadrant streak of bacteria isolated from A: *Navicula directa* & B: *Cylindrotheca closterium*

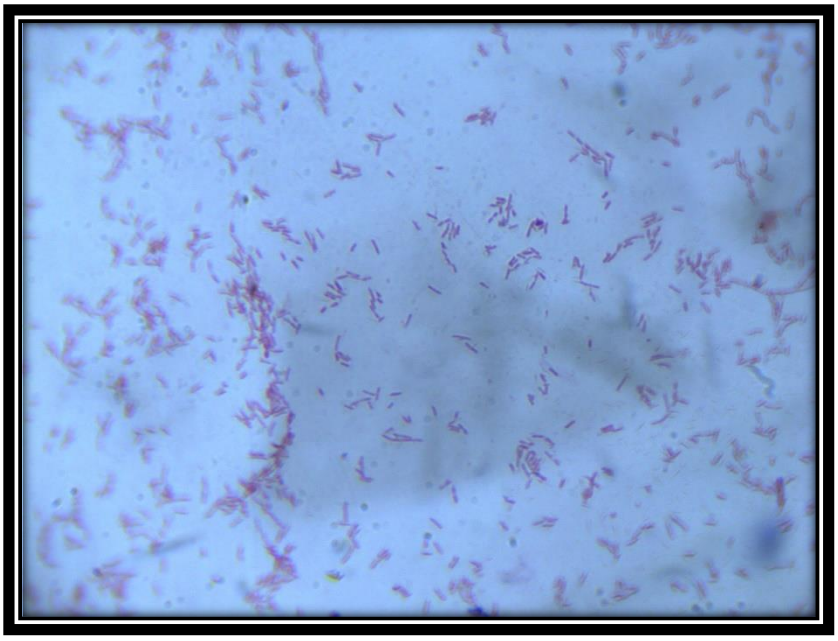

Figure 26. Gram negative rod shaped bacteria isolated from phytoplankton monocultures

4.6. SCREENING OF PIGMENTS AND ITS ANTIOIXDANT ACTIVITY

4.6.1 EXTRACTION OF BIOMASS

The biomass was harvested by centrifugating 50ml of culture media and dried and it's weight was calculated from subtracting the pre-weighed butter paper.

Table 3: Dry biomass in mg obtained from various Phytoplankton cultures

CULTURE	**BIOMASS(mg)**
Dunaliella	59.8
Amphiphora	59.8
Navicula distans	40.4
Amphora ovalis	42.4
Navicula transitions var.derasa f. Delicatula	40.3
Amphora	46.1
Navicula directa	90
Cylindrotheca closterium	49.8

4.6.2 SCREENING OF PIGMENTS & ITS ANTIOXIDANT ACTIVITY

In the case of 90% acetone extract (Fig.27) of phytoplankton the highest content of Chlorophyll *a* was observed in *Amphora ovalis* (0.82335 mg/l) and lowest in *Navicula distans* (0.02145mg/l). Cartenoids contents of 90% acetone extracts were highest in *Navicula transitions var.derasa f. delicatula* (0.49922mg/l). The Chloroform:methanol extract (Fig.28) of phytoplankton cultures showed highest concentration of Chlorophyll *a* in *Navicula transitions var.derasa f. delicatula* (14.15mg/l) followed by *Cylindrotheca closterium*. Diatom *Amphiphora* gave the highest amount of Carotenoid (5mg/l) content using Chloroform:methanol extract of phytoplankton.

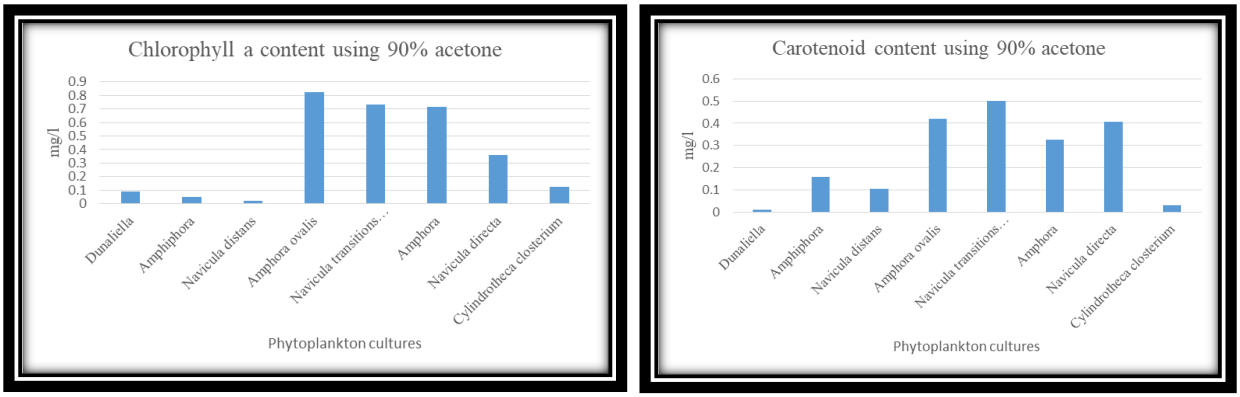

Fig.27 Chlorophyll *a* & carotenoid content obtained from 90% acetone extracts of phytoplankton cultures

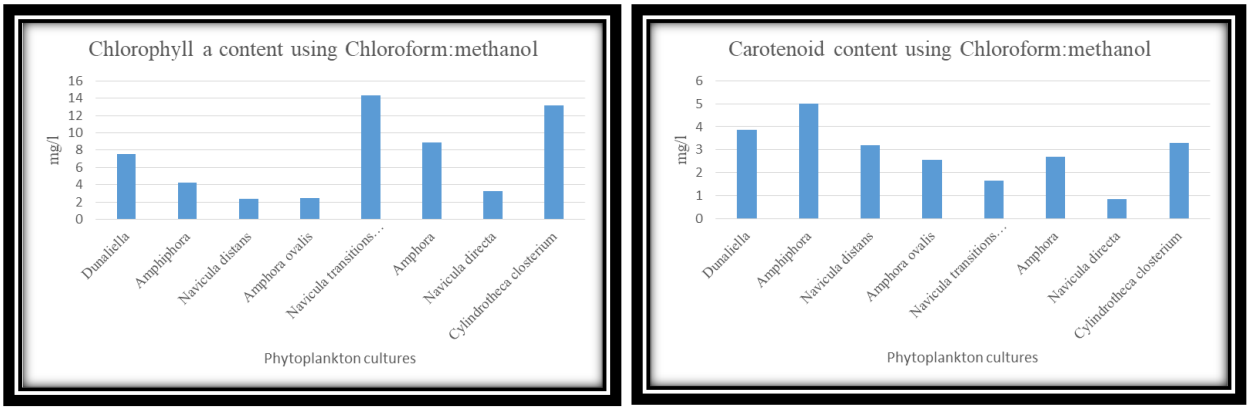

Fig.28 Chlorophyll *a* & carotenoid content obtained from Chloroform: methanol extracts of phytoplankton cultures

A standard test to screen for antioxidant activity using Ascorbic acid was performed (Fig.29). DPPH assay of the test samples were performed and the absorbance was taken at 520nm. The DPPH scavenging activity was calculated using the formula given below (Karthikeyan et al., 2013; Huang et al., 2016; Zakaria et al., 2017).

Scavenging percentage (%) = $[(A_0 – A_1) / A_0] \times 100$ where A_0 is the absorbance of the blank & A_1 is the absorbance of the extracts.

The extracts of 90% acetone and chloroform:methanol of phytoplankton cultures were tested for its antioxidant activity. All the cultures showed antioxidant activity in extracts of 90% acetone (Fig.30). The highest antioxidant activity was observed to be 83.40% in case of *Cylindrotheca closterium* followed by *Dunalliella*, *Navicula directa*, *Navicula transitions var.derasa f. Delicatula* (48.72%), *Amphiophora* (46.59%), *Navicula distans* (37.87%) and lowest was observed in case of *Amphora ovalis* & another sp. of *Amphora* using 90% acetone (15.95%). Using Chloroform:Methanol (Fig.31) as a extraction solvent only *Amphora sp.* showed antioxidant activity. The scavenging activity of *Amphora sp.* was observed to be 5.31%. The bacteria associated with phytoplankton were also screened for their antioxidant properties. However, none of the bacteria isolates showed antioxidant activity.

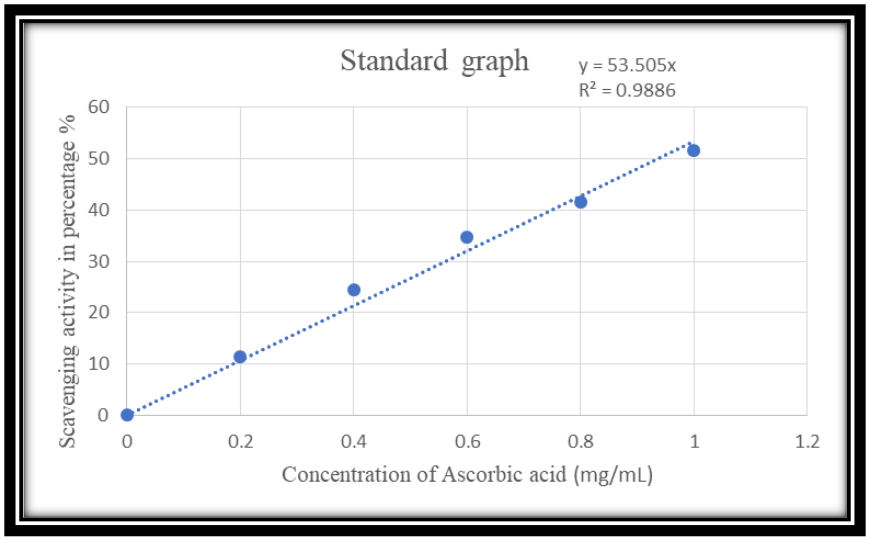

Fig 29. Standard graph for DPPH assay using ascorbic acid as a standard antioxidant

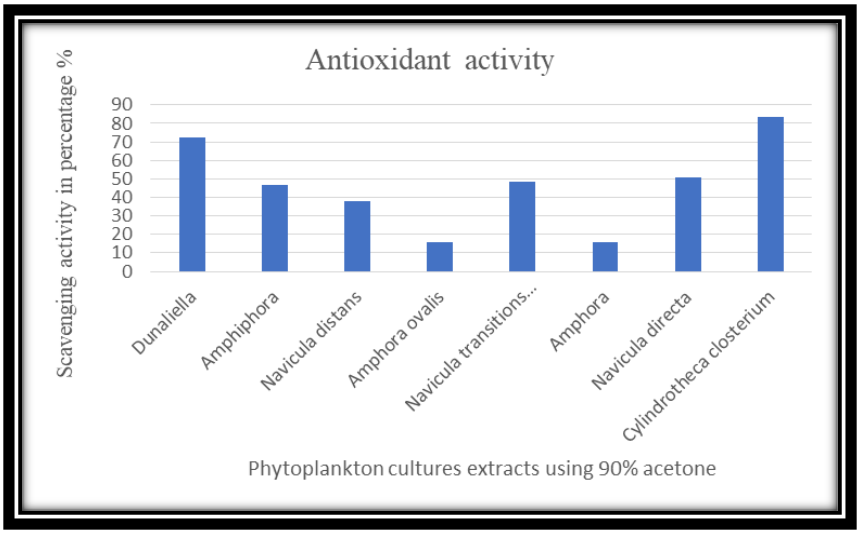

Fig.30 Scavenging activity observed from 90% acetone extracts of phytoplankton monoculture

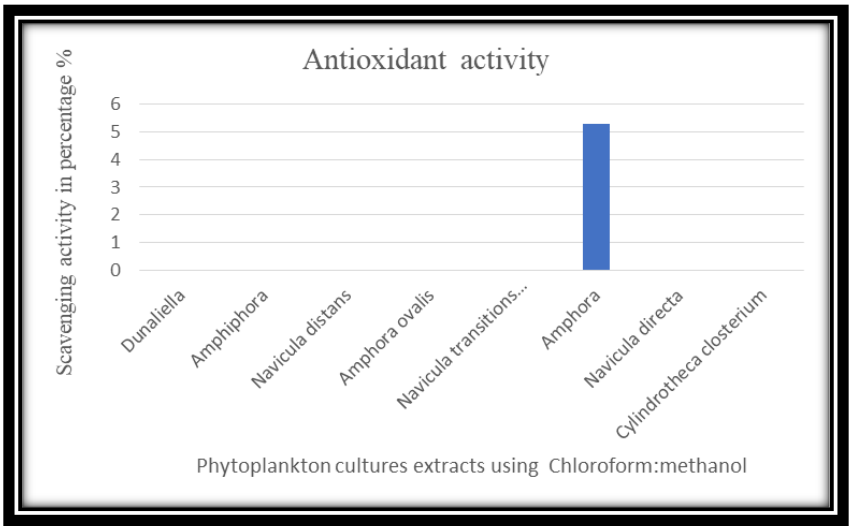

Fig.31 Scavenging activity observed from chloroform: methanol phytoplankton monoculture extracts

Numerous distinct bioactive chemical components can be found in abundance in marine phytoplankton (Plaza et al., 2009). Microalgae's chemical composition is very variable and mostly dependent on environmental elements like light, salinity, temperature, and availability of nutrients (Plaza et al., 2009). Seasonal variations affect most environmental parameters, and these shifts in ecological settings can either promote or impede the production of various nutrients (Herrero et

al., 2006). One of the primary natural sources of β-carotene is *Dunaliella salina*, a green, unicellular marine microalga. Furthermore, it has been noted that *D. salina* can develop noticeably high levels of xanthophylls, specifically zeaxanthin, when exposed to irradiance stress (Yokthongwattana et al., 2005). Diatoms account for over 40% of marine production, and as the oceans encompass roughly 70% of the planet's surface, they significantly boost output worldwide (Falkowski et al., 1998). The constant turbulence in the water causes constant changes in the marine environment, particularly for planktonic species. Depending on the depth, the cells are exposed to different light spectrums and intensities. Diatoms are therefore ideally suited to these environments due to their effective photon collection, CO_2 uptake, and quick reaction to intense light to avoid photodamage. Additionally, diatoms' pigment profile which includes Chl *a*, Chl *c*, β-*car*, *Ddx*, *Dtx*, and *Fx* allows them to harvest light more efficiently than do green and red algae, which contributes to their ecological success as one of the most significant groups of planktonic species (Haxo et al., 1950; Nicklisch A 1998; Falkowski and Knoll, 2011). Thus Chlorophyll *a* content and carotenoid content of acetone and chloroform:methanol extract were evaluated and screened for their antioxidant activity for the phytoplankton isolated from the Chorao mangrove ecosystem. In this study the concentration of Chlorophyll *a* and carotenoids were found to be lower in case of acetone extracts whereas the concentrations were higher incase of chloroform: methanol extracts. On the contrary the antioxidant activity of acetone extracts were found to be higher than chloroform:methanol extracts. Karthikeyan et al., reported lesser correlation of phenolic pigment and antioxidant activity. Banskota et al., 2019 found lipids with higher levels of unsaturated fatty acids (UFAs), especially polyunsaturated fatty acids (PUFAs), enhanced the antioxidant properties of microalgal lipid extracts. Callejón et al., 2020; Ryckebosch et al., 2012; Grima et al., 2013 have used acetone and chloroform in extraction of lipids. The antioxidant activity of the isolates may

be contributed due to higher contents of proteins, lipids or carbohydrates which have to be evaluated (Chen, Y., & Vaidyanathan, S., 2013). Carotenoid production in *Dunaliella* was observed to increase under stressful condition using acetone as extraction solvent (Pisal et al., 2005). A similar study was conducted on *Navicula incerta* to optimise growing conditions for pigments production and its antioxidant capacity (González-Vega et al., 2021). In both studies it was observed that less concentration of $NaNO_3$ was optimal to produce high chlorophyll and carotenoid and antioxidant activity with methanol and acetone as extraction solvents. The DPPH free radical was effectively scavenged by the n-hexane extract of *Amphora coffeaeformis* (67.4 %) and the chloroform extract of *Navicula sp* (63.2%) (Lee et al., 2009). In the current study the radical scavenging activity in *Navicula distans, Navicula transitions var.derasa f. Delicatula, Navicula directa*. *Amphora ovalis & Amphora* were found to be 37.87%, 48.72%, 50.85%, 15.95% and 15.95% respectively using 90% acetone. Rijstenbil in 2004 reported high β-carotene:chl a in UVB exposed and salt-stressed cells of *Cyclindrotheca closterium* indicating the pigment was used in scavenging singlet oxygen. In the current study using 90% acetone extract *Cyclindrotheca closterium* was observed to yield highest antioxidant activity (83.40%) amongst all the isolates. Although, high scavenging activity has been recorded with isolate *Cylindrotheca Closterium* it can be further added that standardization of solvent systems for the extraction of antioxidant compounds for individual isolates can improve the screening methodology.

4.7. SCREENING OF ANTIMICROBIAL ACTIVITY

Extracts of phytoplankton were obtained using hexane (Fig.32) and chloroform:methanol (Fig.33) and tested against three pathogenic cultures: *Escherichia coli, Pseudomonoas aeruginosa*, and *Shigella*. However, no zone of inhibition was observed. Similarly, cultures without extraction

(Fig.32) were tested against the three pathogenic cultures, and no zone of inhibition was observed. Bacteria isolated from phytoplankton (Fig.34) were also tested against the same pathogens, but they showed no antimicrobial activity. Hexane, chloroform:methanol & methanol extracts showed no zone of inhibition. Tetracycline, chloramphenicol & streptomycin were observed to give zone of inhibition.

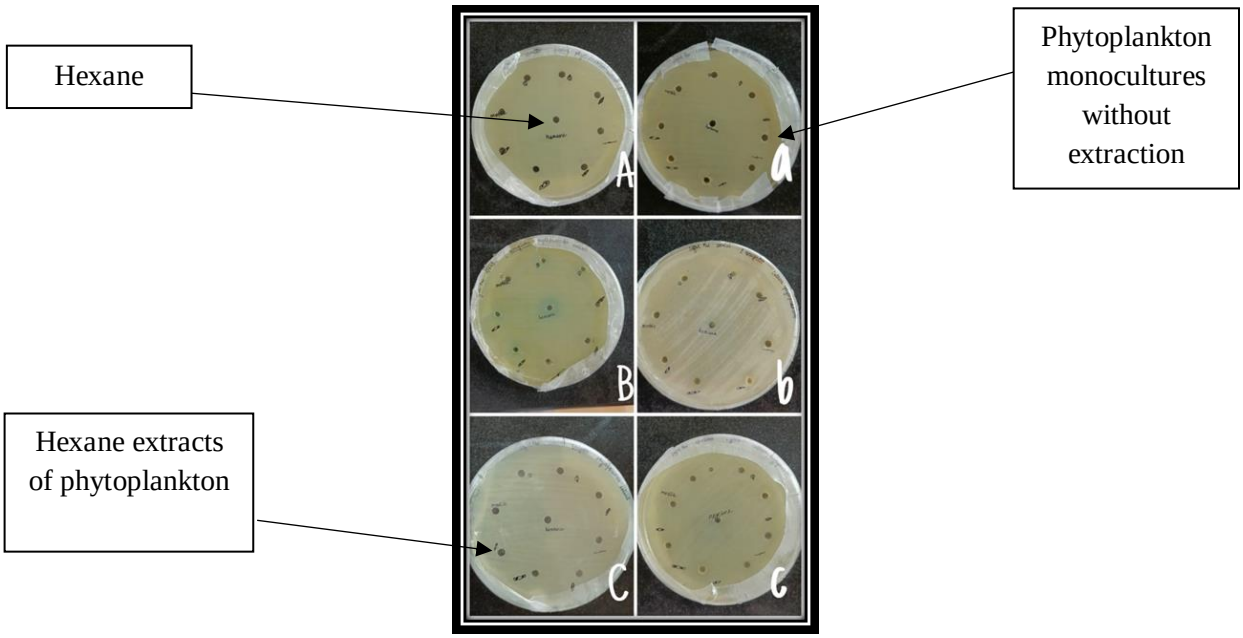

Fig.32 Antimicrobial activity against A: *E. coli*, B: *P. aeruginosa* and C: *Shigella* using hexane extracts obtained from Phytoplankton monocultures, a: *E. coli*, b: *P. aeruginosa* and c: *Shigella* using Phytoplankton monocultures without extraction. No zone of inhibition observed.

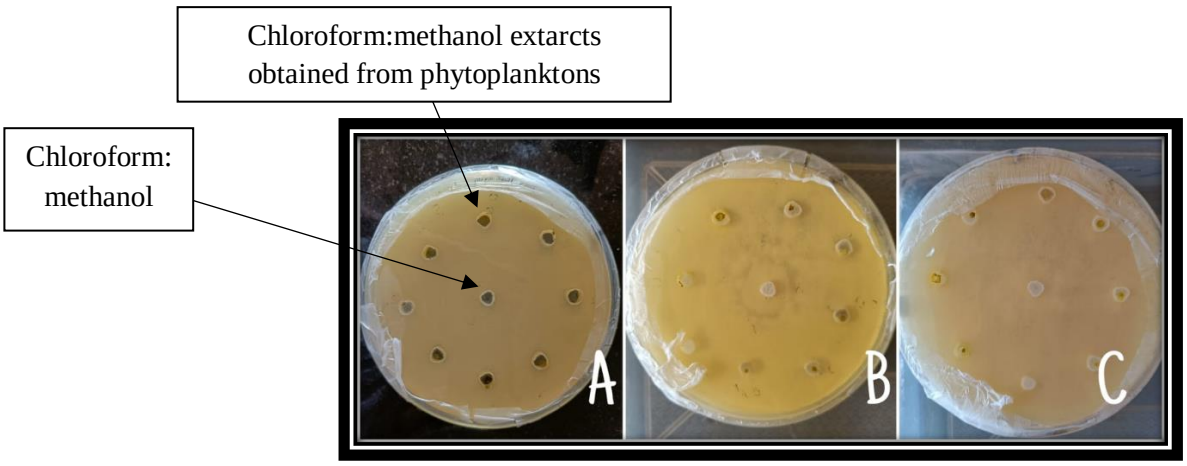

Fig.33 Chloroform: methanol extracts obtained from Phytoplankton cultures showing no zone of inhibition tested against A:*E.coli*, B: *P.aeruginosa* & C: *Shigella*.

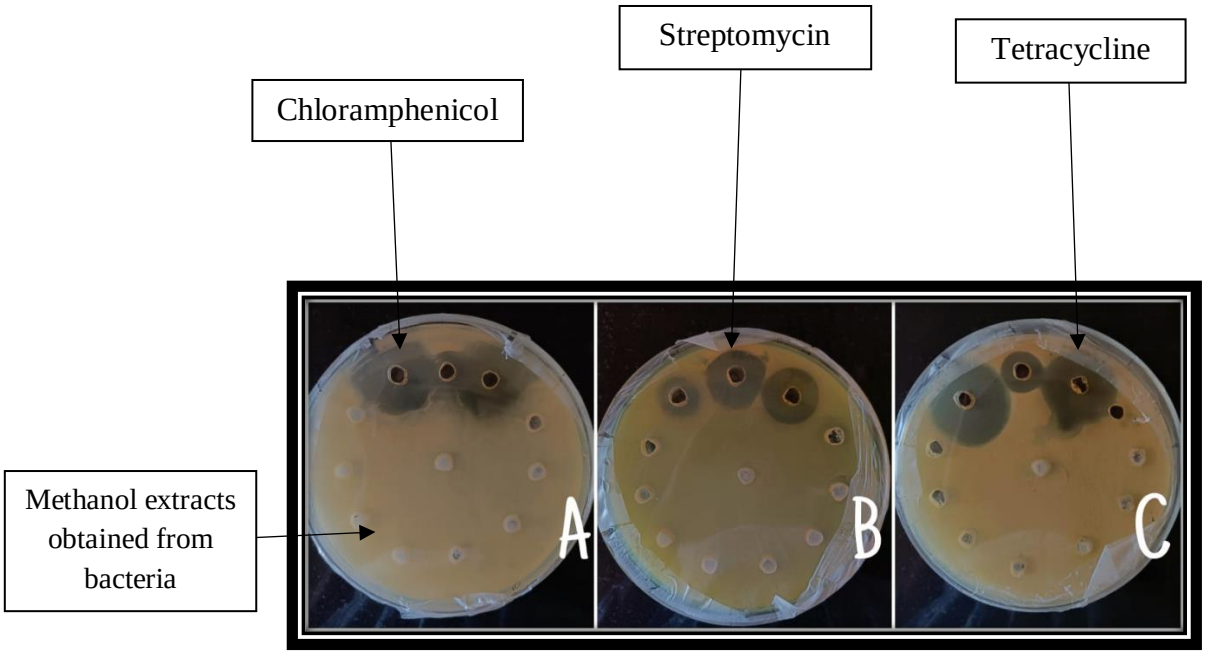

Fig.34 Methanol extracts obtained from Bacteria isolated from Phytoplankton cultures showing no zone of inhibition tested against A: *E.coli*, B: *P.aeruginosa* & C: *Shigella*. Zone of inhibition against the tested bacteria was shown by Chloramphenicol, Streptomycin and Tetracycline.

The Bacillariophyta (Diatom) is regarded as the primary category of phytoplankton on which a substantial amount of research has been conducted because it produces a wide range of biologically active chemicals that can be considered candidates for the synthesis of antibiotics (Findlay, J. A., & Patil, A. D. (1984), Naviner, M., Bergé, J. P., Durand, P., & Le Bris, H. (1999). It is concluded that there are significant differences in the chemical composition of diatom extracts grown in different aquatic environments. These differences may be attributed to the different nutrients supplied in freshwater versus saltwater environments, which alter the diatom's metabolic pathways to produce different compounds. According to a study conducted on *Navicula Incerta*, freshwater diatom extract was shown to be more effective against pathogenic strains of bacteria than salt water diatom extract (Al-Jbory, M. J., & Al-Mayaly, I. K. 2019). Elkomy et al., reported that *Navicula f. delicatula* methanol extract showed antimicrobial activity against *Staphylococcus aureus* and *Micrococus luteus*, *Serratia marcescens*, *Pseudomonas aeruginosa* and unicellular fungus *Candida albicans* but not against *E.coli*. Pigment mediated biogenic synthesis of silver nanoparticles using diatom *Amphora sp.* have strong antibacterial properties against both Gram-positive and Gram-negative microorganisms (Jena et al., 2015). Certain growth characteristics, including growth phase, cell density, exponential growth rate, mean doubling time, and dry weight, are significant in the antibacterial activity assays because they can affect the antibacterial activity (Saavedra et al., 2010). Because biological activity among strains of the same species might vary, strain selection is crucial (Pesando 1990). In the current study the hexane and chloroform:methanol extracts of phytoplankton monoculture did not show antimicrobial activity against the three pathogenic cultures *E.coli*, *P.aeruginosa* & *Shigella*. It is possible that the extracted active component may have had trouble passing through the outer membrane that it known to prevent drug inflow and a multiple-drug pump efflux system that removes the majority of pharmaceuticals

from the bacteria. Another possible reason would be the need to optimise growth conditions ideal for production of antimicrobial activity and using different combinations of extraction solvents (Saavedra et al., 2010). The antimicrobial activity of the isolates can be further studied to extract specific compounds responsible for antimicrobial activity under optimum conditions and use the isolate as feed for aquaculture to reduce transmission of bacteria to humans on consumption.

4.8. PRELIMINARY SCREENING OF LIPIDS

Isolated phytoplankton cultures were observed for lipids under epifluorescence microscope stained with Nile red solution. Lipid bodies were observed for all the cultures (Fig.35-37) whereas cells without Nile red solution showed no fluorescence. Faint lipid bodies were observed in case of *Dunaliella, Amphora* & *Navicula transitions var.derasa f. delicatula* cultures when stained with Nile red.

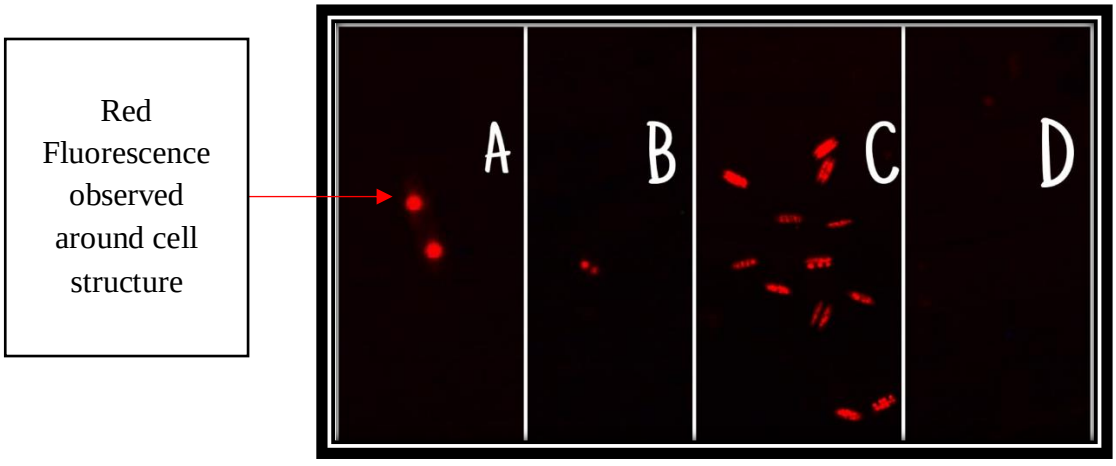

Fig.35 Lipid bodies stained with Nile red A: *Navicula distans*, B: *Amphora ovalis*,C: *Amphiphora* & D: *Dunaliella*

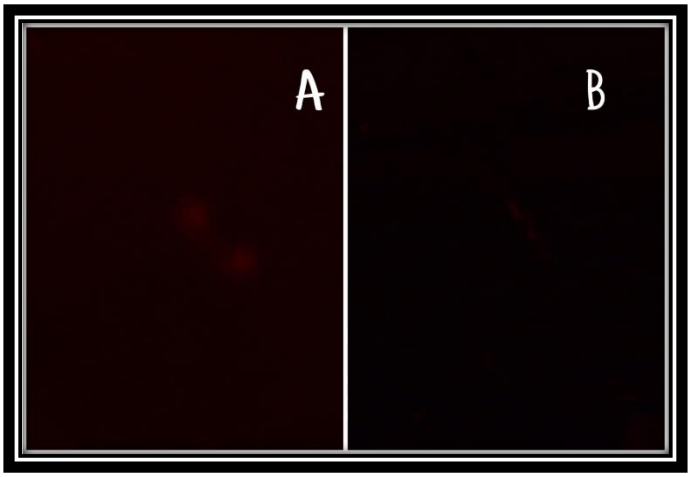

Fig.36 Lipid bodies stained with Nile red A-B: *Amphora* & *Navicula transitions var.derasa f. delicatula*

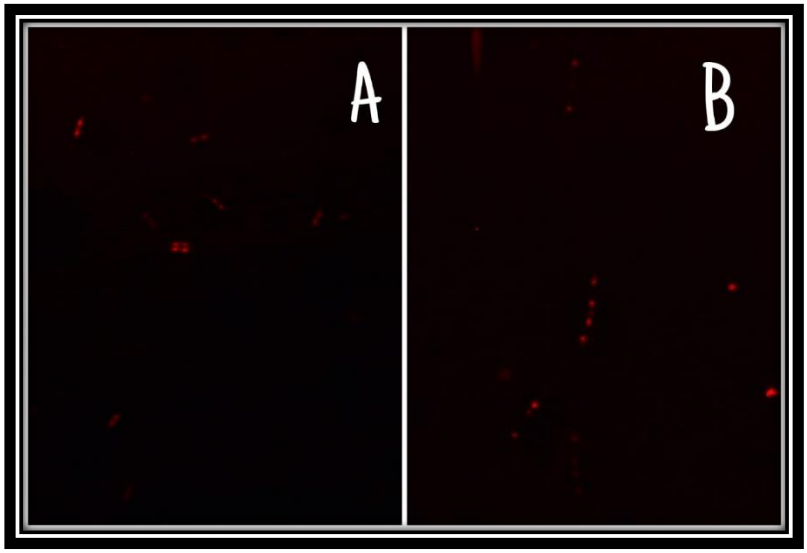

Fig. 37 Lipid bodies stained with Nile red A: *Navicula directa* & B: *Cylindrotheca closterium*

Ponomarenko et al., 2004 reported that microalgae have been found to have greater diversity of sterols. In order to identify the location among lipid-containing phytoplankton cells, the Nile red stain was first investigated using qualitative studies. Nile red is a dye that is not soluble in water and has properties that cause it to change color based on the environment it is in.

Specifically, it emits a deep red to strong yellow gold color in hydrophobic (water-repellent) environments. This dye is often used to stain and visualize hydrophobic materials, such as lipid bodies in cells, due to its fluorescence properties in these environments (Greenspan et al., 1985).

Large cells with distinct cellular bodies and organelles demonstrate that most of the Nile Red fluorescence takes place in specific areas of the cell when examined visually. Researchers utilized nile red, a fluorescent dye that specifically stains neutral lipids, to investigate the levels of neutral lipids in different cells within natural communities of microalgae found in Antarctic sea ice. The microalgae *Navicula sp, Nitzchia sp.* & *Amphora* exhibited wider cells with larger lipid droplets, narrower cells with smaller lipid droplet & light spheres are neutral lipid droplets respectively (Priscu et al., 1990).

A study using chloroform: methanol extract revealed 12 sterols separated from *D. tertiolecta* and *D. salina* from the GC technique successfully (Francavilla et al., 2010). The isolated *Dunaliella* culture screened for lipid body using Nile red showed faint fluorescence under epifluorescence microscope. The possible reasons could be higher cellulose content than diatoms and the need to extract the lipid bodies using cell disruption techniques and suitable solvents. In a study conducted on *Navicula sp.* , high shear in the presence of hexane caused the cells to rupture in-situ, which significantly speed up the lipid extraction process and made it possible to recover high amounts of neutral lipid (>95%) from newly harvested cells in less than five minutes (Yatipanthalawa et al., 2020). *Navicula cryptocephala* yielded higher oil content in sonication method followed by gas chromatography showing the presence of Palmitic acid, Oleic acid, Palmitolic acid and linoleic acid. *Navicula distans* & *Navicula directa* isolated from Chorao mangrove ecosystem showed presence of fluorescence lipid bodies when stained with Nile red in the present study. Chtourou et al. (2015) state that *Amphora* genus lipid rates can reach up to 24%

of DW at 20°C, suggesting that temperature may be a significant effect. Based on their quantified lipid productivity by Cointet et al., (2019) the *Amphora* genus seemed to be a promising option for prospective uses involving lipids. The two *Amphora* species in the current studies also showed presence of lipid bodies when stained with Nile red.

Therefore the diatoms isolated can be further studied for extraction of lipids and purified to identify the lipids using chromatography techniques which could be then used as potential candidates in the pharmaceuticals, aquaculture feed and biorefineries due to small size and less cellulosic content than algae making it energy efficient process at industry scale.

CONCLUSION

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The study conducted on phytoplankton isolated from the mangrove ecosystem in Chorao, Goa, provides valuable insights into their potential applications and ecological significance. The isolation of 10 phytoplankton species (*Navicula distans, Amphora ovalis, Amphiphora, Dunaliella, Amphora, Navicula transitions var.derasa f. Delicatula, Navicula directa* & *Cylindrotheca closterium*) underscores the biodiversity of these ecosystems. Analysis of water samples indicated significant variation in environmental parameters such as salinity, temperature, DO, phosphates, silicates & nitrites over the sampling months, highlighting the dynamic nature of these environments. The isolated phytoplankton species were subjected to screening of pigments & its antioxidant activity, screening of antimicrobial activity & preliminary screening of lipids, revealing their potential as sources of natural antioxidants and lipids with diverse applications. *Cylindrotheca closterium* exhibited the highest scavenging activity, indicating its potential for further study and purification to identify the molecule responsible for its antioxidant properties. Lipid bodies observed in the phytoplankton under the microscope using Nile red stain suggest the potential for biofuel production. Further research and development in this area could lead to the discovery of many other bioactive compounds and sustainable solutions in pharmaceuticals, biotechnology, and environmental conservation. This study highlights the importance of preserving mangrove ecosystems and their biodiversity for future exploration and utilization of their valuable resources.

FUTURE PROSPECTS

FUTURE PROSPECTS

A standard protocol can be designed to maintain the isolated phytoplankton monocultures under axenic conditions without any contamination from other microorganisms. This controlled conditions will allow to study the phytoplankton's behavior and bioactive compound production more accurately. Previous studies have shown that stressful conditions, such as variations in salinity, temperature, or nutrient availability, can induce phytoplankton to produce more bioactive compounds.

To further explore the antioxidant activity observed in the culture extracts, they can be purified further to identify compounds responsible for this activity. This purification process involves isolating the specific biomolecules from the extract and identifying their chemical structure. Once identified, these compounds can be tested for safety and efficacy in human supplementation and laboratory experiments. Another potential application is the extraction of lipids from the phytoplankton. Lipids are organic molecules that can be converted into biofuels, such as biodiesel, through a process called transesterification. Phytoplankton lipids have shown promise as a source of biofuel due to their high lipid content and fast growth rates. This application aligns with the principles of green technology, as biofuels are a renewable and environmentally friendly alternative to fossil fuels.

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APPENDICES

1. PREPARATION OF f/2 MEDIUM (Guillard & Ryther 1962, Guillard 1975)

The seawater was adjusted to the sample salinity and autoclaved. To the autoclaved seawater the stock solutions were added accordingly.

COMPONENT	STOCK SOLUTION
$NaNO_3$	75 g/L dH_2O
$NaHPO_4.H_2O$	5 g/L dH_2O
$Na_2SiO_3.9H2O$	30 g/L dH_2O
Trace metal solution	-
Vitamin solution	-

All the stock components were stored at 4 ℃ except for vitamin stock that is to be stored at -20° C.

1.1.Preparation of Trace Metal solution:

COMPONENT	PRIMARY STOCK SOLUTION
$FeCl_3.6H_2O$	-
$Na_2EDTA.2H_2O$	-
$CuSO_4.5H_2O$	9.8 g/L dH_2O
$Na_2MoO_4.2H_2O$	6.3 g/L dH_2O
$ZnSO_4.7H_2O$	22.0 g/L dH_2O
$CoCl_2.6H_2O$	10.0 g/L dH_2O
$MnCl_2.4H_2O$	180.0 g/L dH_2O

1.2.Preparation of Vitamin solution:

COMPONENT	PRIMARY STOCK SOLUTION
Thiamine HCl (vit. B_1)	-
Biotin (vit. H)	0.1 g/L dH_2O
Cyanocobalamin (vit. B_{12})	1.0 g/L dH_2O

2. DETERMINATION OF DO

I. Manganese (II) chloride (Winkler's A): 60 g of $MnCl_2 .4H_2O$ was dissolved in 100 mL with distilled water.

II. Alkaline iodide (Winkler's B): 60 g of KI and 30 g of KOH were dissolved separately in a minimum amount of water and combined. The solution was made up to 100 mL with distilled water, and stored in an amber-coloured bottle.

III. Sulphuric acid: 50mL of concentrated sulphuric acid was added carefully to 50mL of distilled water and mixed under cold conditions.

IV. Sodium thiosulphate 0.2 mol/L stock : 49.5 g of $Na_2S_2O_3. 5H_2O$ was dissolved and made up to 1 L with distilled water.

V. Sodium thiosulphate, 0.02 mol/L: The 0.02 mol/L working solution is dilution of 1 : 10 of 0.2 mol/l sodium thiosulphate solution.

VI. Starch solution: 1 g of soluble starch was added in l00 mL of distilled water. The solution was heated to boiling point.

3. ESTIMATION OF SILICATES (Grasshoff et al., 1999):

I. Acid molybdate reagent: 38 g of ammonium heptamolybdate tetrahydrate was dissolved in 300 mL of distilled water. The solution was added to 300 mL of sulphuric acid (reagent 1). Stored in ambered coloured bottle.

II. Oxalic acid solution:10g of oxalic acid dihydrate was dissolved in 100 ml of distilled water.

III. Ascorbic acid solution: 2.8g of ascorbic acid was dissolved in 100 mL of pure water & stored in an amber glass bottle at < 8°C.

IV. Standard stock solution: Add 5.188 mg of hexafluorosilicate in 100 mL of distilled water in a plastic beaker. This solution contains 10 mmol/L. The standard is stable for at least one year.

4. ESTIMATION OF PHOSPHATES (Grasshoff et al., 1999):

I. Sulphuric acid (Reagent 1) - 250mL of concentrated sulphuric acid was added to 750 mL of pure water. Allowed to cool and diluted to 1 L. Stored in a poly- ethylene bottle.

II. Phosphate standard solution: 136.09 mg of Potassium dihydrogen phosphate, KH_2PO_4, was dissolved in distilled water to 0.2 mL of sulphuric acid (reagent 1) was added and made up to 100 mL. Stored under cold conditions. This standard stock solution contains 10 mmol/L phosphate.

III. Ascorbic acid solution: 10 g of ascorbic acid was dissolved in 50 mL of distilled water followed by addition of 50 mL of sulphuric acid (reagent 1) and stored in dark in a brown bottle at < 8 °C.

IV. Mixed reagent: 12.5 g of ammonium heptamolybdate tetrahydrate was dissolved in 125 mL of distilled water. 0.5 g of potassium antimony tartrate was dissolved in 20mL of pure water.The molybdate solution was added to 350mL of sulphuric acid (reagent 1) while continuously stirring. Followed by addition of the tartrate solution and mixed well. Stored in a laboratory glass bottle.

5. ESTIMATION OF NITRITE (Grasshoff et al., 1999):

I. Sulphanilamide: 10 g of crystalline sulphanilamide was dissolved in 100 mL of concentrated hydrochloric acid in about 600 mL of distilled water. After cooling, the solution was made up to 1 L with distilled water. Stored in the dark at < 8℃.

II. N-(1 -naphthyl)-ethylenediamine dihydrochloride: 0.5 g of the amine dihydrochloride was dissolved in 500mL of pure water. The solution was stored in a brown bottle at < 8℃.

III. Nitrite standard solution: 0.690 g of Anhydrous sodium nitrite ($NaNO_2$) was dissolved in 1 L of distilled water. This solution contains 10 mmol/L of nitrite and was stored cool and dark place.

6. PREPARATION OF REAGENTS FOR DPPH ASSAY

I. Ascorbic acid stock 1mg/l: 10mg ascorbic acid was weighed and dissolved in ethanol and stored at < 8℃.

II. 0.1Mm DPPH: 0.0078864g of DPPH was weighed and dissolved in 20ml ethanol. The solution was stored in ambered coloured bottle.

7. PREPARATION OF CULTURE MEDIA (himedia, M385-500G, GRM026-500G,M391-500G)

   I. To prepare ZMA: 40.25g of ZMB and 20g of bacteriological agar was added to 1000ml of water. The mixture was gently shake and autoclaved at 121 ℃ 15psi for 20 minutes.

   II. To prepare MHA: 21.0g of MHB was weighed and 20g of bacteriological agar was added to 1000ml of water. The mixture was gently shake and autoclaved at 121 ℃ 15psi for 20 minutes.

8. PREPARATION OF ANTIBIOTICS

10mg/ml stock solutions of Tetracycline, Chloramphenicol & Streptomycin were prepared and filtered sterilized.

9. Nile red solution

0.1mg/ml of Nile red stock solution was prepared and dissolved in acetone.