Effect of Temperature and Nutrients on Physiological Behavior in Fresh & Marine Water *Nostoc* Spp.: A Comparative Study

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DECLARATION

*I, **Ms. Prabha Tiwari**, hereby declare that this thesis represents work which has been carried out by me and that it has not been submitted, either in part or full, to any other University or Institution for the award of any research degree.*

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CERTIFICATE

*I hereby certify that the above declaration of the candidate, **Ms. Prabha Tiwari** is true and the work was carried out under my supervision.*

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Ms. Prabha Tiwari

Dedicated

to

My Family

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ABBREVIATIONS

APC	Allophycocyanin
ARA	acetylene reductase activity
$AlCl_3$	aluminum chloride
ASN III	Artificial Sea Nutrient-III
APX	Ascorbate peroxidase
A_SA	Ascorbate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
$Ca_5(PO_4)_3OH$	Hydroxyapatite
CH_4	Methane
CAT	Catalase
Chl_3	Triplet Chlorophyll state
$(Ca)_3(PO_4)_2$	Calcium phosphate
CO_2	Carbon dioxide
CO	Carbon monoxide
Chl a	Chlorophyll a
COX	Cytochrome c oxidase
Cyt b6f	Cytochrome b_6f
Cys	Cystine
C_2H_2	Ethylene
$CaCl_2$	Calcium chloride
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea
DN	Double the concentration of nitrogen
DNA	Deoxyribonucleic acid
DNPH	2,4-dinitrophenyl hydrazine
DP	Double the concentration of phosphorus
DNP	Double the concentration of nitrogen and phosphorus
ETC	Electron transport chain
ETR	Electron transport rate
$FePO_4$	Iron phosphate
Fe_3O_4 NPs	Iron oxide nanoparticles
F_m	Maximum fluorescence
F_v	Variable fluorescence
F_v/F_m	Maximal quantum yield of PSII photochemistry
Fe-S	Iron-sulfur
FNR	Ferredoxin-$NADP^+$ reductase
FAD	Flavin adenine dinucleotide
FAMEs	Fatty acid methyl esters
GHGs	Greenhouse gases

GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulphide (oxidized)
G3P	Glyceraldehyde 3 phosphate
GC-MS	Gas chromatography- mass spectrophotometery
GAA	Glacial acetic acid
GC	Gas chromatography
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
HSP	Heat shock protein
HPLC	Higher performance liquid chromatography
IPCC	Intergovernmental panel on climate change
LP	Lipid peroxidation
Lys	Lysine
MDA	Malondialdehyde
MDHA	Mono-dehydroascorbate
MDHAR	Mono-dehydroascorbate Reductase
Mo	Molybdenum
MUFA	Monosaturated fatty acids
Met	Methionine
MoFe	Molybdenum-iron
MRP	Mussorie rock phosphate
N_2O	Nitrous oxide
NO_2	Nitrogen dioxide
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NDH-1	NAD(P)H dehydrogenase
NFMC	National Facility for Marine Cyanobacteria
NEM	N-ethylmaleimide
NBT	Nitro blue tetrazolium
N. spongiaeforme	*Nostoc spongiaeforme*
N. calcicola	*Nosotc calcicola*
NIST	National Institute of Standard and Technology
(-)N	Nitrogen starvation
(-)NP	Combined starvation of nitrogen and phosphorus
OEC	Oxygen evolving complex
OH^-	Hydroxyl anions
$OH^{\cdot}$	Hydroxyl radical
$^1O_2$	Singlet oxygen
O_2	Oxygen
$O_2^{\bullet -}$	Superoxide radical
PSI	Photosystem I
PSII	Photosystem II
Pi	Inorganic phosphate
Pht	Phosphate transporters
Pst	Phosphate specific ABC like transporters

PBS	Phycobilisome
3-PGA	3-phosphoglyceric acid
PQ	Plastoquinone
PUFA-OO$^\bullet$	Peroxyl radical
PUFA$^\bullet$	PUFA alkyl radical
PUFA	Polyunsaturated fatty acids
Pro	Proline
PAMS	Pulse amplitude modulating system
PC	Phycocyanin
(-)P	Phosphorus starvation
RNA	Ribonucleic acid
RT-PCR	Real-time polymerization chain reaction
RT	Room temperature
RuBP	Ribulose-1,5-bisphosphate
RuBisCO	RuBP carboxylase/oxygenase
ROS	Reactive oxygen species
SEM	Scanning electron microscope
SOD	Superoxide dismutase
SDH	Succinate dehydrogenase
SFA	Saturated fatty acids
TBA	Thiobarbituric acid
TPC	Total phenolic content
TFC	Total flavonoid contents
TAG	Triacyl glycerides
TCA	Trichloroacetic acid
Thr	Threonine
TCP	Tricalcium phosphate
UFA	Unsaturated fatty acid
VOCs	Volatile organic compounds

ABSTRACT

The burgeoning human population has increased the consumption of fossil fuels and excessive emission of GHGs resulting in escalating global temperature. The intergovernmental panel on climate change, 2021, has suggested that by 2100, the Earth's temperature is anticipated to increase by 2-4ºC, and the sea level would rise between 0.3 m to 0.6 m. The growing human population's food requirement has increased deforestation and, consequently, polluting fresh and marine waterbodies due to runoff from point and non-point sources, which may alter the nutrient concentration of these aquatic bodies. These factors, increasing global temperature, and nutrient alterations in waterbodies affect various ecosystems, including agriculture and fisheries. There have been several studies to ascertain the effect of these two factors on crop productivity; however, very few studies have been on the productivity of aquatic ecosystems (fresh and marine). Cyanobacteria are multi-functional bio-agents for eco-friendly agriculture and environmental sustainability as they enrich the soil with carbon and nitrogen, enhance the bioavailability of phosphorus, excellent accumulators and degraders of environmental pollutants, and capture & store CO_2 mitigating global warming. Cyanobacteria are one of the prokaryotic photosynthetic organisms that are severely affected by climate change. In the present thesis, we attempt to study the effect of a range of temperatures and nutrients, specifically phosphorus and nitrogen content, in the growth medium in a freshwater, *N. spongiaeforme,* and a marine, *N. calcicola,* both nitrogen-fixing gram-negative cyanobacteria on morphological, physiological, biochemical, and molecular parameters for better understanding of their behavior under changing temperature and nutrient regime. Cultures of *N. spongiaeforme* and *N. calcicola* were grown with their respective culture media at growth temperatures ranging from 10-40ºC and with different compositions of N and P along with their respective controls (cultures grown under normal nutrient strength at 30ºC). Our study observed that at the optimum temperature of 30ºC and under DP, DN, and (-)N, both the *Nostoc* species maintained their optimum growth rate, morphological structure, and other physiological parameters. While suboptimal and supra-optimal temperatures, (-)P and (-)NP caused a decline in the growth and biomass as *Nostoc* species morphologically suffered severe distortion, which resulted in a significant decline in the photosynthetic

pigment content, lower photochemical efficiency of PSII (Fv/Fm ratio), oxygen evolution and oxygen consumption in both the *Nostoc* species. *Nostoc* species grown at suboptimal and supra-optimal temperatures, (-)P and (-)NP, generated excess ROS, resulting in oxidative damage indicated by lipid peroxidation and protein oxidation. A higher generation of ROS in both the *Nostoc* species was responded to by increased activity and expression of enzymatic antioxidants (SOD and CAT). Non-enzymatic antioxidants such as ascorbate and proline also showed similar results in combating ROS. Our study also showed higher expression of HSP in *N. calcicola* at 40ºC, allowing it to adapt better than *N. spongiaeforme*. Temperature and nutrient variations also affected the phenolic compounds content in both the *Nostoc* species. Nitrogenase activity of both the *Nostoc* spp., was decreased at sub and supra-optimal temperatures but increased when grown under N-deficient conditions, both being diazotrophic species. No increase in nitrogenase was seen in either species when grown with (-)P, (-)NP, or DP, or DN. The expression of *nif H* gene also corroborated the nitrogenase activity pattern. GC-MS analysis showed qualitative and quantitative variation in the fatty acid composition of both the *Nostoc* species under sub and supra-optimal temperature and due to variations in the nutrients. Temperature affected the shift in the phase transition, but no such observation in phase transition was seen with nutrients. The observations suggest an influence of both these factors on cell membrane stability, probably with regard to sol-gel status, specifically with temperatures for optimal functions like photosynthesis, ion permeability, and respiration. Our study concludes that among the two *Nostoc* species studied, *N. calcicola* showed better growth at a high temperature of 40ºC. Suboptimal temperature, however, equally affected the growth of both the *Nostoc* species. Nutrient starvation, mainly (-)P affected the physiological and biochemical parameters of both the *Nostoc* species but starvation of nitrogen alone did not cause any deleterious effect on any of the studied parameters except for higher population of heterocyst, increased activity of nitrogenase and expression level of *nif H* gene.

1. Cyanobacteria

The name cyanobacteria originated from their color (Greek word "kuanos," meaning blue) due to the presence of phycobilin pigments, phycocyanin, and thus are commonly known as blue-green algae. It belongs to the bacterial Kingdom Monera (gram-negative), Division Eubacteria, and Class Cyanobacteria. Cyanobacteria evolved during the era of precambrian around 3.5 billion years ago (Schopf & Packer, 1987) and are characterized as an oxygenic photosynthetic organism that possesses pigments such as chlorophyll a, phycobiliprotein (phycocyanin, allophycocyanin, and phycoerythrin), and carotenoid. Cyanobacteria are thought to be the photosynthetic ancestors of plastids in eukaryotic algae and plants, which were formed by the endosymbiosis of a phototrophic prokaryotic cell. Among all the photosynthetic prokaryotic organisms, cyanobacteria display the utmost diverse and intricate morphologies, such as unicellular, colonial, unbranched, and branched filamentous forms with or without heterocysts. Its cell size range between 1 mm for unicellular and over 30 mm for multicellular species, which is larger than most bacteria.

The cyanobacteria cells are enclosed within a cell wall (containing an outer peptidoglycan layer surrounded by a mucilaginous sheath), cell membrane (which separates the cytoplasm from periplasm), thylakoids membrane, carboxysomes, ribosomes, and nucleoids. Unlike plants, where respiration and photosynthesis occur in separate organelles, i.e., mitochondria and chloroplast, cyanobacteria share its thylakoid membrane for photosynthetic and respiratory redox-active protein complexes and do not form grana as it does in algae and plants. Moreover, among cyanobacteria, there is a genus *Gloeobacter* that lacks thylakoids and photosynthetic pigments of which are associated with cytoplasmic membrane (Nakamura et al., 2003) (Fig. 1.1).

Cyanobacteria are ubiquitous autotrophs with numerous traits, such as fixing atmospheric nitrogen, a fundamental metabolic process of cyanobacteria, giving them the simplest nutritional requirements among all living organisms. They use the enzyme nitrogenase complex to convert N_2 into NH_4 (a form through which nitrogen enters the food chain) (e.g., *Anabaena*, *Nostoc*) (Veaudor et al., 2020). In addition to nitrogen fixation, cyanobacteria have the capability to sequestrate extra phosphorus intracellularly (Isvánovics et al., 2000). Along with a high affinity for nitrogen and phosphorus,

cyanobacteria also have high pH optima (Dokulil & Teubner, 2000). In addition, they produce gas vesicles, which are cytoplasmic inclusions with gas-filled and cylindrical structures that regulate buoyancy and enable cyanobacteria to adjust their vertical position in the water column (Walsby, 1987).

Another trait of cyanobacteria is the formation of akinetes, a thick-walled, nonmotile cells that differentiate from vegetative cells of cyanobacteria under unfavorable conditions and serve a perennating role as they preserve the ability to recommence morphology and functions of vegetative cells after a long period of dormancy (Sukenik et al., 2019). Cyanobacteria also produce a variety of secondary metabolites, and some have been identified as potent cyanotoxins, such as hepatotoxins, cytotoxins, neurotoxins, dermatoxins, and irritant toxins (Wiegand & Pflugmacher, 2005) in the aquatic bodies (Gobler et al., 2007). All these characteristics have enabled cyanobacteria to colonize various niches in the aquatic and terrestrial ecosystem, demonstrating their pioneering ancestral capacities as the earliest earth inhabitants (Moreira et al., 2013).

Cyanobacteria are also often associated with other organisms, forming microbial mats, benthic communities, and biofilms. Sometimes these associations are predominant and only life forms in specific extreme habitats. In addition to surviving under different abiotic stresses, cyanobacteria have also attracted attention towards their wide range of applications in various sectors.

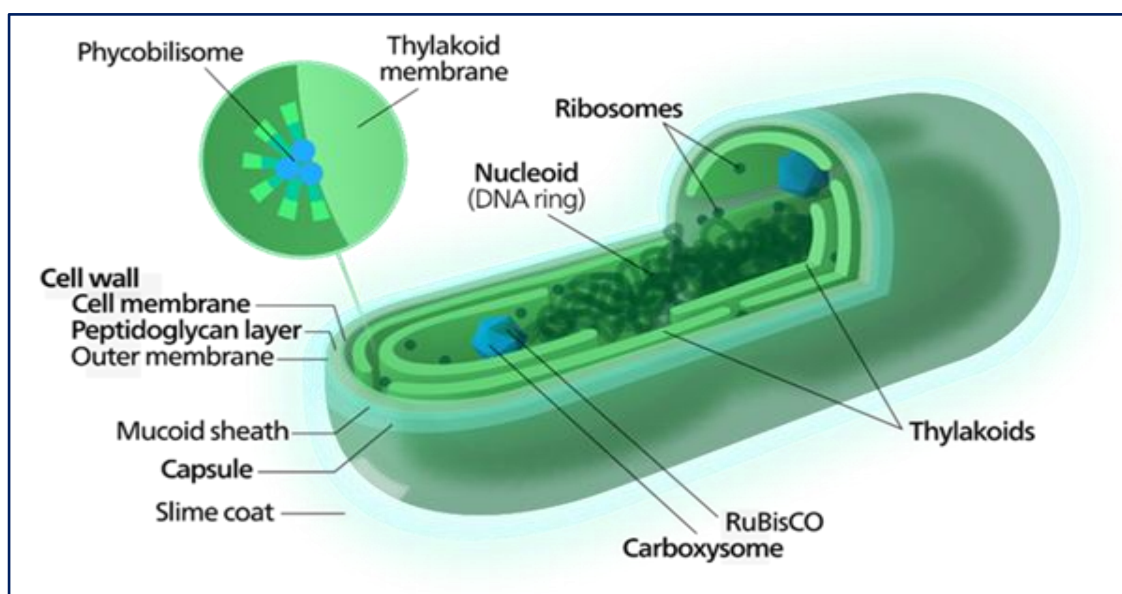


Fig. 1.1 Internal structure of cyanobacteria cell (adopted from shorturl.at/npJ37).

1.2 Application of cyanobacteria

Cyanobacteria, one of the largest and multipurpose groups of photosynthetic prokaryotes, are known for enormous applications and have drawn huge attention in the last few decades.

Carbon-dioxide sequestration and biofertilizer: Cyanobacteria have a 10-50 times faster carbon dioxide fixation rate than terrestrial plants, and 20-30% of global primary photosynthetic productivity originates from them. This corresponds to the yearly fixation of about 20-30 Gt of CO_2 into biomass which can be used for several purposes like, biofertilizer and biofuel production, and the release of about 50-80 Gt of O_2 in the atmosphere (Veaudor et al., 2020). Hence, cyanobacteria are considered an effective approach to cutting down the concentration of atmospheric CO_2, thereby helping mitigate global warming. In addition, many cyanobacteria can fix atmospheric N_2 using heterocysts (modified thick-walled cells), a site for enzyme complex nitrogenase which catalyze the conversion of biological N_2 into a reduced form of ammonia and released into the soil either by secretion or by microbial degradation after their death. Apart from heterocystous cyanobacteria, there are several unicellular and filamentous non-heterocystous cyanobacteria that are responsible for fixing atmospheric nitrogen. They contribute 20-30 kg N ha^{-1} and organic matter to the soil and provide great benefits over chemical nitrogen fertilizers (Issa et al., 2014). Some examples of cyanobacteria that are used as effective biofertilizers are *Anabaena variabilis*, *Nostoc muscorum*, *Aulosira fertissima*, and *Tolypothrix tenuis*. Along with nitrogen fixation, they can improve the bioavailability of phosphorus in the plants by solubilizing and mobilizing the insoluble organic phosphates present in the soil with the help of phosphatase enzymes. They can also solubilize the insoluble form of $(Ca)_3(PO_4)_2$, $FePO_4$; $AlPO_4$, and hydroxyapatite $[Ca_5(PO_4)_3OH]$ in soils and sediments (Wolf et al., 1985; Cameron & Julian, 1988). Once an inorganic phosphate is solubilized, the resulting PO_4^{3-} is scavenged by the growing population of cyanobacteria for their own nutrition needs, and after their death, the cell-locked PO_4^{3-} is released in the soils and easily available to plants and other organisms following mineralization (Mandal et al., 1992). Hence, improve soil quality by enhancing nutrient solubilization, mobility,

soil aggregation, and water permeability, thus, improving the overall soil physio-chemical properties and crop production.

Reclamation of salt-affected lands: Cyanobacteria play a potential role in the reclamation of salt-affected, commonly known as Usar land soil, arid or sub-arid soils. Basically, salt-affected soils are less productive and impervious to water due to the excessive salts in the upper layers. Depending on the salt content salt-affected soil can be classified as alkaline or saline. The alkaline soil is categorized by a high pH, measurable amounts of carbonates, and high exchangeable Na^+, and it undergoes extensive clay dispersion. The poor hydraulic conductivity and reduced soil aeration make the soils infertile. While saline soil is characterized by a high amount of soluble salts, imparting high osmotic tension to plant roots for absorption of nutrients and water (Pandey et al., 1992) and for improvement of these salt-affected soil, chemical methods of using gypsum, sulfur, or excessive irrigation are applied (Dhar & Mukherji, 1936), which are not so cost-effective or eco-friendly. Therefore, the reclamation of these lands can be done using cyanobacteria which form a thick stratum on the soil surface, binds the soil particles and help to conserve the organic C, P, N, and moisture and convert the Na^+ clay to Ca^{2+} clay and this method is cost-effective as well as eco-friendly. The addition of N and organic matter by the cyanobacteria in such soil aid in the binding of the soil particles and hence, improve soil aeration and permeability (Singh, 1961; Nisha et al; 2007). Subsequently, they solubilize nutrients from insoluble carbonate nodules through the secretion of oxalic acid (Singh, 1961; Fritsch, 1945); cyanobacteria improve the physico-chemical quality of alkali and saline soils such as soil aggregation by lowering the pH, hydraulic conductivity and electrical conductivity (Kaushik & Subhashini, 1985). There are a few physiological advantages associated with cyanobacteria that enable them to endure these stresses: (a) accumulation of inorganic (K^+ ion) or organic osmoregulators (b) curtailment of Na^+ influx (Apte et al., 1987; Reed et al., 1984). Cyanobacterial species such as *Anabaena oscillarioides*, *Anabaena aphanizomenoides*, and *Microcystis aeruginosa* displayed 7-15 g L^{-1} salt tolerance ability (Coutinho & Seeliger, 1984; Moisander et al., 2002).

Bio-control agents: Cyanobacteria are also used as bio-control agents producing a wide array of biologically active compounds of antibacterial, antifungal, and antiviral potential (Teuscher et al., 1992; Dahms et al., 2006). These compounds belong to the

group of amides, alkaloids, polyketides, lipopeptides, fatty acids, and indoles (Abarzua et al., 1999; Burja et al., 2001). In addition, they also produce anti-algal compounds that constrain the growth of pathogens by disrupting their physiological and metabolic activities (Dahms et al., 2006). The cell extract of cyanobacteria is known to reduce the occurrence of *Erysiphe polygoni* producing powdery mildew on turnips*, Botryti scinerea* on strawberries, and damping-off disease in tomato seedlings (Kulik, 1995). *Nostoc muscorum* produces antifungal compounds against soil fungi, especially those causing damping-off (De Caire et al., 1990). *Nostoc* sp., is a known potential cryptophycin producer, a source of natural pesticides against fungi, insects, and nematodes (Biondi et al., 2004). *Nostoc muscorum* also inhibits the growth of other fungi producing the "wood blue stain," a grayish or bluish discoloration of the sapwood caused by certain dark-color fungi (*Alternaria*, *Aureobasidium*, *Cladosporium*, etc.) on the surface and inside of the wood (Zulpa et al., 2003).

Plant growth promoters: Cyanobacteria provide a wide scope for commercial application as plant growth promoters due to their simple nutritional requirements, mainly water, sunlight, and CO_2, and fast cell growth (Ruffing, 2011). They produce extracellular growth-promoting substances such as hormones like auxin (*Anabaenopsis* and *Anabaena*) (Ahmad & Winter, 1968), *Glactothece and Cylindrospermum* (Selykh & Semenova, 2000), *Nostoc* and *Plactonema* (Sergeeva et al., 2002); gibberellins (*Cylindromum* and *Anabaenopsis*) (Singh & Trehan, 1973); cytokinin (*Anabaena*, *Chlorogloeopsis*, and *Calothrix*) (Rodgers et al., 1979; Selykh & Semenova, 2000); abscisic acids (Marsalek et al., 1992); amino acid (Vorontsova et al., 1988); vitamin B (Grieco & Desrochers, 1978); and antibiotics (Marsalek et al., 1992; Selykh & Semenova, 2000). Co-inoculation of cyanobacteria with wheat enhances the root dry weight and chlorophyll content (Obreht et al., 1993).

Bioremediation: Bioremediation is the treatment of contaminated water, soil, and subsurface material, by using microorganisms that degrade the target pollutants. One of the emerging bio-remediator is cyanobacteria. Cyanobacteria have several advantages over other microorganisms due to their photosynthetic and nitrogen-fixing nature, which makes them self-sufficient for growth, maintenance, and adaptability to endure in polluted environments (Sorkhoh et al., 1992). Due to the high multiplication rate and metal sorption

capacity, cyanobacteria play a vital role in detoxifying numerous industrial effluents from breweries and distilleries, sugar mills, oil refineries, paper mills, dye, and pharmaceutical industries. Cyanobacteria also help in mitigating metal toxicity and eutrophication problem as they are used for tertiary treatment of urban, agro-industrial effluents (Vílchez et al., 1997). *Synechococcu selongatus*, *Anacystis nidulans,* and *Microcystis aeruginosa* degrade many organo-chlorine and organo-phosphorus insecticides from polluted aquatic systems (Vijayakumar, 2012). *Lyngbya* sp., *Microcystis* sp., *Anabaena* sp., and *Nostoc* sp., degrade organo-phosphorous herbicide glyphosate, and the mineralized glyphosate is used as a phosphorus source (Forlani et al., 2008).

Source of bio-energy: The simple cell structure and minimum requirement of nutrients make cyanobacteria a unique group of photosynthetic bio-agents and have the capacity to produce bio-energy, including bio-diesel, bio-hydrogen, and bio- or syngas (Kumar & Singh, 2016). Cyanobacteria convert the carbon dioxide (CO_2) taken through photosynthesis to carbon-rich lipids that can be used to produce biofuels. Cyanobacteria such as *Calothrix*, *Oscillatoria*, *Anabaena*, *Nostoc*, *Cyanothece*, *Synechococcus*, *Gloeobacter*, *Microcystis*, *Aphanocapsa*, *Microcoleus*, and *Chroococcidiopsis*, also produce hydrogen (Parmar et al., 2011; Masukawa et al., 2001; Nozzi et al., 2013). Cyanobacteria biomass can also be used for the production of biogas via fermentation or anaerobic digestion. In the cyanobacterial biomass, the organic biopolymers are hydrolyzed, broken down into monomers, and subjected to aerobic digestion to yield biogas (a mixture of methane and carbon dioxide). During biogas production, carbon dioxide is the second principal component (approximately 25-50%) and can be removed to obtain bio-methane (Hankamer et al., 2007). Bio-methane can be used as compressed natural gas in automobiles, which will be more eco-friendly than the fossil fuels like gasoline/petrol and diesel (Hankamer et al., 2007).

Food supplements: Cyanobacteria such as *Anabaena*, *Spirulina*, and *Nostoc* are consumed as food in many countries, including Mexico, Peru, Chile, and the Philippines. Cyanobacteria are used as food supplements in different forms, such as capsules, tablets, and liquid (Radmer, 1996), and can act as a nutritional supplement or signify a source of natural food colorants (Branen et al., 2002; Muller-Feuga, 2000; Becker, 2004; Bhaskar et al., 2005). *Spirulina* (*Arthrospira*) is the most common cyanobacterial strain commercially

used for human nutrition. It contains more than 60% proteins, rich in thiamine, beta-carotene, and riboflavin, and is the richest source of vitamin B12 (Bohm et al., 1995; Rimbau et al., 2001; Kedar et al., 2002; Benedetti et al., 2004; Romay et al., 2003 and Subhashini et al., 2004).

Thus, cyanobacteria are multi-functional bio-agents for safe and eco-friendly agriculture and environmental sustainability, used as food supplements and enrich the soil with organic carbon and nitrogen, enhancing the bioavailability of phosphorus to the plants. They are excellent accumulators and degraders of various environmental pollutants, capture, and store CO_2, thus mitigating climate change.

1.3 Temperature

The natural greenhouse gases (CO_2, CH_4, and water vapor) maintain the Earth's temperature for the physiological and biochemical functions of living organisms. The burgeoning temperature of the earth, due to the emission of greenhouses gases (GHGs) such as carbon dioxide (CO_2), nitrous oxide (N_2O), methane (CH_4), fluorinated (F) compounds, tropospheric ozone, has resulted in significant changes in the biotic communities, including a reduction in biodiversity both in terrestrial and aquatic ecosystems. The emission of GHGs has caused continuous heating of the global climate causing global warming, first noticed during the Industrial Revolution between 1850 and 1900. The increase in GHGs is due to an increase in the consumption of fossil fuels (coal and oil) as a result of the expansion of industries, transportation, agriculture, and the global population causing great risk to the environment (Fig. 1.3A). Fossil fuels are known to emit GHGs like CO_2, which contributes to approximately 65% of global warming. CH_4 generated due to the production and transport of coal, oil, natural gas, livestock rearing, sewage treatment, and agriculture practices contribute another 16% to global warming. Fluorinated (F) compounds such as perfluorocarbons, hydrofluorocarbons, nitrogen trifluoride, and sulfur hexafluoride are produced through refrigeration, air conditioning, medicine, and electronic equipment, produced in smaller quantities and are known as "high global warming potential gases" contribute additional 2% to global warming. Tropospheric ozone, produced as a result of the reaction between nitrogen dioxide (NO_2), carbon monoxide (CO), and volatile organic compounds (VOCs), contributes to a further 11% of

global warming. In addition, nitrous oxide (N_2O) added due to agricultural activities, sewage treatment, and chemical production contributes 6% of global warming, which traps and emits heat in the thermal infrared range (Fig. 1.3B).

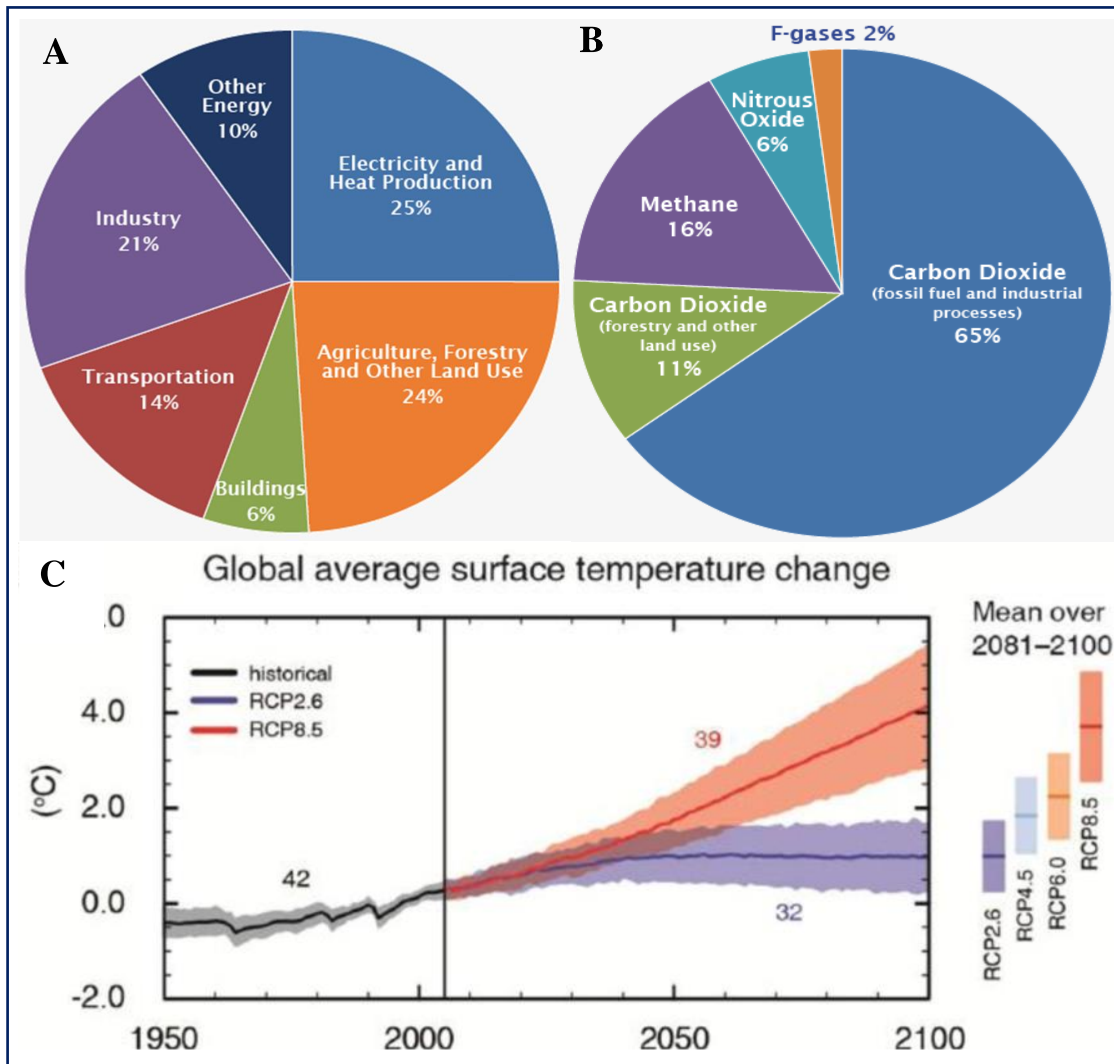


Fig. 1.3 Emission of greenhouse gases by different sectors (A), Overview of greenhouse gases emission (B) (Adopted from https://tinyurl.com/yckk4abb), Global average surface temperature change (C) (Source: IPCC 2013).

The present increase in GHGs has altered Earth's climate faster than at any time in the history of modern civilization. This drastic climate change has caused an overall

change in the Earth's atmosphere, not only in thermal conditions but also in micro and macro-environment that influence all organisms living on land and aquatic ecosystems (Hoegh-Guldberg & Bruno, 2010; Walther et al., 2002). The intergovernmental panel on climate change, 2021, reported that by 2100, the Earth's temperature is anticipated to increase by 2-4ºC, and the sea level would rise between 0.3 m to 0.6 m (Fig. 1.3C). The upsurge in sea level due to the melting of glaciers and shrinkage of ice caps has altered or destroyed the coastal wetlands to a significant level, which will further cause shifts in geographical distribution, development, reproduction, and behavior of aquatic plants (Thackeray et al., 2010; Scheffers et al., 2016; Hoegh-Guldberg & Bruno, 2010). Global warming has caused an increase in the sea surface temperature, ocean acidification, and a decrease in oxygen concentration (Bindoff et al., 2019; Schmidtko et al., 2017; Doney et al., 2016). Along with the marine ecosystem, global warming has also affected the freshwater ecosystems causing larger lakes to warm up at an average rate of 0.045 ± 0.011ºC yr^{-1} and small lakes at an average of 0.34ºC $decade^{-1}$ from 1985-2009 (Sharma et al., 2015; Schneider & Hook, 2010).

Global warming also results in an invasion of salt water into the freshwater system leading to the relocation or demise of some key species of that habitat and alteration in the food web. Along with aquatic and wildlife, the rise in temperature has also affected agriculture by changing the optimal temperature and rainfall (Rosenzweig & Parry, 1994).

1.4 Nutrients

Nutrients are chemicals required by organisms for growth and development. They are classified into two groups, i.e., macronutrients (N, K, Ca, Mg, P, and S) and micronutrients (Cl, Fe, B, Mn, Zn, Cu, Mo, and Ni). An adequate amount of macronutrients and micronutrients are needed for the function of healthy terrestrial and aquatic ecosystems. However, as a consequence of global warming, climate change has affected the concentration of these nutrients in the soil and aquatic bodies. Clearing lands for agriculture, animal husbandry, and urbanization have amplified the impervious surface area, resulting in an increased runoff in addition to excess rainfall, affecting the nutrient concentration of waterbodies (Chiew & McMahon, 2002).

Runoff contains various nutrients, which come from point-source pollution and nonpoint-source pollution. A point source is any source that drains directly into a waterway, such as oil refineries, paper mills, municipal wastewater treatment plants, and various industries. A nonpoint source is any source where runoff does not empty directly into a waterway, such as fertilizer from various farm lands, uses of detergents, oil tank spills, or runoff from residential or agricultural areas. The various nutrients added to the waterbodies are chlorides, sodium, iron, lead, cadmium, zinc, sulfur, chlorine, nickel, chromium, bromine, aluminum, nitrogen, and phosphorus (Liu et al., 2020). As these sources drain the effluents in the waterbodies, nutrient concentrations near the mouth of different waterbodies are much higher than the open waterbodies, which display far less amount of nutrient content (Jankowiak et al., 2019). Among these nutrients, phosphorus and nitrogen are the primary nutrients that affect the survival of organisms.

1.4.1 Phosphorus (P) is an essential mineral nutrient and is taken as a phosphate ion by organisms for their growth and development. It is an essential element required for the synthesis of genetic material, proteins, energy molecules, and cell membranes. It also plays a vital role in photosynthesis, respiration, and nutrient transport.

DNA and RNA are made up of a long chain of nucleotides, each consisting of nitrogenous base, ribose sugar, and phosphate group. RNA is, in turn, used to synthesize proteins and enzymes, and the crucial energy molecule ATP consists of an adenine rest attached to the sugar ribose and three phosphate groups. Phosphorus is also an important part of the cell membrane. Phospholipids, a key component of all cell membranes made up of two fatty acids and one phosphate group, which maintains the integrity of cell membranes. Omega-3 fatty acids are incorporated into phosphorus compounds to form cell membrane structures required for allocating nutrients into the cells and moving waste products out of the cells (Fig. 1.4.1A).

The phosphorus movement through the lithosphere, hydrosphere, and biosphere has four major components, tectonic uplift and weathering of phosphorus-bearing rocks (Buendía et al., 2010), physical erosion, chemical (oxidation), and biological weathering (Buendía et al., 2010), riverine and subsurface runoffs to the ocean, and sedimentation of

phosphorus associated with organic matter and oxide or carbonate minerals in lakes, rivers and sea (Ruttenberg, 2003) (Fig. 1.4.1B).

River runoff of soil phosphorus buried in lake sediments and atmospheric phosphorus deposition is a major contributor to water bodies. In addition, dissolved inorganic phosphorus, mainly orthophosphate (PO_4^{3-}), assimilated by algae and transformed into organic phosphorus compounds, also contributes to surface seawater (Paytan & McLaughlin, 2007).

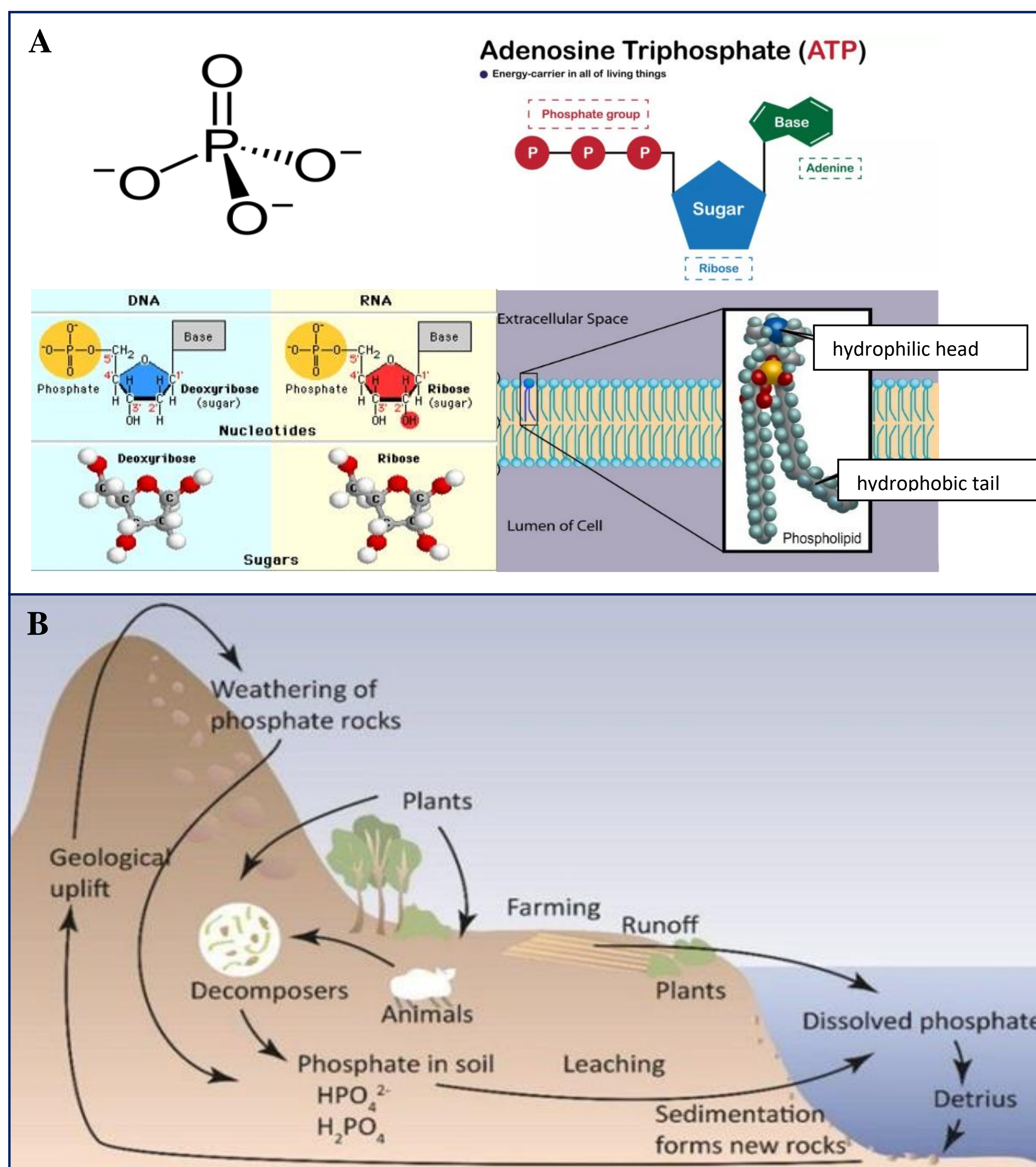


Fig. 1.4.1 Diagrammatic representation of phosphate group in nucleic acid, ATP, and lipid membrane(A)(https://tinyurl.com/2p9xpncz;https://tinyurl.com/53yrsfdf;https://tinyurl.com/errz75nz https://tinyurl.com/yzrv5cfs); Phosphorus cycle (B) (adopted from encyclopedia of ocean sciences)

Remineralization and sedimentation lock the phosphate in waterbodies, making it unavailable to flora. A series of diagenetic processes such as microbial respiration of organic matter in sediments, microbial reduction and dissolution of iron and manganese oxides, abiotic reduction of iron oxides by hydrogen sulfide, and discharge of iron-associated phosphorus, phosphate associated with calcium carbonate and, the transformation of iron oxide-bound phosphorus to vivianite play key roles in this process of phosphorus sedimentation in the marine environment.

Factors influencing the phosphorus content in soil and waterbodies

The healthy and productive soil contains phosphorus concentrations between 20-100 mg kg^{-1}. Its availability in the soil gets influenced by various factors such as pH, compaction, aeration, moisture, temperature, cation exchange capacity, and organic matter. Aeration reduces the oxygen flow to plant roots which reduces the uptake of phosphorus by 50%. In the water bodies, the natural levels of phosphate range from 0.005 to 0.05 mg L^{-1} (Kotoski, 2010) and are influenced by three pathways: Surface runoff, leaching, and sub-surface flow.

Surface runoff: Water-assisted downhill transport process that causes phosphorus to move with water either in dissolved or particulate form from the top two inches of soil. The amount of phosphorus transported through runoff gets affected by vegetation and land cover as well as the intensity and distribution of rainfall.

Leaching: Downward movement of water along with a soil profile. Percolating water transfers the dissolved phosphorus deeper into the soil profile. Continuous addition of phosphorus can decrease the retention capacity of lower soil horizons for phosphorus, which causes the phosphorus to leach out into the groundwater.

Sub-surface: Phosphorus loss happens when phosphorus dissolved in percolating water travels horizontally beneath the soil surface but above the water table through lateral flow processes.

Uptake of phosphorus in plants and cyanobacteria

Phosphorus, in an adequate amount, plays an important role in maintaining the health of plants and cyanobacteria. Its deficiency and excess both affect their growth and development. Plants suffer from phosphorus deficiency when grown in compacted, poorly aerated soil that is low in organic matter, causing the falling of premature leaves and fruits, stunted plant growth, and prolonged seed dormancy. Similarly, cyanobacteria growth, photosynthesis, chlorophyll pigments, nitrogen fixation, and cell membrane are also compromised under phosphorus deficiency (Collen et al., 2004). The excess phosphorus reduces the plant's ability to take up micronutrients, particularly iron and zinc, which causes poor growth or death of plants. However, the growth of diazotrophic and non-diazotrophic cyanobacteria are favored in the presence of excessive phosphorus in the waterbodies (Silveira & Odebrecht, 2021). As plant growth is limited by the availability of inorganic phosphate (Pi), they have evolved a range of adaptive strategies that increase the acquisition of Pi and improve the efficiency of internal utilization of Pi (Smith et al. 2000). Amongst these adaptations is the formation of cluster roots by a few species of plants. These combine a high density of determinate lateral roots in a localized soil volume for gathering Pi with a synchronized release of organic acids and acid phosphatases that increase the availability of Pi in the vicinity of these roots (Dinkelaker et al., 1995). Apart from the morphological adaptation strategy, specific transporter systems are also employed by plants for the uptake of Pi and its internal redistribution. The Pht1 family of plant phosphate transporters transport Pi from the soil solution into the plant's root. Once the Pi enters the root, its internal cycling is done through Pht2:1 transporter, which is primely expressed in shoot tissues.

Under excess phosphorus conditions, phosphorus absorbed by the root is transported in the xylem to younger leaves. In Pi-deficient plants, the restricted supply of Pi to the shoots from the roots via the xylem is supplemented by increased mobilization of stored phosphorus in the older leaves and translocation to both the younger leaves and

growing roots. However, cyanobacteria cells under excess phosphorus concentration, employs polyphosphate synthetase for the formation of polyphosphate using ATP (Rai & Sharma, 2006). Under phosphorus deficiency, along with accessing the stored intracellular polyphosphate bodies, cyanobacteria increase the phosphorus ion uptake rate and synthesize extracellular phosphatases for scavenging phosphorus ions from various substrates present in the surroundings to cope with this stress. During this, clusters of genes involved in phosphate metabolism are regulated through a two-component signal transduction system known as pho-regulon. Pst system (phosphate-specific ABC-like transporters) and phosphatases, both play an important role in the transport and release of phosphorus from complex organic compounds (Suzuki et al., 2004).

The sensing of phosphate availability in the external medium and response of the cyanobacterial cell is regulated by a two-component regulatory system SphS-SphR. SphS, a histidine kinase, senses the inorganic phosphate level in the environment, mediated by the Pst transport system and SphU. SphR, a response regulator, regulates the expression of genes involved in the assimilation of complex organic forms of phosphorus (Hirani et al., 2001).

1.4.2 Nitrogen is an essential nutrient required by organisms for their growth and development, which is a vital component of catalysts and intermediates of primary metabolism. It is found in nucleic acids, amino acids, proteins, chlorophylls, and phycobiliproteins (Andrews et al., 2013). It plays an essential role in all structural and functions of plants, and it accounts for 7-10% of cell dry weight (Hu, 2004). In the atmosphere, nitrogen occurs as dinitrogen (N_2), in which the two nitrogen are bonded together by triple covalent bonds which is fixed into the soil by physical and biological processes and making it available for the plants and other organisms.

Factors influencing the nitrogen content in soil and waterbodies

The amount of nitrogen requires to maintain a healthy soil range from 25-125 mg kg^{-1}. Its availability in the soil gets influenced by drainage, soil texture, moisture, soil aeration, salt content, slope steepness, rainfall, and temperature. In the waterbodies, nitrogen concentration varies from 1-2 mg L^{-1}. However, this is influenced by various

factors, such as leaching, runoff from various points, nonpoint sources, and groundwater baseflow discharge.

Leaching: Loss of soluble nitrate as it moves with soil water below the root zone and enters groundwater or surface water through tile drainage system. Coarse textured soils have a lower water-holding capacity and have more potential to lose nitrate from than fine textured soils.

Precipitation: The leaching loss of nitrate is related with precipitation, and more precipitation leads to large leaching loss. As the precipitation varies with the seasons to a large extent, the total concentration of nitrogen in the infiltrated water also changes accordingly. After the rainy season, the concentration of nitrate in groundwater rises sharply, even reaching the highest value. Precipitation and its intensity are the two main reasons that determine the leaching and downward migration of nitrate in the soil (Tong et al., 2005).

Deep groundwater: Nitrate in groundwater mainly comes from surface nitrogen pollution sources and enters shallow groundwater through vadose zone with rainfall or irrigation. Nitrogenous compounds will undergo a series of complex physical, biological and chemical reactions during the infiltration process, and finally exists in groundwater with the form of salt nitrogen. The variation of nitrate in groundwater has a certain correlation with the depth of regional groundwater level as the concentration of nitrate in groundwater decreases significantly as the depth of groundwater increases (Jing et al., 2013).

Sewage irrigation: Sewage discharge can increase groundwater nitrate pollution (Jing & wang, 2021). In this process, the pollutant sources carrying nitrate mostly from livestock manure, industrial residues and domestic sewage. Healthy balance of water bodies and the natural environment is threatened by the dry and wet sedimentation, leaching infiltration, and discharge. The drip irrigation method makes it easier for a large amount of nitrate to enter the deep soil, thereby polluting the groundwater body.

Uptake of nitrogen in plants and cyanobacteria

Nitrogen plays an important role in maintaining the physiological processes of plants and cyanobacteria. Its deficiency and excess affect their physiological and

biochemical processes. Nitrogen deficiency impacts chlorophyll, and photosynthesis causing the yellowing of older leaves, eventually wilting or death of leaves, and stunted plant growth. Among cyanobacteria, non-nitrogen fixing cyanobacteria get affected due to nitrogen limitation, causing a loss in pigments, lipids, and protein synthesis. Excess nitrogen causes plants to be too leafy, lose or no flowering and fruiting, dehydrate the roots, and stunt root growth. It also blocks the absorption of other nutrients. Whereas nitrogen increases the growth of cyanobacteria and reduces the heterocysts formation in nitrogen-fixing cyanobacteria.

The atmospheric nitrogen gets converted through the physical and biological process into other forms making it obtainable for absorption by plants and other organisms. Among these processes, biological nitrogen fixation is done by bacteria and cyanobacteria with the help of the nitrogenase protein complex. Nitrogen sources used by cyanobacteria and plants are in the form of atmospheric N_2, nitrate, nitrite, ammonium, urea, arginine or glutamine, and organic nitrogen, respectively (Fig. 1.4.2A). In cyanobacteria, the uptake of nitrate, nitrite, urea, arginine, and glutamine involves a multicomponent ABC (ATP-binding cassette)-type and ABC-type permeases uptake transporters, respectively (Valladares et al., 2002). Intracellular nitrate is consecutively reduced to nitrite and ammonium by nitrate reductase and nitrite reductase and incorporated into carbon skeletons through the glutamine synthetase–glutamate synthase pathway (Fig. 1.4.2B) (Flores & Herrero, 2005).

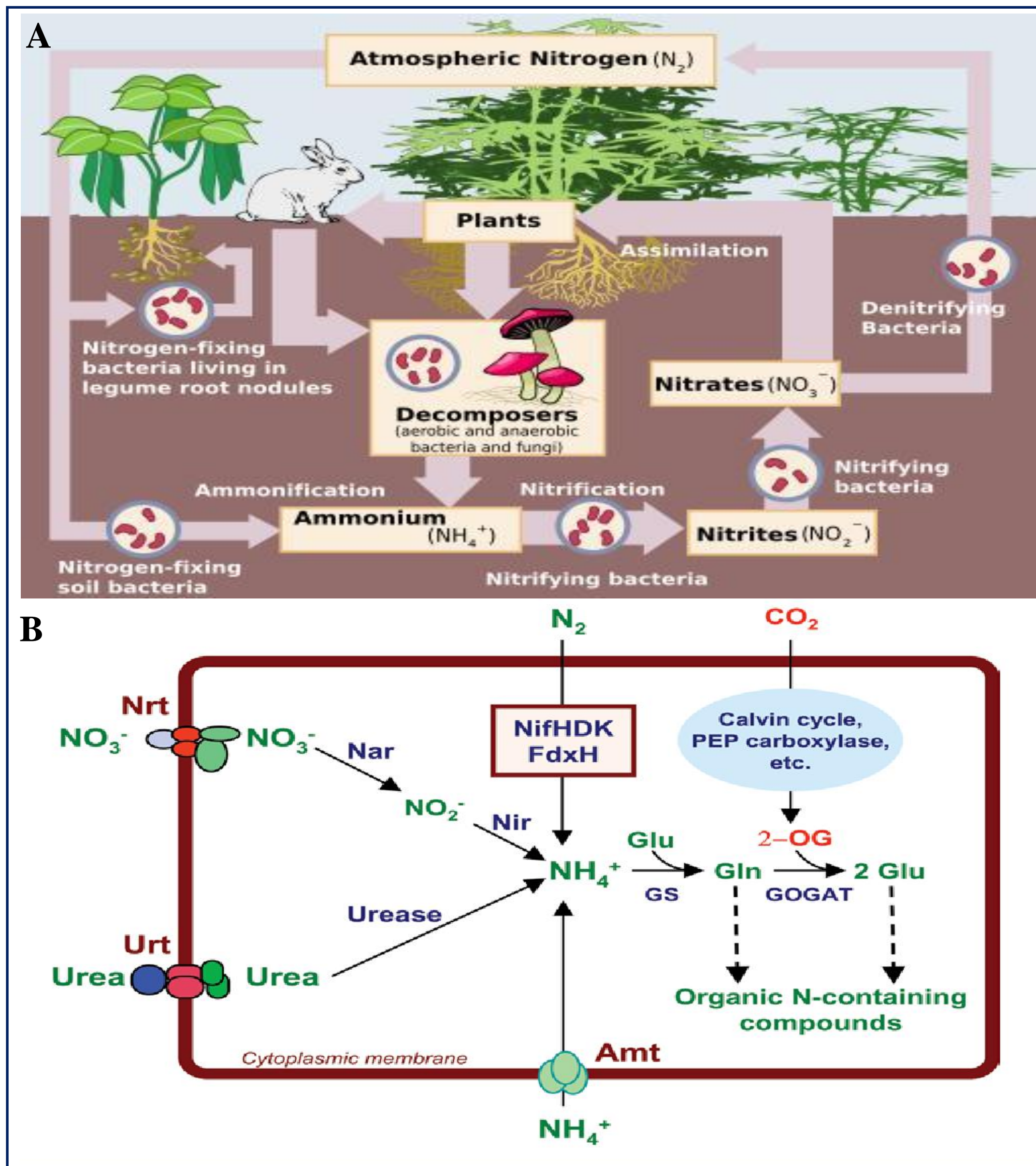


Fig. 1.4.2 Nitrogen flow through the ecosystem (A) (adopted from https://tinyurl.com/5d6afdzy) nitrogen assimilation pathways in cyanobacteria (B) (adopted from Flores & Herrero, 2005).

1.5 Effect of temperature and nutrient stress on the physiological and biochemical processes of cyanobacteria

Alteration in temperature and nutrients affects various physiological and biochemical processes in cyanobacteria, most importantly photosynthesis, respiration, pigment contents, nitrogen fixation, nutrient uptake, fatty acid profile, membrane lipid phase transitions, membrane damage, protein oxidation, heat shock proteins, antioxidants

(non-enzymatic and enzymatic) activities and expression of antioxidants genes. Thus, the rise in temperature and loading or deficiency of nutrients into waterbodies have attracted the attention of researchers on cyanobacteria, as temperature and nutrient concentration may affect their primary productivity and communities (Beardall & Raven, 2004; Paerl & Paul, 2012).

1.6 Electrogenic membrane: photophosphorylation and oxidative phosphorylation

Cyanobacteria being autotrophic, convert light energy into chemical energy via photosynthesis. The process of photosynthesis is a two-stage process: the light reaction, where light plays a direct role, and the light-independent reaction. The light reaction in cyanobacteria takes place in multiprotein pigment complexes, PS II, Cytochrome b_6f complex, and PS I, embedded in the thylakoid membrane (Fig. 1.6).

PS II, an intrinsic core subunit, consists of 32 kD D1, D2 protein, and CP43, and CP47. The D1 polypeptide consists of redox-active tyrosine, Yz, QB plastoquinone, pheophytin, and QA. Structurally, photosystem II comprises more than 25 polypeptides guarded by chl *a* and PBS binding proteins that capture the PS II complex light in the red region (680 nm) (Komenda et al., 2012). Photosystem I is made up of PsaA and PsaB heterodimeric complex of protein, a binding site for P700, which absorb light in the far-red region (700 nm) and functions as a trimer where each monomer comprises 12 protein subunits and 129 cofactors along with chlorophylls, iron-sulfur (Fe-S) clusters, β-carotene, phylloquinone, and lipids (Nelson & Yocum, 2006).

The first event of light reaction begins with capturing of light energy by phycobilisomes (PBS), a light-harvesting protein complex system attached to the thylakoid membrane (Fig. 1.6), and passes it to the reaction center complexes, i.e., PSII and PSI (MacColl, 1998). The PSII uses light energy to oxidize water to oxygen and to reduce the electron acceptor plastoquinone to plastoquinol. The plastoquinol pool transfers the electrons to the cytochrome b_6f complex. Cytochrome b_6f oxidizes plastoquinol to plastoquinone and reduces plastocyanin which reduces the PS I reaction center. Further, PS I reduce the Ferredoxin (Fd). Ferredoxin is used by the ferredoxin-$NADP^+$ reductase (FNR) enzyme to reduce $NADP^+$ to NADPH (Fig. 1.6).

Respiration serves to maintain a proton gradient across the thylakoid membrane, wherein both the photosynthetic and respiratory electron transport chains intersect (Fig.1.6) NAD(P)H dehydrogenase (NDH-1) is the major respiratory electron transport route into the PQ pool and accepts electrons from Fd, reduced by PS I (Bernát et al., 2011), cytochrome c oxidase (COX), reduce molecular oxygen to water and couple electron transfer with proton translocation across the membrane where ATP synthase utilizes the proton motive force (pmf) generated across the thylakoid membrane to catalyze the ATP formation from ADP (Von Ballmoos et al., 2009). Another important components of the respiratory electron chain to PQ pool is succinate dehydrogenase (SDH). Plastoquinone (PQ) pool, Cytochrome b_6f (Cyt b_6f), Soluble electron carriers (PC and cytochrome c_{553}) are involved in both photosynthesis and respiration. Plastoquinone (PQ) pool is a lipophilic membrane-bound carrier in the thylakoid membrane that shuttles electrons from both photosynthetic and respiratory electron transport chains to the Cyt b_6f complex (Cooley & Vermass, 2001). Cytochrome b_6f (Cyt b_6f), function as oligomeric quinol cytochrome c/PC oxidoreductases capable of proton translocation across the thylakoid membrane (Cramer & Zhang, 2006). Soluble electron carriers (plastocyanin and cytochrome c_{553}) are soluble metalloproteins that act as alternative electron carriers between Cyt b_6f and PS I.

The NADPH and ATP produced during the light reaction are then utilized for CO_2 fixation by the Calvin-Benson cycle. The Calvin cycle has three phases: a) Carboxylation: CO_2 is accepted by ribulose-1,5-bisphosphate (RuBP) to produce two molecules of 3-phosphoglyceric acid (3-PGA), a three-carbon compound, in the presence of RuBP carboxylase/oxygenase (RuBisCO); b) reduction: 3-PGA is converted into Glyceraldehyde 3-phosphate (G3P) by using ATP and NADPH and c) regeneration: G3P molecules are recycled to regenerate the ribulose-1,5-bisphosphate (Fig. 1.6) (Benson & Calvin 1950).

1.6.1 Effect of temperature stress on photosynthesis

Temperature stress, both sub-optimal and supra-optimal, causes de-stacking and reorganization of the thylakoid membrane, resulting in ion leakage and change in the phase transition of the energy membrane, causing damage to the photosynthetic machinery and affecting the photosynthetic electron transport rate of several algae species *Scytonema*

javanicum, *Amphistegina radiata*, *Heterostegina depressa*, *Calcarina hispida*, *Spirulina platensis*, *Arthrospira platensis*, *Cyanidioschyzon merolae*, seagrasses etc. (Wahid & Shabbir, 2005; Sharkey, 2005; Mohanty et al., 2002; Zhu et al., 2020; Schmidt et al., 2011; Zhao et al., 2008; Campbell et al., 2006; Mackey et al., 2013; Tang & Vincent, 2000; Breitbarth et al., 2007; Vonshak & Novoplansky, 2008; Nikolova et al., 2017; Venkataramanaiah et al., 2003). Reports with suboptimal and supra-optimal temperatures also revealed a significant reduction in the pigment content of several algal species (Chalanika & Asaeda, 2017; Giannuzzi et al., 2016; Kim et al., 2019, Reddy et al., 2019). However, there is a conflicting report that high-temperature stress at 40ºC causes no change in the pigment content (Zhang & Liu, 2016).

Several reports working with various algae suggest that temperature in combination with other stresses such as salinity (Xiao et al., 2016), light (Chaneva et al., 2007; Jodlowska and Latala, 2013; Kim et al., 2019), carbon dioxide level (Kang & Kim, 2016), pH (Brutemark et al., 2015), herbicides (Chalifour & Juneau, 2011; Chalifour et al., 2014; Yeo et al., 2018) affect chlorophyll fluorescence, oxygen evolution, electron transport chain, pigment content, and growth.

1.6.2 Effect of nutrient stress on photosynthesis

Variability in phosphorus and nitrogen concentrations affects cyanobacteria's photosynthesis (Jaanus et al., 2009). Phosphorus deficiency causes a decline in the photosynthetic electron transport rate in *Synechocystis* sp., *Phaeodactylum tricornutum*, *Thalassirosira weissflogii*, *Chaetoceros affinis*, *Symbiodinium sp*, *Chlorella vulgaris*, *Cylindrospermopsis raciborskii*, *Thalassirosira weissflogii*, and several other algal species (Fuszard et al., 2013; Liu et al., 2011; Lin et al., 2013; Shih et al., 2015; Rosset et al., 2017; Jiao et al., 2017; Wu et al., 2012; Qi et al., 2013). Phosphorus deficiency also reduces the pigment content of *Chaetoceros muelleri*, *Thalassirosira weissflogii*, *Cylindrospermopsis raciborskii*, *Phaeodactylum tricornutum*, *Tetraselmis suecica,* and *Chlorella vulgaris* (Lovio-Fragoso et al., 2021; Lin et al., 2013; Wu et al., 2012; Goiris et al., 2015). However, reports of excess phosphorus concentrations to increase the photosynthetic electron transport rate and pigment content in *Gracilaria lemaneiformis*,

Chlorella vulgaris, *Platymonas subcordiformis*, and *Heterosigma akashiwo* are shown (Xu et al., 2010; Jiao et al., 2017; Qi at al., 2013).

A few reports are available on limiting nitrogen concentration in culture media showing a decline in the photosynthetic activity (Zhao et al., 2017; Liefer et al., 2018; Pancha et al., 2014; da Silva et al., 2009; Salomon et al., 2013) and pigment content (Barker-Astrom et al., 2005; Zhao et al., 2016) while excess nitrogen in the culture media increases the photosynthetic and respiratory rate in algae (Zou and Gao, 2014; Martins et al., 2011). *Dunaliella salina* grown in nitrogen, phosphorus, and sulfur-deficient media exhibit a decline in photosynthetic activity and pigment content (Srinivasan et al., 2018).

Combine treatment of nitrogen starvation with low (35ºC) and high temperatures of 45ºC and 55ºC significantly reduces the photosynthetic efficiency of PS II and the pigment content of *Thermosynechococcus* (Li et al., 2021). Nitrogen starvation at a low temperature of 35ºC has been reported to decline growth rate and biomass productivity of *Thermosynechococcus,* while the same species' growth rate and biomass productivity were not influenced by combined nitrogen starvation and high temperatures (Li et al., 2021). In a study by Chen et al. (2011), high phosphorus concentration and temperature reported to increase the pigment content of *Scenedesmus obliquus* and *Microcystis aeruginosa*. Report of high nutrient supply used in the form of fishpond effluents enhancing the electron transport rate and chlorophyll in *Ulva lactuca* (Figueroa et al., 2009) but in another study with high nitrogen, using ammonia as a source, *Cholrella* strains displayed a reduction in photosynthetic activities (Wang et al., 2018). Several studies (Kumar et al., 2003; Collen et al., 2004; Degerholm et al., 2006; Conley et al., 2009; Xu et al., 2010; Liefer et al., 2018; Peng et al., 2018; Jankowiak et al., 2019) exhibited that limitation or excess of phosphorus and nitrogen concentration influences the photosynthesis of micro and macroalgae, which subsequently affect their growth.

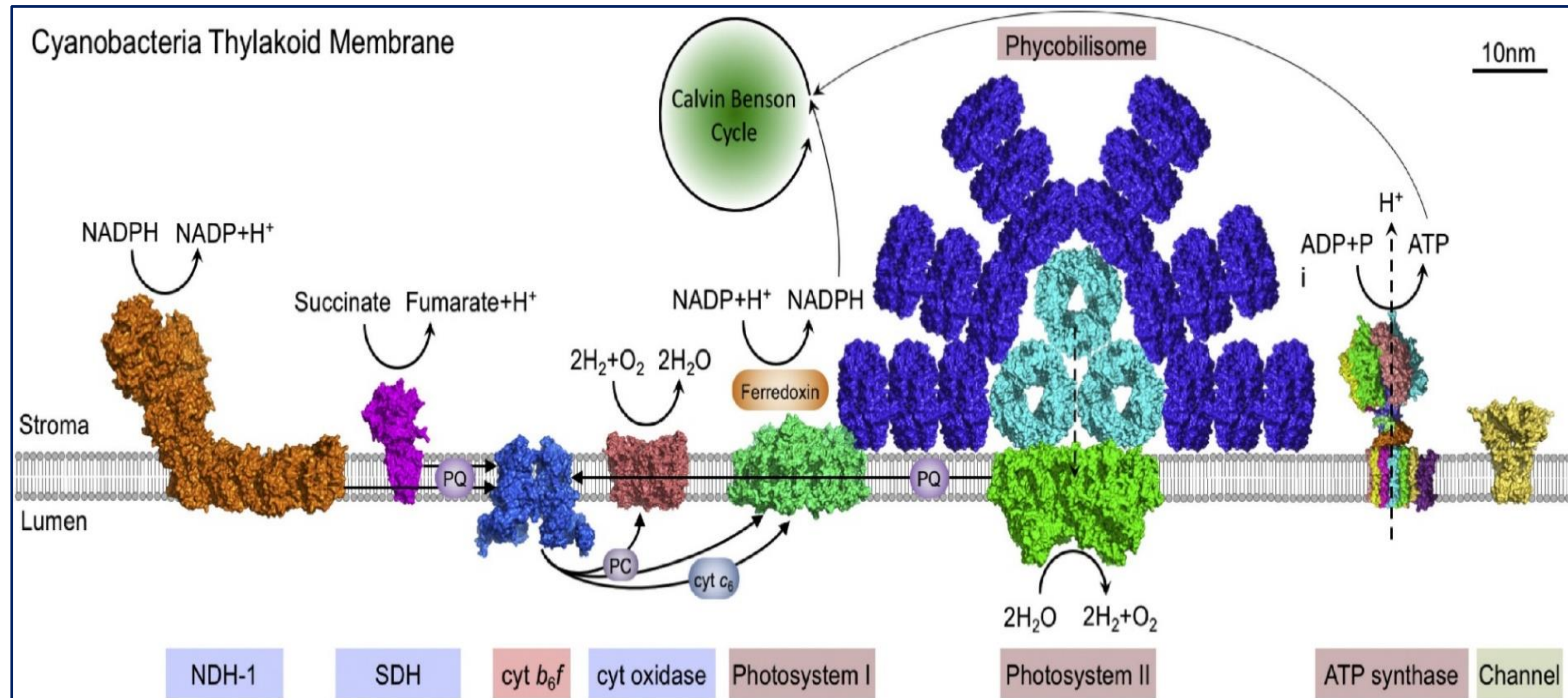


Fig. 1.6 Schematic model of cyanobacterial thylakoid membrane (based on knowledge of *Synechocystis* 6803 thylakoids), showing the interplay of photosynthetic and respiratory electron transport component in the same membrane. Photosynthetic electron transfer complexes include phycobilisomes, PS II and PS I, cyt b_6f, and ATPase. Complexes specific for respiratory electron transport chain are NDH-1, SDH and cyt oxidase. Some components, such as the cyt b_6f, PQ, and PC are shared by both electron transport pathways (Figure adapted from Liu, 2016).

1.7 Biochemical responses

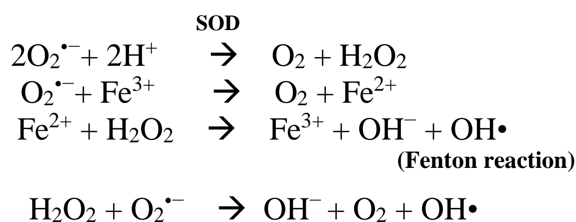
1.7.1 Reactive oxygen species (ROS) production

Life originally began on Earth under a reducing atmosphere (Dietrich et al., 2006) that became oxidized due to the proliferation of cyanobacteria, which supported the development of aerobic conditions, billions of years ago (Brocks et al., 1999). Concurrently, these organisms had to endure the damaging effects of oxygen on the metabolic networks due to the production of ROS as a byproduct of oxidation (respiration) and reduction (photosynthesis) of O_2 (Latifi et al., 2009) (Fig. 1.7). The various types of ROS produced by organisms are superoxide radical ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), hydroxyl anions (OH^-), and hydroxyl radical (OH•), more so under stress conditions.

a) *Superoxide radical* ($O_2^{\bullet-}$): Superoxide radical is formed by the reduction of oxygen during an over-reduced photosynthetic electron transport chain where electrons are leaked to molecular oxygen. It is the first ROS to be formed with a short half-life of 2-4 µs and moderate reactivity.

b) *Hydrogen peroxide* (H_2O_2): is formed when the superoxide radical undergoes protonation as well as univalent reduction (dismutation). It is a moderately reactive ROS with a half-life of 1 ms and is only ROS in gaseous form. Due to its longer half-life and gaseous nature, it can traverse longer distances across cell membranes and cause greater oxidative damage to biomolecules.

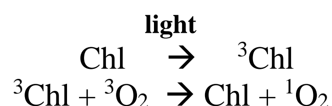
c) *Hydroxyl anions* (OH^-) *and Hydroxyl radical* (OH•): OH• is the neutral form of the OH^-, which is the most reactive and toxic member of ROS with a half-life of 1 ns. Hydroxyl radicals and hydroxyl anions are generated during the Fenton reaction between hydrogen peroxide and superoxide radicals in the presence of any transition metals like Cu (Cu^{2+}, Cu^{3+}) and Fe (Fe^{2+}, Fe^{3+}).



$$H_2O_2 + O_2^{\bullet-} \rightarrow OH^- + O_2 + OH^\bullet$$

OH• is highly reactive and capable of causing lipid peroxidation, protein damage, membrane destruction, and, finally causing, cell death (Pinto et al., 2003) (Fig. 1.7).

d) *Singlet oxygen* ($^1O_2$): is generated when the triplet state of chlorophyll in the antenna system transfers energy to an oxygen molecule.



$$Chl \xrightarrow{\textbf{light}} {}^3Chl$$
$$^3Chl + {}^3O_2 \rightarrow Chl + {}^1O_2$$

The short half-life of $^1O_2$ is about 3 µs and is capable of causing damage to both the photosystem, PS I and PS II, pigments, lipids, proteins, nucleic acids, and cellular death.

ROS at lower concentrations acts as a regulatory signal for physiological processes like photosynthesis, photorespiration, stomatal movement, senescence, cell cycle, attainment of tolerance to biotic and abiotic stresses, and programmed cell death (Kwak et al., 2003; Neill et al., 2002; Miller et al., 2010; Noctor et al., 2002; Peng et al., 2005; Tanou et al., 2009a,b).

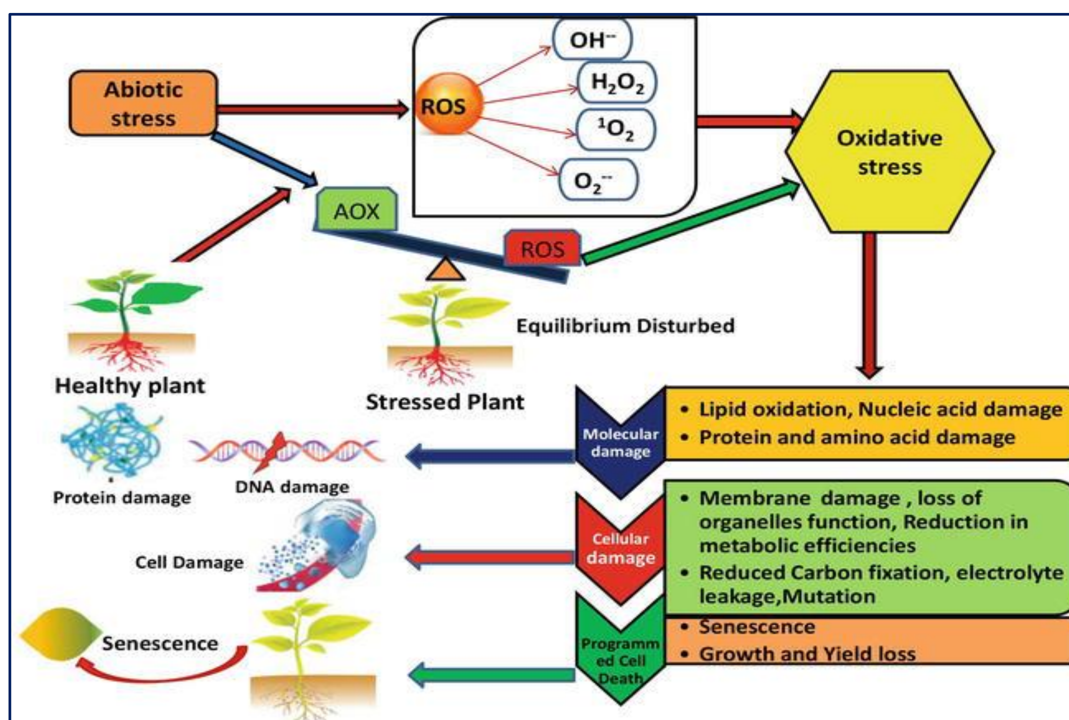


Fig. 1.7 Abiotic stresses induce oxidative stress in photosynthetic organisms (adopted from Kumari et al., 2021).

1.8 Oxidative damage caused by ROS

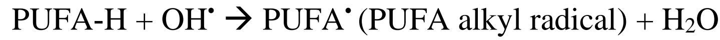
The production of ROS under abiotic stresses is responsible for damaging various biomolecules.

1.8.1 Lipid peroxidation

Lipids include fats, phospholipids, waxes, hydrocarbons, steroids, free higher fatty acids, and salts. All cell membrane constitutes membrane lipids as a major chemical component which are mainly represented by phospholipids and sterols, except thylakoid membranes which mainly contain galactolipids. They are amphipathic and most stable in a bilayer configuration (López et al., 2011; Tetlow et al., 2005; Robinson & Mant, 2005). The amphipathic nature of membranes permits the formation of membranous sheets that self-anneal their edges into a compartment and make it highly sensitive to the external environment. The changes in the membrane lipids act as stress markers and help the plant to
change with the potential impacts of abiotic stress (Barkla & Pantoja, 2011; Furt et al., 2011; Murphy et al., 2011). Due to the presence of a greater number of double bonds in polyunsaturated fatty acids (PUFA) results in easy removal of a hydrogen atom. PUFA is more vulnerable to oxidative damage due to ROS, leading to the formation of lipoperoxides than in saturated fatty acids (SFA) or monosaturated fatty acids (MUFA) (Porter et al., 1995). The main products of lipid peroxidation are moieties containing hydroperoxyls, hydroxyls, ketones, aldehydes [such as malondialdehyde (MDA)], and trans double bonds (Borchman & Sinha, 2002), which in turn causes severe damage to cells by binding free amino groups of amino acids of proteins (Sochor et al., 2012).

The overall mechanism of ROS-mediated lipid peroxidation consists of (a) initiation (activation), (b) propagation (distribution), and (c) termination (cleavage).

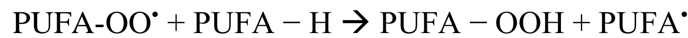
Initiation step begins with energizing the O_2 molecules, which further produce superoxide radicals and hydroxyl radicals. This radical reacts with the methylene group of polyunsaturated fatty acid (PUFA), yielding lipid alkyl radical, peroxyl radical, conjugated dienes, and hydroperoxide (Smirnoff, 2000).



$$PUFA\text{-}H + OH^{\bullet} \rightarrow PUFA^{\bullet} \text{ (PUFA alkyl radical)} + H_2O$$

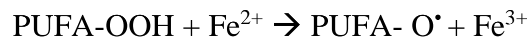
$$PUFA^{\bullet} + O_2 \rightarrow PUFA\text{-}OO^{\bullet} \text{ (Peroxyl radical)}$$

Elongation PUFA peroxyl radical disseminates the LP by removing the H atom from the adjacent PUFA.



$$PUFA\text{-}OO^{\bullet} + PUFA - H \rightarrow PUFA - OOH + PUFA^{\bullet}$$

The lipid hydroperoxide experienced breakage by reacting with reduced metals (Fe^{2+}).



$$PUFA\text{-}OOH + Fe^{2+} \rightarrow PUFA\text{-} O^{\bullet} + Fe^{3+}$$

Lipid hydroperoxide undergoes decomposition to form various reactive species, for instance, lipid alkoxyl radicals, alkanes, aldehydes, lipid epoxides, and alcohol.

Termination of LP occurs by forming different lipid dimers triggered by various lipid-derived radicals.

Generally, lipid peroxidation is responsible for exaggerating membrane fluidity, makes the membrane leaky for the substances that otherwise enter the cell through specialized channels, disable the membrane receptors, deteriorate the membrane proteins, ion channels, and membrane-localized enzymes (Das & Roychoudhury, 2014) (Fig. 1.8.1).

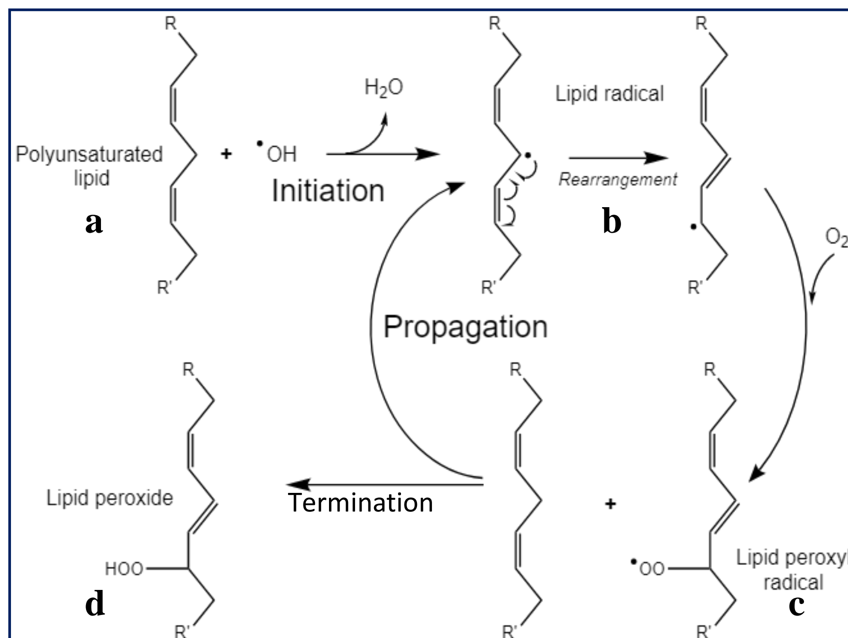


Fig. 1.8.1 Mechanism of lipid peroxidation: Unsaturated lipid (A); radical lipid (B); lipid peroxyl radical (C); lipid peroxide (D) (Adopted from González-Minero et al., 2020).

1.8.2 Protein oxidation

Proteins play a crucial role in the majority of cellular events; thus, to survive under abiotic stress conditions, functional conformations of cellular proteins are maintained, and the aggregation of non-native proteins is prevented (Ghosh and Xu, 2014; Timperio et al., 2008). Though proteins are continuously oxidized even under normal physiological conditions, the same is greatly enhanced under stress conditions (Johansson et al., 2004; Job et al., 2005). The dysfunction of proteins and inhibition of their synthesis are among the initial metabolic responses to stresses in plants (Timperio et al., 2008). However, due to their great abundance and high-rate constants of an array of reactive radicals, proteins are one of the main cellular constituents targeted by ROS (Davies, 2005). ROS damage the proteins by modifying their covalent bonds or by directly oxidizing amino acids, such as the oxidation of Met residues to form Met sulfoxide, oxidation of Cys residues to form disulfide bonds, and oxidation of Arg, Pro, Lys, and Thr residues and thus creating carbonyl groups in the side chains (Rhoads et al., 2006). ROS affects the thiol groups or sulfur-containing amino acids, causing direct and indirect modification: In a direct modification, the protein activities are affected through several processes such as nitrosylation, formation of disulfide cross-links, glutathionylation, and carbonylation of specific amino acid residues (Davies, 2005). Indirect modification of proteins occurs by conjugation with the breakdown products of fatty-acid peroxidation. Protein carbonylation involves the oxidation of the protein backbone and some amino acid side chains (especially histidine, arginine, and lysine), yielding ketone or aldehyde derivatives. In addition, protein carbonylation may also be caused by the reaction of lipid-peroxidation products with proteins and protein conjugation with reducing sugars (glycation) or their oxidation products (glycoxidation) (Richter et al., 2005). The protein-carbonyl content is the most commonly used marker for ROS - mediated protein oxidation under stress in autotrophic organisms (Qiu et al., 2008) (Fig. 1.8.2).

1.8.3 Damage to DNA

DNA is the genetic material of the cells and also experiences oxidative damage mainly due to the production of hydroxyl radical (OH•), which causes oxidation of

deoxyribose sugar residue, modification of nucleotide bases, an abstraction of nucleotide, breaking of DNA strands, cross-linking of DNA (Halliwell, 2006) (Fig. 1.8.3).

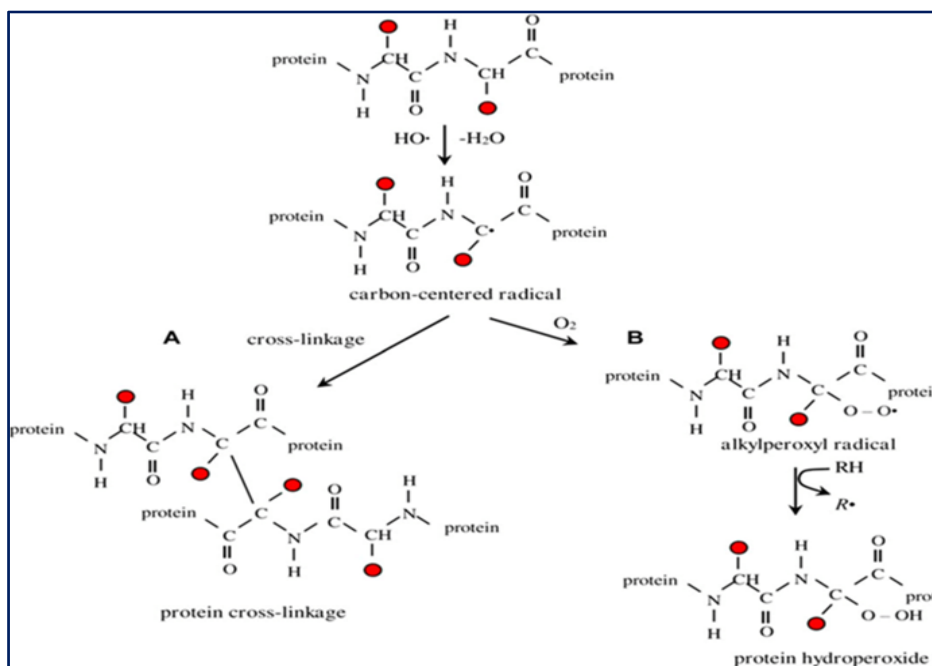


Fig. 1.8.2 Protein oxidation caused by ROS: Formation of an interprotein cross-linkage (A); Formation of a backbone protein hydroperoxide (B) (adopted from Papuc et al., 2017).

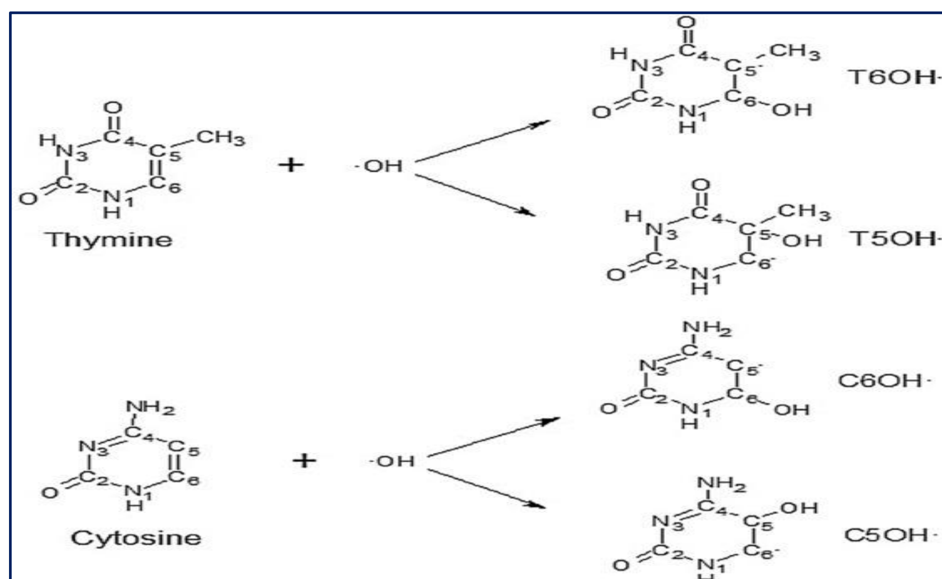


Fig. 1.8.3 Oxidation of DNA by hydroxyl radical (adopted from Borràs et al., 2019).

1.8.4 Temperature and oxidative damage

The optimum temperature of 25ºC to 30ºC cause a lower level of ROS generation and oxidative damage to the cell membrane in *Microcystis aeruginosa* (Zheng et al., 2020; de la Rosa et al., 2020). Several reports also suggest that suboptimal and supra-optimal temperatures increase the ROS production and lipid peroxidation in *Synechocystis* sp., *Potamogeton crispus*, *Elodea nuttallii*, *Vallisneria asiatica*, *Acutodesmus dimorphus*, *Microcystis aeruginosa* and *Anabaena doliolum* (Chalanika and Asaeda, 2017; Chokshi et al., 2020; Giannuzzi et al., 2016; Zheng et al., 2020; Mishra et al., 2005 and Reddy et al., 2019). When the temperature is coupled with other stresses such as carbon dioxide/pH in *Dolichospermum* sp., *Anabaena doliolum* (Brutemark et al., 2015), salt and copper in *Anabaena doliolum* (Srivastava et al., 2006), UV-B in *Microcystis aeruginosa* (Babele et al., 2017), light in *Cladophora glomerata, Enteromorpha ahlneriana*, *Scenedesmus quadricauda* FACHB-45 (Choo et al., 2004; Wu et al., 2008), herbicides in *Pseudokirchneriella subcapitata* (Yeo et al., 2018) cause an exaggeration in ROS generation and oxidative damage.

1.8.5 Nutrient and oxidative damage

Micro and macroalgae also experience ROS generation and oxidative damage under starvation and excess nitrogen and phosphorus concentration (Peng et al., 2017; Wu et al., 2012; Moussa et al., 2017; Yilancioglu et al., 2014; Al-Rashed et al., 2016; Fan et al., 2014; Chokshi et al., 2017; Hamid & Sibi, 2018; Yang & Wang, 2019). Reports of phosphorus deficiency causing an increase in the ROS and MDA content in *Cylindrospermopsis raciborskii* and *Microcystis aeruginosa* are available (Wu et al., 2012, Peng et al., 2017). Replete phosphorus conditions also increase H_2O_2 production and MDA content in *Tetraselmis marina* (Moussa et al., 2017). Some conflicting reports showing less production of MDA content in *Cylindrospermopsis raciborskii* under excess phosphorus concentration are also there (Wu et al., 2012).

Similarly, nitrogen-limiting culture media have exhibited greater ROS production and MDA content in *Dunaliella salina*, *Oscillatoria willei*, *Spirulina platensis*, *Acutodesmus dimorphus*, *Porphyridium cruentum*, *Microcystis aeruginosa* and *Chlorella sorokiniana*

(Yilancioglu et al., 2014; Al-Rashed et al., 2016; Kumar et al., 2003; Chokshi et al., 2017; Zhao et al., 2017; Peng et al., 2017; Zhang et al., 2013). Another study with nitrogen-replete conditions exhibited an increase in the accumulation of H_2O_2 and MDA content in *Tetraselmis marina* (Moussa et al., 2017).

Deficiency of phosphorus, nitrogen, iron, and sulfur also reveale an increase in ROS generation and MDA content in *Chlorella pyrenoidosa*, *Dunaliella salina*, and *Chlamydomonas reinhardtii* (Fan et al., 2014; Srinivasan et al., 2018; Çakmak et al., 2015). While combine deficiency of phosphorus and nitrogen also shown an increase in oxidative damage in *Chlorococcopsis minua* (Hamid & Sibi, 2018).

A combination of excess phosphorus with Bisphenol A in *Microcystis aeruginosa* (Yang & Wang, 2019), titanium dioxide nanoparticles in *Chlorella Ellipsoides* (Matouke et al., 2018), iron in *Halothece* sp. (Fernández-Juárez et al., 2020) and excess nitrogen with an herbicide in *Raphidiopsis raciborskii* (Brêda-Alves et al., 2021) displayed an excess production of ROS and lipid peroxidation.

1.9 Antioxidants

Excessive ROS generation induces oxidative damage to various biomolecules, and if the damage continues, cell death can happen. Thus, to maintain homeostasis, the cell employs various enzymatic and non-enzymatic antioxidants to scavenge excessively produced ROS. The various enzymatic antioxidants produced by the plants and lower photosynthetic organisms are superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and glutathione peroxidase (GPX) (Gill & Tuteja, 2010) (Fig. 1.9).

Superoxide Dismutase (SOD) is a metalloenzyme that provides the first line of defense against elevated ROS levels. It catalyzes the scavenging of $O_2^{\bullet-}$ by dismutating it into oxygen and hydrogen peroxide via the Haber-Weiss-type reaction and Mehler's like reaction. In cyanobacteria, SOD is classified into Fe-SOD, Ni-SOD, Cu/Zn-SOD, and Mn-SOD; however, Cu/Zn-SOD is rare in cyanobacteria (Priya et al., 2007). Each type of SODs displays characteristic intracellular localization. Both cytoplasmic and thylakoid membranes consist of MnSOD, FeSOD, and NiSOD in the cytosol, while Cu/ZnSOD is found in the periplasmic. Most cyanobacteria have two types of SOD, MnSOD and FeSOD or FeSOD and

Cu/ZnSOD or NiSOD and Cu/ZnSOD (Li et al., 2002; Regelsberger et al., 2004; Blot et al., 2011; Dupont et al., 2008).

Catalase (CAT) is a tetrameric heme-containing enzyme that removes H_2O_2 from organisms by dismutating H_2O_2 into water and oxygen. It has a high affinity for H_2O_2, but lesser specificity for organic peroxides (R-O-O-R). It has a very high turnover rate (6×10^6 molecules of H_2O_2 to H_2O and O_2 min^{-1}) and is unique amongst antioxidant enzymes in not requiring a reducing equivalent (Mittler, 2002).

Ascorbate peroxidase (APX) belongs to class I heme-peroxidase and forms an essential constituent of the Ascorbate-glutathione cycle and plays a vital role in detoxifying H_2O_2 to H_2O and DHA by using ascorbate as an electron donor (Das & Roychoudhury, 2014).

Heat shock proteins (HSPs) upon temperature increase, a set of proteins known as the heat shock proteins (Hsps) are induced by cyanobacteria through transcriptional activation (Rajaram & Apte, 2003). The magnitude of induction of Hsps depends on the growth temperature and the degree of temperature increase (Lehel et al., 1993). The most predominant Hsps that cyanobacteria cells accumulate under heat stress are GroEL, GroES, and small Hsps (Rajaram & Apte, 2003).

Non-enzymatic antioxidants: Apart from enzymatic antioxidants, the other antioxidants are non-enzymatic antioxidants which constitute ascorbate (AsA), proline, glutathione (GSH), α-tocopherol, carotenoids, phenolics, and flavonoids (Fig. 1.9). They play an essential role in protecting plants from various oxidative damages as well as in plant growth and development (de Pinto & De Gara, 2004).

Ascorbate (AsA) is one of the most prodigious and intensely studied non-enzymatic antioxidants. It is involved in donating electrons to a broad range of non-enzymatic and enzymatic reactions; hence it is considered the most powerful component among the non-enzymatic antioxidants. AsA removes OH•, O_2•−, and $^1O_2$ and reduces H_2O_2 to H_2O through APX reaction (Blokhina et al., 2003). AsA also carries out many non-antioxidant functions in the cells, for instance, cell division and cell cycle development from G_1 to S phase (Liso et al., 1988; Smirnoff, 2000) and elongation of the cell (De Tullio et al., 1999).

Tocopherols (vitamin E) have an aromatic ring and a long hydrocarbon chain and play a role in oxidation-reduction reactions. It destroys reactive types of oxygen and protects unsaturated fatty acids from oxidation. There are four tocopherols (α-, β, ϒ, and ϐ-tocopherols); among these, α-tocopherol is a crucial antioxidant as it can suppress $^1O_2$ and reduce $O2^{\cdot -}$ and lowers the lipid peroxidation reaction (Takenaka et al., 1991).

Proline is a proteinogenic amino acid employed in the biosynthesis of proteins. Under stress conditions, it acts as a signaling molecule, antioxidative defense molecule, a metal chelator, and an osmoprotectant. Proline can prevent LPO due to its compelling nature of removing OH• and $^1O_2$ (Verbruggen & Hermans, 2008).

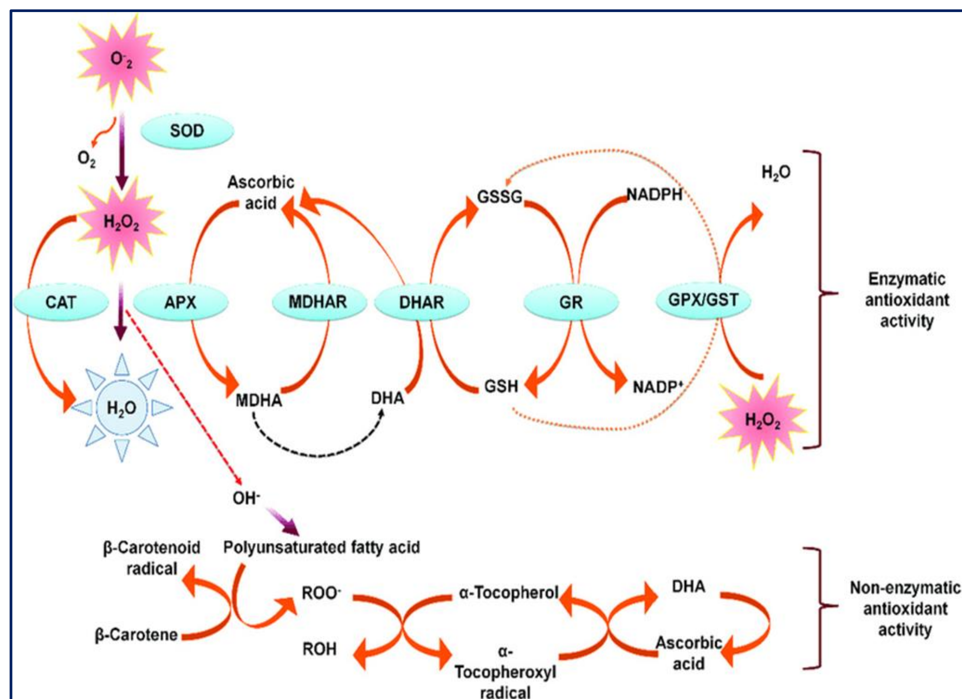


Fig. 1.9 Detoxification of ROS by enzymatic antioxidant and non-enzymatic antioxidant. SOD-Superoxide dismutase; CAT- catalase; APX- ascorbic peroxidase; GPX- glutathione peroxidase; GST-glutathione S-transferase; MDHAR- monodehydroascorbate reductase; DHAR- dehydroascorbate reductase; GR- glutathione reductase; β-carotene, Ascorbic acid, α-tocopherol and GSH- reduced glutathione (adopted from Chourasia et al., 2021).

1.9.1 Temperature and antioxidants

Cyanobacteria are reported to produce antioxidants to scavenge ROS and help to withstand oxidative damage (Choo et al., 2004; Chalanika & Asaeda, 2017; Yang et al., 2019; Chokshi et al., 2020). There are several reports demonstrating an increase in the activity of SOD, CAT, APX, and proline content at low and high temperatures in *Arthrospira platensis* Kenya, Antarctic plankton and temperate phytoplankton, *Arthrospira platensis*, *Anabaena doliolum*, *Acutodesmus dimorphus*, *Microcystis aeruginosa* and several other algae species (Chien & Vonshak, 2011; Perelman et al., 2006; Ismaiel & Piercey-Normpore, 2021; Chokshi et al., 2020; Giannuzzi et al., 2016; Chalanika &Asaeda, 2017; Reddy et al., 2019; Choo et al., 2004; Gacheva et al., 2013; Babele et al., 2017; Mishra et al., 2005; Xing et al., 2022). However, a study by Mishra et al. (2005) at 43℃ showed an increase in SOD, CAT, and APX enzymes, while the activities of these enzymes decline with a further rise in temperature.

1.9.2 Nutrients and antioxidants

Nutrient deficiency and acquisition also influence the antioxidant response in several algal species. Phosphorus-limitation conditions reveal an increase in the activity of enzymatic antioxidants in *Microcystis aeruginosa* and ascorbic acid in three microalgae species (Peng et al., 2017; Goiris et al., 2015), while higher phosphorus concentration causes a reduction in antioxidant activity (Yang & Wang, 2019). Microalgae grown under nitrogen starvation conditions reveal an increase in the activities of antioxidant enzymes SOD, CAT, and APX (Peng et al., 2017; Yilancioglu et al., 2014; Zhang et al., 2013).

Deficiency of phosphorus, nitrogen, iron, sulfur and combined starvation of nitrogen and phosphorus also reveal an increase in enzymatic antioxidant activity and proline content in several algal species (Fan et al., 2014; Ruiz-Domínguez et al., 2015; Salbitani et al., 2015; Srinivasan et al., 2018; Hamid & Sibi, 2018). Conflicting reports show that nitrogen, phosphorus, and sulfur deficiency cause a decline in the activity of antioxidants in *Chlamydomonas reinhardtii*, which was increased when supplemented with nitrogen and zinc (Çakmak et al., 2015). The nutrient limitation also causes a decline in the phenolic content and other non-enzymatic antioxidants in various algae (Goiris et al., 2015).

1.10 Nitrogenase activity

Nitrogen is abundantly available in Earth's atmosphere in the form of dinitrogen (N_2) gas and biologically fixed into the soil with the help of free-living bacteria (*Azotobacter*, *Agrobacterium*, *Gluconobacter*, *Azospirillum*, *Flavobacterium*, and *Herbaspirillum*) or through a symbiotic association between bacteria and plants or fungi (*Rhizobium* with root nodules or *Anabaena* with *Azolla* or lichen) and cyanobacteria (heterocystous and non-heterocystous). These organisms fix atmospheric nitrogen using the nitrogenase enzyme which is a two-component anaerobic system composed of the molybdenum-iron (MoFe) protein (dinitrogenase or component I) and electron transfer Fe protein (dinitrogenase reductase or component II). The MoFe protein contains two copies each of two metalloclusters designated as FeMo-co (iron-molybdenum cofactor), representing the site of substrate reduction and P-cluster, involved in electron transfer from Fe protein to FeMo-co. The nitrogenase enzyme catalyzes the reduction of nitrogen to ammonia using ATP (Priscu et al., 1998; Prabaharan et al., 2010; Amarouche-Yala et al., 2014; Winckelmann et al., 2015).

All heterocystous forms of cyanobacteria are capable of fixing nitrogen. In the absence of combined nitrogen, 5-10% of their vegetative cells differentiate into heterocysts cells (Bergman et al., 1997). The heterocysts provide an anaerobic environment suitable for the functioning of nitrogenase enzymes. Unlike vegetative cells, heterocysts are photosynthetically inactive as it lacks O_2 evolving PS II and possess PS I and depend on the supply of reducing equivalents from vegetative cells, which is in the form of carbohydrates (Curatti et al., 2002). They also have a thick glycolipid cell wall that serves as an effective gas diffusion barrier, limiting the diffusion of oxygen into the cell (Walsby, 2007). Heterocysts also have a high respiration rate, thus removing any oxygen entering the cell. Although the heterocysts provide a favorable environment for nitrogenase activity, nitrogen fixation in heterocystous cyanobacteria is affected by environmental conditions (oxygen, light, pH, temperature, and nutrients) (Premanandh et al., 2009; Staehr & Jensen, 2006).

1.10.1 Temperature and nitrogenase activity

Temperature variations affect the activity of nitrogenase enzymes of various cyanobacteria (Kumar et al., 1992; Gallon et al., 1993; Compaoré & Stal, 2010).

Sub and supra-optimal temperatures are reported to decrease the nitrogenase enzyme activity in various species of algae (Thangaraj et al., 2017; Reddy et al., 2019; Rajaram & Apte, 2003; Waughman, 1977; Stal, 2017; Zielke et al., 2002). A combination of high-temperature stress of 40-50ºC with elevated CO_2 of ~ 6% positively influences the nitrogen fixation of *Anabaena fertilissima* (Chinnasamy et al., 2009).

1.10.2 Nutrients and nitrogenase activity

The nutrient acquisition also influences the nitrogenase activity in various cyanobacteria. Excess phosphorus are reported to increase the activity of the nitrogenase enzyme in various algae (Stewart & Alexander, 1971). The nitrogenase activity of *Anabaena variabilis* and *Westiellopsis prolifica* was increased under 20 mg of TCP than 10, and 30 mg P L^{-1} of Mussorie rock phosphate (MRP) and tricalcium phosphate (TCP) (Yandigeri et al., 2011).

A double dose of potassium or phosphorus and iron reported to increase the nitrogenase activity of *Nostoc* speices (Nisha et al., 2015). In disparity, the exogenous supply of nitrogen sources negatively affects the nitrogenase activity of *Nostoc* sp., *Anabaena doliolum* (Nisha et al., 2015). Similarly, under high pH (>8.5), dipotassium hydrogen phosphate (>0.06 g L^{-1}), iron (>6 mg L^{-1}), and nitrogen concentration (>0.5 g L^{-1}), *Scytonema coactile* showed a decline in nitrogenase activity (Shewli et al., 2013).

1.11 Fatty acids

Lipids are a heterogeneous group of molecules and hydrophobic in nature. Lipids display an array of structure variations, from simple short hydrocarbon chains to more complex molecules, including triacylglycerols, phospholipids, sterols, and their esters. Lipids within each class may vary in the number of carbon atoms and double bonds, position and orientation of double bonds, branching of the hydrocarbon chain, and addition of polar groups such as choline, inositol, ethanolamine, and glycosylation. While fatty acids are hydrocarbon chains with 2-30 carbon lengths and degrees of unsaturation, with a carboxyl group at one end and a methyl group at the other end. Fatty acids are characterized into saturated fatty acid (SFA), monosaturated fatty acid (MUFA), and polyunsaturated fatty acids (PUFA). In PUFA, the sequence of double bonds is usually interrupted by alternating methylene groups.

Alteration in the double bonds number, position, and orientation can curve the fatty acid chain, thus changing its packing in cell membranes which modifies its biophysical properties, for instance, melting temperature (Kenyon &Stanier, 1970).

Membrane lipid exists in various organized structures (polymorphic form) whose structure depends on the type of fatty acid chain and other factors like temperature, pH, pressure, and ionic strength (Lewis & McElhaney, 2013). Among all the factors mentioned above, temperature is a crucial factor that affects the phase separation state of the lipid membrane (thylakoid membrane and plasma membrane). Under relevant physiological conditions, depending on the amount of saturated or unsaturated fatty acids, a lipid membrane exists in the bilayer phase, i.e., the gel phase (solid) and liquid crystalline state. In-gel state bilayer, the hydrocarbon chain exists in a rigid state, expanded, and trans-conformation state (Ragoonanan et al., 2008). Whereas, in liquid crystalline bilayer**,** the hydrocarbon chain poses various unorganized conformers, and lipid molecules, due to an increase in the head group spacing, display an increased rate and amplitude of intramolecular and intermolecular motion resulting in a disordered lipid acyl chain, reducing the thickness of a bilayer (Ragoonanan et al., 2008). The process of conversion from gel to liquid crystalline involves lateral expansion accompanied by a reduction in the bilayer's thickness and a slight elevation in the lipid molecule's total volume. Studies have demonstrated that at optimal temperature, both the thylakoid membrane and plasma membrane exist in the liquid crystalline state, and as the temperature decreases below the optimum level, the thylakoid membrane enters a phase-separated state. Whereas the plasma membrane enters the phase separated state at nearly 10ºC below the phase separation of the thylakoid membrane (Lewis & McElhaney, 2012). A decrease in the growth temperature usually increases the degree of membrane lipids' unsaturation, a crucial factor that impacts these membranes' phase transition temperature (Cossins, 1994). Another study on *Geobacter sulfurreducens*, showed a decrease in gel phase lipid order under hyperosmotic shock (Ragoonanan et al., 2008).

1.11.1 Temperature and fatty acid

The lipid and fatty acid composition of cyanobacteria are directly affected by temperature, whereas nature and the rate of this effect vary from species to species (Nalley et

al., 2018; Converti et al., 2009). Cyanobacteria cells modify their cell membrane fatty acid composition with changing ambient temperatures. Cyanobacteria accumulate high unsaturated FA at low temperature to confer higher membrane fluidity and saturated FA at high temperature to increase the cell membrane's rigidity (Nalley et al., 2018; Renaud et al., 2002). Studies investigated that low-temperature stress causes an increase in the lipid and unsaturated fatty acid content in *Chlamydomonas reinhardtii*, *Scenedesmus acutus*, and *Phaeodactylum tricornutum* and other algal species (Chalifour et al., 2014; El-Sheekh et al., 2017; Jiang and Gao, 2004; Renaud et al., 2002; Sushchik et al., 2003; Ye et al., 2015; Zhao et al., 2020; Jiang & Chen, 2000).

In addition, several studies with high-temperature stress reveal an increase in the lipid content, accumulation of neutral lipids and saturated fatty acid in the cell membrane of *Acutodesmus dimorphus*, *Scenedesmus acutus*, *Cryptomonas* sp., *Rhodomonas* sp. and *Spirulina platensis* (Chokshi et al., 2015; El-Sheekh et al., 2017; Renaud et al., 2002; Sushchik et al., 2003). However, few conflicting reports show that a high temperature of 32-40ºC decreases the SFA content and increases the PUFA in chlorophytes, *Tetraselmis suecica*, and *Nannochloropsis* sp. (Nelly et al., 2018; Chaisutyakorn et al., 2018).

1.11.2 Nutrients and Fatty acids

Nutrient compositions also result in altered lipid content in algal species. However, conflicting report exists on the effect of a nutrient on lipid content in algal species (Xin et al., 2010; Rios et al., 2015; Solovchenko et al., 2008). Phosphorus limitation conditions cause an increase in the overall lipid content in *Monodus subterraneus*; *Scenedesmus obliquus*, and several other algae (Khozin Goldberg & Cohen's, 2006; Mandal and Mallick, 2009; Yang et al., 2018; Patel et al., 2017; Roopnarain et al., 2014; Feng et al., 2012). Phosphorus-deficient conditions also exhibited a decrease in overall lipid content in *Chlamydomonas reinhartdtii* (Sato et al., 2000) and *Scenedesmus* sp., (Xin et al., 2010). While sufficient phosphorus and nitrogen starvation with adequate phosphorus reveals the highest lipid productivity in *Microcoleus* and *Chlorella vulgaris* (Kumar et al., 2017; Chu et al., 2013).

Depleting nitrogen in the cultivation medium causes an increase in the lipid productivities of *Namnochloropsis*, *Desmodesmus* sp., *Parietochloris incisa*, and several algal

species (Rodolfi et al., 2009; Rios et al., 2015; Solovchenko et al., 2008; Xu et al., 2001; Zhu et al., 2014; Breuer et al., 2012; Anand et al., 2015; Cointet et al., 2019; Delgado et al., 2020; Li et al., 2008). Conflicting report of excess nitrogen concentration causing an increase in lipid content in *Microcoleus* sp., (Kumar et al., 2017) and a decrease in the fatty acid in phytoplankton (Yodsuwan et al., 2017) are present. Another study with sufficient nitrogen concentration showed an increase in the PUFA content of algae species compared to nitrogen deprivation, while the concentration of SFA and lipid content increased under nitrogen deprivation (Zarrinmehr et al., 2019; Zhu et al., 2014; Jia et al., 2015; Pancha et al., 2014; Feng et al., 2012; Savvidou et al., 2020; Delgado et al., 2020).

In another study, combining low nitrogen and phosphorus displayed a relatively higher lipid content in *Scenedesmus* sp., and *Chlorella* sp., (Zhang and Hong, 2014; Singh et al., 2015; Fan et al., 2014). Also, a study combining low temperatures with the nitrogen-replete and depleted condition shows an increase in the lipid content in *Chlamydomonas malina* (Morales-Sánchez et al., 2020).

1.12 Aim and Objectives

The burgeoning temperature of the Earth and amplified impervious surface area have increased the runoff into the aquatic bodies from various point and nonpoint sources. These factors are causing alterations in water bodies, which subsequently affect algae's physiological and biochemical processes. Thus, there is a need for an understanding of the effect of temperature and nutrient stress on physiological parameters such as growth, photosynthesis, respiration, pigment contents, and biochemical processes such as nitrogen fixation, fatty acid profile, membrane lipid phase transitions, membrane damage, protein oxidation, heat shock proteins, antioxidants (non-enzymatic and enzymatic) activities and expression of antioxidants genes. Change in the physiology of cyanobacteria under temperature and nutrients has been investigated but for a few cyanobacteria.

In this study we look at the effect of range of growth temperature and phosphorus and nitrogen content in the growth medium on morphological, physiological, biochemical, and molecular parameters in *N. spongiaeforme* a freshwater and *N. calcicola* a marine

cyanobacterial nitrogen-fixing gram-negative bacterial species belonging to order Nostocales and family Nostocaceae is used as a model organism.

Following objectives were set out to study:

OBJECTIVE:1 To study the effect of temperature and nutrients on morphology, growth, photosynthesis, and photosynthesis pigments

OBJECTIVE:2 To study the effect of temperature and nutrients on biochemical parameters

OBJECTIVE:3 To study the effect of temperature and nutrients on phenolic compounds and nitrogenase activity

OBJECTIVE:4 To study the effect of temperature and nutrients on protective processes of biochemical and molecular analysis

2.1 Cyanobacteria and Growth Conditions

Nostoc spongiaeforme Agardh (a freshwater cyanobacterium provided by Dr. Deepak Vyas, Dr. Hari Singh Grover University, Sagar, Madhya Pradesh), and *Nostoc calcicola* BDU 180601 Brebisson ex. Bornet & Flahault, (a marine cyanobacterium, procured from National Facility for Marine Cyanobacteria (NFMC), Bharathidasan University, Tiruchirappalli, Tamil Nadu, India) were used for the comparative account of their physiology and molecular parameters with regard to different growth temperatures and nutrient concentrations. Cultures of *Nostoc spongiaeforme,* were routinely grown in sterile BG-11 culture medium adjusted to pH 7.5 (Table. 2.1.1), and *Nostoc calcicola*, grown in ASN-III (Artificial Sea Nutrient-III) culture medium adjusted to pH 7.8 (Table. 2.1.2) following the method of Rippka et al. (1979). Cultures were maintained in 100 ml conical flasks filled to 40% of their volume and kept on a temperature-regulated shaker (Scigenics Biotech) with 150 rpm at temperature of 30ºC under continuous fluorescent white light (80 µmol photon $m^{-2}s^{-1}$) and photoperiod of 16:8 h. The growth of *Nostoc* spp., was monitored every alternate day by taking optical density (O.D) at 680 nm using UV-Visible spectrophotometry (UV-2450, Shimadzu model, Japan) and measuring chlorophyll fluorescence (Fv/Fm ratio) using a Pulse amplitude modulation system chlorophyll fluorometer (PAM, Walz, Germany) till both the *Nostoc* spp., achieved their exponential stage (Wang et al., 2012). The culture of *Nostoc spongiaeforme* reached its exponential stage on the 13th day and *Nostoc calcicola* on the 25th day at 30ºC. All experiments were conducted with 13 and 25 days grown *N. spongiaeforme* and *N. calcicola* cultures.

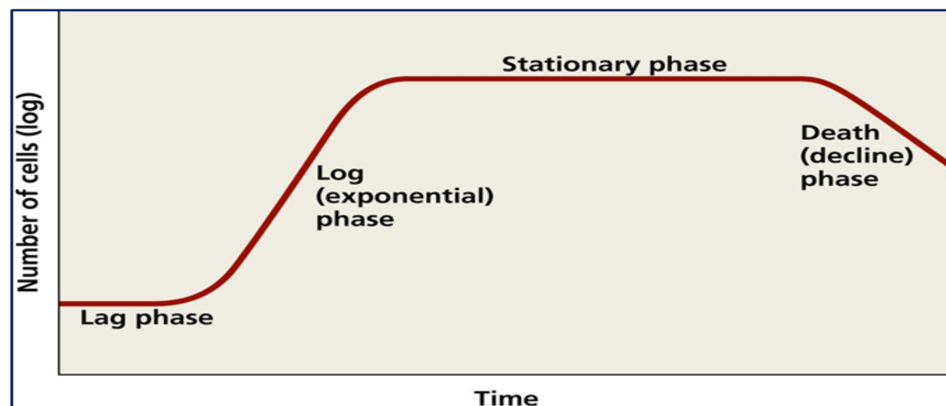


Fig. 2.1 Graphical representation of four phases of growth curve (adopted from https://tinyurl.com/2p8eadhy)

2.2 Temperature Treatment

Once the exponential phase of 13 days for *N. spongiaeforme* and 25 days for *N. calcicola* was determined, on the basis of O.D at 680 nm, and Fv/Fm ratio, both the *Nostoc* species were grown for temperature treatment at 10, 15, 20, 25, 30, 35, and 40ºC with other growth condition being same as mentioned in section 2.1 for their respective exponential growth phase of 13 and 25 days.

Table. 2.1.1 BG-11 media composition (pH 7.5)

Sr. No	Macronutrient	Concentration g L^{-1}
1	Sodium nitrate ($NaNO_3$)	1.5
2	Potassium hydrogen phosphate ($K_2HPO_4 \cdot 3H_2O$)	0.04
3	Magnesium sulphate anhydrous ($MgSO_4 \cdot 7H_2O$)	0.075
4	Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.036
5	Citric acid	0.006
6	Sodium carbonate anhydrous ($NaCO_3$)	0.02
7	Ferric ammonium citrate	0.006
8	EDTA (Di sodium salt)	0.001
9	Trace minerals	1 ml
10	Distilled water	1 L
	Micronutrient	Concentration g L^{-1}
1	Boric acid (H_3BO_3)	2.86
2	Manganese chloride ($MnCl_2 \cdot 4H_2O$)	1.81
3	Zinc sulphate ($ZnSO_4 \cdot 7H_2O$)	0.222
4	Sodium molybdate ($NaMoO_4 \cdot 2H_2O$)	0.39
5	Copper sulphate ($CuSO_4 \cdot 5H_2O$)	0.079
6	Cobalt nitrate hexahydrate ($Co(NO_3)_2 \cdot 6H_2O$)	49.4
7	Distilled water	1.0 L

Table. 2.1.2 ASN-III media composition (pH 7.8)

Sr. No	Macronutrient	Concentration g L^{-1}
1	Sodium chloride (NaCl)	25
2	Magnesium sulphate ($MgSO_4.7\ H_2O$)	3.5
3	Magnesium chloride ($MgCl_2.\ 6H_2O$)	2
4	Sodium nitrate ($NaNO_3$)	0.75
5	Potassium hydrogen phosphate (K_2HPO_4)	0.02
6	Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.5
7	Potassium chloride (KCl)	0.5
8	Sodium carbonate anhydrous ($NaCO_3$)	0.02
9	Citric acid	0.003
10	Ferric ammonium citrate	0.003
11	EDTA (Di sodium salt)	0.0005
12	Trace minerals	1 ml
13	Distilled water	1 L
	Micronutrient	Concentration g L^{-1}
1	Boric acid (H_3BO_3)	2.86
2	Manganese chloride ($MnCl_2 \cdot 4H_2O$)	1.81
3	Zinc sulphate ($ZnSO_4 \cdot 7H_2O$)	0.222
4	Sodium molybdate ($NaMoO_4 \cdot 2H_2O$)	0.39
5	Copper sulphate ($CuSO_4 \cdot 5H_2O$)	0.079
6	Cobalt nitrate hexahydrate ($Co\ (NO_3)_2 \cdot 6H_2O$)	49.4
7	Distilled water	1.0 L

2.3 Nutrient treatment

Nostoc spongiaeforme and *N. calcicola* were grown at 30ºC with culture and growth conditions being same as mentioned in section 2.1 except for the amount of phosphorus and nitrogen. The cultures were treated (grown) with nutrient medium containing double the

concentration of nitrogen or phosphorus (DN or DP), nitrogen starvation (-N), phosphorus starvation (-P), and combined starvation of nitrogen and phosphorus (-NP) for their respective exponential growth phase i.e., 13 days for *N. spongiaeforme* and 25 days for *N. calcicola.*

The parameters studied included the study of morphology (scanning electron microscope and light microscope), growth, biomass, chlorophyll fluorescence, photosynthetic and respiratory rate, pigment analysis, phenolic compounds, reactive oxygen species (ROS), i.e., H_2O_2 and $OH^{\cdot}$ lipid peroxidation, protein oxidation, enzymatic antioxidant activity (SOD, APX, and CAT), non-enzymatic antioxidant (proline and ascorbate), lipid-phase transition, acetylene reduction assay, fatty acid analysis, and expression of genes for antioxidants enzymes (SOD, CAT), heat shock proteins (HSP), and nitrogen fixing gene (*nif H*).

2.4 Morphological studies

2.4.1 External morphological study

The alterations in surface morphology of *Nostoc* cells under different temperatures and nutrient concentrations were studied using scanning electron microscopy (SEM) (Carl Zeiss EVO 18, Germany). The *Nostoc* culture (50 ml) was centrifuged at 4,000 *g* and suspended in preservation buffer (5% glutaraldehyde prepared in 2 ml 0.1 M phosphate buffer with pH 7.2) for 4 h. Then, the cells were washed thrice with preservation buffer and dehydrated using graded ethanol in the ascending series of 35, 50, 75, 95, and 100%. *Nostoc* cells were centrifuged, the pellet was collected and loaded onto a copper-coated grid, and cells were examined under the SEM.

2.4.2 Internal morphological study

Internal morphological studies were carried out using a light microscope (Nikon, eclipse, E200). Fresh cultures were mounted on the slide and observed under 100X magnification.

2.5 Measurement of growth and growth rate

The growth was determined using a UV-Visible spectrophotometer (UV2450, Shimadzu model, Japan) according to wang et al. (2012). The 20 ml of *Nostoc* culture was homogenized in a glass homogenizer for approximately 15 min to create a uniform suspension and measuring the absorbance at 680 nm. The growth rate (μ, day^{-1}) was calculated following Widdel, (2010) formula:

$$\mu = \ln (O.D.f) - \ln (O.D.i) / Tf - Ti \qquad (1)$$

Where μ (day^{-1}) is the specific growth rate, O.D.*f* is the optical density at the final time (T*f*), and O.D.*i* is the O.D at the initial time (Ti) of the log phase of the growth curve.

Biomass and biomass productivity: The biomass concentration (g L^{-1}) was measured by dry weight determination. *Nostoc spongiaeforme* and *N. calciocla* at their exponential phase were harvested by centrifuging at 6,700 *g* for 10 min. The fresh weight of the pellet was measured and same pellet was dried at 60ºC for 72 h for dry weight measurement. The biomass productivity was calculated following equation (2) according to Song et al. (2013).

$$\text{Biomass productivity } (g\ L^{-1}\ day^{-1}) = \mu \times \text{biomass} \qquad (2)$$

Where μ is the growth rate, and biomass concentration was calculated from the difference in fresh weight and dry weight.

2.6 Photosynthetic measurement

2.6.1 Chlorophyll fluorescence (Fv/Fm ratio) measurement

The Fv/Fm ratio, an indicator of photosynthetic efficiency, was determined using a Pulse amplitude modulation system chlorophyll fluorometer (PAM, Walz, Germany) according to Bhandari & Sharma, (2006) (Fig. 2.6A). The culture was dark adapted for 10 min to allow all the reaction centers to be in the open state. The dark-adapted culture was exposed to a modulated light of 6μmol m^{-2} s^{-1} to measure initial fluorescence (Fo), followed by exposure to a saturating pulse of the white light of 4,000 μmol $m^{-2}s^{-1}$ to estimate the maximum fluorescence (Fm). Variable fluorescence (Fv) was determined by subtracting the initial fluorescence from maximal fluorescence (Fv = Fm - Fo), and the maximal quantum efficiency yield (Fv/Fm) ratio was calculated.

2.6.2 Measurement of photosynthetic and respiratory rate

The photosynthetic and respiratory rate were measured according to Delieu & Walker, (1972) using a Clark-type oxygen electrode (Oxygraph, Hansatech Instrument Ltd., Norflok, UK) (Fig. 2.6B). During the measurement, 1 ml homogenized culture, and 1 ml 0.1 M sodium bicarbonate ($NaHCO_3$) was added into the reaction cuvette, with adequate stirring for 2 min. The photosynthetic rate was measured by exposing the culture to white light of 1,200 µmol $m^{-2}s^{-1}$ PAR while the respiratory rate was measured in the dark by covering the oxygraph chamber with a black cloth and expressed in nmoles of O_2 evolved or consumed, ml^{-1} of culture respectively.

2.6.3 Extraction and analysis of photosynthetic pigments using High-Performance Liquid Chromatography (HPLC)

The culture of *N. spongaieforme* and *N. calcicola* were harvested at 6,700 *g* for 10 min and washed twice with sterile BG-11 and ASN-III media, respectively, and again centrifuged for 10 min at 6,700 *g*. The pellet was pooled out in a 10 ml glass vial and allowed to dry by lyophilizing at -108ºC for 8 h using a freeze dryer. Lyophilized algal tissue (0.05 g) was ground in 80% methanol (Merck, HPLC grade), making a final volume of 2 ml, and incubated at 4ºC for overnight and centrifuged at 4,000 *g* for 10 min at 4ºC (Z32HK, HERMLE Labortechnik GmbH, Germany). The supernatant was collected and filtered using 0.2 µm Millipore filter (Ultipor®N66®Nylon membrane filter, PALL Life Sciences, USA). The filtered sample of 10 µl was injected and analyzed on the HPLC system (Water, USA) (Fig. 2.6C) according to Sharma & Hall, (1996). The mobile phase used consisted of a linear gradient of ethyl acetate and acetonitrile: water, (9:1) at the flow rate of 1.2 ml min^{-1} for 30 min run time. C18 column (Waters Spherisorb ODS2-250 mm x 4.6nm x 5 µm) was used to separate the pigments. The peak was determined at 445 nm based on RT and spectral characteristics with a Water 2996 photodiode array detector. *β*-carotene was used as an external standard for the relative quantification of pigments (Sharma & Hall, 1996).

2.6.4 Extraction and analysis of phycobilisomes pigments

Lyophilized culture of 0.05 g was used to extract phycobilisomes pigments by suspending in 2 ml of 50 mM phosphate buffer (pH 6.7) with repeated freezing and

thawing (9 cycles) for total of 10 min and centrifuged at 6,700 *g* for 15 min at RT. The supernatant was collected and absorbance was read at 615 and 652 nm, using phosphate buffer as blank, using a UV-Visible spectrophotometer (UV2450, Shimadzu model, Japan). The concentration of phycocyanin (PC) and allophycocyanin (APC) was calculated using the formula given by Johnson et al. (2014).

$$\mathbf{PC\ (\mu g\ ml^{-1}) = \frac{A615 - 0.474\ (A652)}{5.34}}$$

$$\mathbf{APC\ (\mu g\ ml^{-1}) = \frac{A652 - 0.208\ (A615)}{5.09}}$$

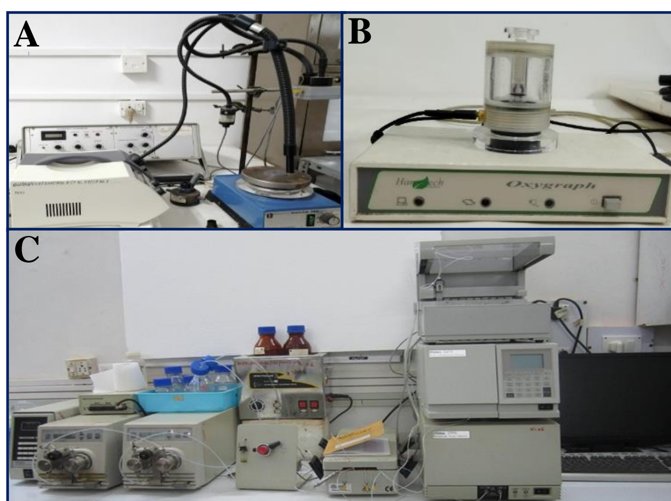


Fig. 2.6 Tools to measuring photosynthetic responses of cyanobacteria under temperature and nutrients stress: Pulse amplitude modulating system (A); Oxygraph system (B); High-performance liquid chromatography (C).

2.7 Extraction and estimation of phenols and flavonoids

The *Nostoc* cells were harvested by centrifuging at 6,700 *g* for 10 min at 4ºC, and lyophilized. 0.05 g of the pellet was suspended in 2 ml of 70% ethanol and sonicated using an Ultrasonic bath (Spectralab Model UCB 20) to disrupt cells and homogenized at 4ºC in mortar and pestle. The homogenate was centrifuged at 800 *g* for 15 min at 4ºC. The supernatant was collected in a fresh eppendorf tube and centrifuged at 800 *g* for 10 min, and the supernatant was filtered through 0.2 µm Millipore filter (Ultipor®N66®Nylon

membrane filter, PALL Life Sciences, USA), evaporated to dryness and dissolved in 100% methanol for the estimation of phenol and flavonoid.

a) Estimation of phenols

The total phenolic content was estimated by following the Folin-Ciocalteu method according to Lister & Wilson, (2001). The reaction mixture containing 100 µl of the extract in 500 µl of Folin-Ciocalteu reagent (1:10 dilution with water) was mixed and incubated for 3 min at RT. 20% of sodium carbonate (Na_2CO_3) (1.5 ml) was added, mixed, and again incubated for 2 h in the dark, and absorbance was read at 765 nm using a UV-Visible spectrophotometer (UV-2450). Gallic acid was used as a standard for quantitation.

b) Estimation of flavonoids content

The total flavonoids content was estimated based on the formation of complex flavonoid aluminum in the presence of aluminum chloride ($AlCl_3$) using UV-spectrophotometer according to Djeridane et al. (2006). 1 ml extract was mixed with 1 ml of $AlCl_3$ methanolic solution (2% w/v), and incubation for 15 min at RT and absorbance was read at 430 nm. The total flavonoid was assessed using quercetin as standard.

2.8 Determination of ROS generation

2.8.1 Hydrogen peroxide (H_2O_2)

H_2O_2 is formed when $O_2^{\bullet-}$ undergoes univalent reduction, and it is produced in organisms via two possible pathways: dismutation of $O_2^{\bullet-}$ with the involvement of SOD and via oxides such as amines and oxalate oxidases (Murata et al., 2007; Takahashi & Murata, 2008). The total H_2O_2 content was determined according to Sagisaka et al. (1976). Lyophilized tissue of 0.05 g, homogenized in 5 ml of 5% trichloroacetic acid (TCA, Merck), and centrifuged at 3,000 *g* for 10 min at 4℃ (Eppendorf, centrifuge 5804). The supernatant (1 ml) was added with 40 µl of 50% TCA, 20 µl of 2.5 M potassium thiocyanate (Himedia), and 40 µl of 10 mM ferrous ammonium sulfate (Himedia) and incubated for 30 s at RT. Absorbance was taken at 480 nm using a UV-Visible

spectrophotometer (UV2450, Shimadzu, Japan). The concentration of H_2O_2 was determined as µmol g^{-1} sample FW using H_2O_2 as standard.

2.8.2 Hydroxyl radical (OH•)

Hydroxyl radical is generated during the Fenton reaction between hydrogen peroxide and superoxide radicals in the presence of any transition metals**.** Hydroxyl radical (OH•) content was measured according to Liu et al. (2009). Lyophilized tissue of 0.05 g was homogenized in 2 ml of 5% TCA and centrifuged at 6,700 *g* (Eppendorf, centrifuge 5804) for 10 min at 4℃. 500 µl supernatant was added with 500 µl of 50 mM sodium phosphate buffer (pH 7.0) (Himedia) and 1 ml of 25 mM sodium phosphate buffer (pH 7.0) containing 2.5 mM, 2-deoxyribose (Sigma) and incubated in the dark for 1 h at 35℃. After incubation, 1 ml of 1% thiobarbituric acid (TBA) and glacial acetic acid (GAA) (Merck) was added to the reaction mixture and incubated at 95℃ in the dry bath for 10 min (WiseTherm HB-48P), cooled immediately on ice, and absorbance was read at 532 nm using a UV-Visible spectrophotometer (UV2450, Shimadzu, Japan). The OH• content was determined as absorbance units per gram sample fresh weight (Absorbance x 1000).

2.9 Oxidative damage caused by ROS

2.9.1 Lipid peroxidation (MDA)

Lipid peroxidation was determined by measuring the adduct formation of TBA-MDA (thiobarbituric acid-malondialdehyde), which indicates the formation of polyunsaturated fatty acid peroxides in biological systems, according to Bhandari & Sharma, (2007). Lyophilized tissue of 0.05 g was ground in 4 ml of 1% TCA and centrifuged for 5 min at 300 *g* (Eppendorf, centrifuge 5804). The reaction mixture contained 1 ml of supernatant, 1.5 ml of incubation buffer (50 mM Tris HCL, 175 mM NaCl at pH 8.0), and 1.5 ml of 0.5% TBA prepared in 20% TCA. The reaction mixture was incubated at 95℃ in a dry bath for 30 min (WiseTherm HB-48P). The reaction mixture was cooled to RT, and absorbance was measured at 532 and 600 nm using a UV-Visible spectrophotometer (UV-2450, Shimadzu, Japan). Non-specific turbidity was corrected by subtracting absorbance measured at 600 nm from 532 nm. MDA content was calculated

using an extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as µmol gm^{-1} of FW tissue.

2.9.2 Protein oxidation (PO)

The protein-carbonyl content is the most commonly used marker for ROS-mediated protein oxidation under stress. Protein carbonyl content was measured according to Vega-López et al. (2013). Lyophilized *Nostoc* tissue of 0.05 g was ground in 2 ml of 30% TCA (Merck) and centrifuged at 1,000 *g* (Eppendorf, centrifuge 5804) for 5 min at 4ºC. The supernatant, of 1 ml was added with 500 µl of 10 mmol 2,4-dinitrophenyl hydrazine (DNPH) (Himedia AR grade) prepared in 2 N hydrochloric acid (HCl) and vortexed for 30 s. The reaction mixture was incubated for 1 h at RT with regular vortexing every 10 min. The mixture was centrifuged at 1,600 *g* for 5 min at 4ºC, and the pallet was washed with ethanol:ethyl acetate (1:1 V/V) (Merck) to eliminate excess DNPH. The residue dissolved in 1 ml of 6 M urea in 20 mM potassium phosphate at 2.5 pH (Himedia) and incubated at 37ºC for 1 h, and absorbance was read at 366 nm using a UV-Visible spectrophotometer (UV2450, Shimadzu, Japan). The amount of protein was estimated using Bradford’s methods at 595 nm. The protein carbonyl content was estimated using an extinction coefficient of 0.022μM^{-1} cm^{-1} and reported in µmol mg^{-1} of protein.

Protein oxidation (µmol mg^{-1} of protein) = Average/ 0.022μM^{-1} cm^{-1} (a/b)

Where, extinction coefficient = 0.022μM^{-1} cm^{-1}

a = volume of sample used (µl)

b = volume of urea used (µl)

2.10 Measurement of enzymatic and non-enzymatic antioxidants

2.10.1 Protein estimation

The protein estimation was measured according to Bradford, (1976). Lyophilized 0.05 g tissue was homogenized in 2 ml of 0.5 M phosphate buffer (pH 7.8) and centrifuged at 6,700 *g* for 10 min at 4ºC. The resulting supernatant was used for protein estimation. 200 µl sample was mixed with 1 ml Bradford's reagent, vortexed, and incubated for 5 min

at RT, and, absorbance was measured at 595 nm. The protein content was determined using bovine serum albumin (BSA) as standard.

2.10.2 Superoxide dismutase (SOD) activity

SOD provides the first line of defense against elevated ROS levels. It dismutates $O_2^{\bullet-}$ into oxygen and hydrogen peroxide via the Haber-Weiss-type reaction and Mehler's reaction. The SOD activity was measured according to Dhindsa et al. (1981). Lyophilized 0.05 g tissue was homogenized in 2 ml of 0.5 M sodium phosphate buffer (pH 7.5) and centrifuged at 6,700 *g* for 10 min at 4ºC (Eppendorf, centrifuge 5804). The SOD activity was determined by its ability to prevent photochemical inhibition of nitro blue tetrazolium (NBT, Sigma). The reaction mixture contained 1.5 ml reaction buffer containing 0.5 M phosphate buffer of pH 7.5 and 0.1 M phosphate buffer of pH 7.8, 200 µl of 200 mM methionine, 100 µl of 1 mM Na_2CO_3, 100 µl of 2.25 mM NBT, 100 µl of 3 mM EDTA, 100 µl of 60 µM riboflavin (added at the end) and 100 µl of crude enzyme extract. The reaction mixture was shaken and placed under 11 W white fluorescent light for 15 min till the blue color developed. A standard containing only the reaction mixture was also placed along with the sample in light. The reaction mixture, along with enzyme extract, placed in the dark, was considered blank. Further, the reaction was terminated by switching off the white fluorescent light, and placed in the dark (covered with black cloth). After incubation, the absorbance of the sample and standard was measured at 560 nm against blank using a UV-visible spectrophotometer. The change in percentage of reduction in the color between the standard and sample was calculated. 50% reduction in the color was considered as one unit of SOD activity and expressed as Unit mg^{-1} of protein min^{-1}. The protein concentration of enzyme extract was measured using the Bradford method at 595 nm.

2.10.3 Ascorbate peroxidase (APX)

APX plays a vital role in detoxifying H_2O_2 to H_2O and DHA by using ascorbate as an electron donor (Das & Roychoudhury, 2014). The activity of APX was estimated

according to Nakano & Asada, (1981). Lyophilized 0.05 g *Nostoc* tissue extracted in 2 ml of lysis buffer containing 0.1 M potassium phosphate buffer with 7.8 pH, 1% of polyvinylpyrrolidone (PVP), and 1 mM ethylenediamine tetra acetic acid (EDTA). The homogenate was centrifuged at 4,000 *g* (Eppendorf, centrifuge 5804) for 30 min at 4ºC. The reaction mixture contained 1 ml of 50 mM potassium phosphate buffer with pH 7.2, 100 µl of 0.1 mM H_2O_2, 100 µl of 0.5 mM ascorbate, 100 µl of 0.5 mM EDTA, and 100 µl of enzyme extract. The reaction mixture was incubated for 30 s before reading the absorbance at 290 nm using a UV-Visible spectrophotometer (UV2450, Shimadzu, Japan). The APX activity was estimated using the average and extinction coefficient of 2.8 mM^{-1} cm^{-1} (ε=2.8 mM^{-1} cm^{-1}) and expressed as Unit μg^{-1} of protein min^{-1}. The protein concentration of enzyme extract was measured using the Bradford method at 595 nm.

2.10.4 Catalase activity (CAT)

Just like APX, CAT is also responsible for dismutating H_2O_2 into water and oxygen. The activity of CAT was determined according to the method of Aebi, (1984). Lyophilized tissue of 0.05 g extracted in 2 ml lysis buffer (0.1 M potassium phosphate buffer with 7.8 pH, 1% PVP, and 1 mM EDTA) centrifuged at 4,000 *g* (Eppendorf, centrifuge 5804) for 30 min at 4ºC, and the supernatant used for the CAT assay. The reaction mixture contained 1 ml of 50 mM potassium phosphate buffer with pH 7.0, 200 µl of H_2O_2, and 200 µl of enzyme extract. The absorbance was read at 240 nm using a UV-Visible spectrophotometer (UV2450, Shimadzu, Japan), and enzyme activity was expressed as Unit mg^{-1} of protein min^{-1}. The protein concentration of enzyme extract was measured using the Bradford method at 595 nm.

2.10.5 Ascorbic acid (AsA)

Ascorbic acid is a non-enzymatic antioxidant involved in donating electrons to a broad range of non-enzymatic and enzymatic reactions and removes OH•, O_2•−, and $^1O_2$ and reduces H_2O_2 to H_2O through APX reaction (Blokhina et al., 2003). The estimation of AsA was measured according to Kampfenkel et al. (1995). Lyophilized tissue of 0.05 g was homogenized with 2 ml of extraction buffer (0.1 M HCl + 0.1 mM EDTA). The

homogenate was centrifuged at 10,000 *g* (Eppendorf, centrifuge 5804) for 2 min at 4℃, and the supernatant was used for the ascorbate assay. For the ascorbate assay, the reaction mixture contained 100 µl of supernatant, 100 µl of 0.4 M phosphate buffer (pH 7.4), 400 µl of color reagent (containing two solutions [A] TCA (4.6%), H_3PO_4 (15.3%), $FeCl_3$ (0.6%) and [B] 2, 2 dipyridyl (4%) in 70% ethanol, in the ratio of 2.75:1) and 50 µl of 0.5% N-ethylmaleimide. The mixture was thoroughly mixed and incubated for 45 min at 42℃, and absorbance was measured at 520 nm. The ascorbic acid was used as standard, and the ascorbate content was expressed in µmol g^{-1} FW.

2.10.6 Proline content

Proline is a multifunctional molecule that acts as a signaling molecule, an antioxidative defense molecule, a metal chelator, and an osmoprotectant. Proline content was estimated according to Bates et al. (1973). Lyophilized tissue of 0.05 g was ground in 3% sulfosalicylic acid (5ml) and centrifuged at 1,600 *g* for 5 min (Eppendorf, centrifuge 5804). The reaction mixture contained 1 ml of supernatant, 1 ml of acid ninhydrin (a mixture containing 1.25 g ninhydrin, 30 ml of GAA, 20 ml of phosphoric acid), and 1 ml of GAA was incubated for 1 h at 95℃ in the dry bath (WiseTherm HB-48P). After cooling, 10 ml of toluene was added and mixed vigorously. The reaction mixture was further incubated for 30 min at RT, and absorbance was read at 520 nm using a UV-Visible spectrophotometer (2450UV, Shimadzu, Japan). Proline concentration was calculated using the L-Proline as standard and expressed as µmol g^{-1} FW.

2.11 Fatty acid extraction

Fatty acid extraction was measured according to Turnham & Northcote, (1984). Lyophilized tissue of 0.05 g was transferred to 50 ml graduated centrifuge tube with 5 ml of pre-cooled (4℃) 1:2 (v/v) chloroform: methanol, spin-vortex for 3 min and sonicated for 15 min in a Sonicator. The sample was then centrifuged at 1,600 *g* for 5 min at 4℃, and the supernatant was collected in a fresh tube, and 2.5 ml pre-cooled 1:1 (v/v) chloroform: methanol was added to the pellet, which was vortexed and centrifuged at 1,600 *g* for 5 min at 4℃. Again, the supernatant was pooled, and this step was repeated for 4 times. The

pooled supernatant was filtered using Whatman filter paper and washed using an equal volume of potassium phosphate buffer (pH 7.5). The filtrate was centrifuged at 1,600 *g* for 5 min at 4ºC, and the lower organic phase was collected. Total lipids were dried using nitrogen gas, and the total dried lipid was stored at -20ºC for further analysis.

Conversion of total lipid to fatty acid methyl esters (FAMEs) by transmethylation

The fatty acid profile of both the *Nostoc* sp., was achieved using Gas chromatography coupled with a Mass Spectrophotometer (GC-MS). Total lipid was dissolved in 1 ml of methanolic NaOH and kept for drying at 80ºC for 5 h. After drying, 2 ml of 5% methanolic hydrochloric acid was added and vortex for 20 s, followed by drying at 80ºC for 5 h. To this, 1 ml of water (Milli Q) and 2 ml of 100% Hexane (HPLC grade) was added, vortexed for 90 s, and allowed to settle down. Further, the top layer with hexane was transferred into a new vial. This step was repeated thrice, and at the end, the top layer was added with 1 ml saturated sodium bicarbonate, vortexed for 15 s, and allowed to settle down. Again, the upper layer was collected, and 1 ml sterile water was added with a few crystals of $CaCl_2$ and allowed to settle down. Further, the top layer was transferred into a new vial and dried with the help of N_2 gas. Before injecting into the GC-MS injector, the sample was dissolved in 500 µl of 100% hexane.

GC-MS analysis

Chromatography was performed using a Gas chromatography-Mass spectrometer (2010 plus Shimadzu, Japan) equipped with GC-MS-TQ8030 detector, and an auto-sampler (AOC-20s) (Fig. 2.11). An electron ionization system with an ionization energy of 70eV was used for GC-MS analysis. Helium was used as the carrier gas with a flow rate of 1 ml min^{-1}. The column oven temperature was set to 270ºC and the injection temperature to 250ºC. Further, the compound was identified by matching the mass spectra with the inbuilt library from the National Institute of Standards and Technology (NIST).

Fig. 2.11 Gas chromatography-Mass Spectroscopy

2.12 Phase transition of thylakoid membrane

The phase transition of the thylakoid membrane was determined following Chaloub et al. (2003). The culture was centrifuged at 4,000 *g* for 5 min at RT and re-suspended in fresh culture media, and dark-adapted for 5 min in the presence of 15µM 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) to eliminate the influence of the photochemical reactions of photosynthesis on fluorescence yield. The temperature of phase transition was obtained from curves of fluorescence yield versus temperature by exciting chl *a* at 430 nm and detecting the fluorescence at 684 nm using a Fluorescence spectrophotometer, RF-6000 Shimadzu.

2.13 Nitrogenase enzyme

Nitrogenase (ARA, E.C. 1.18.6.1) activity was measured by Acetylene Reduction Assay (ARA) according to Stewart et al. (1967). Glass vial of 13 ml capacity containing 5 ml freshly grown *Nostoc* spp., cultures were bubbled with argon gas for about 2 min and sealed the vials with rubber septa and secured with aluminum cap using a seal crimper. 10% gas phase was withdrawn from the vial using a disposable syringe (26-gauge needle), and injected the same quantity of acetylene gas into it. The vials were then incubated at 30ºC for 2 h. At the end of the incubation period, 0.2 ml of 20% TCA (Merck) was injected into each vial to terminate the enzyme activity.

Fig. 2.13 Gas chromatograph

For the determination of ethylene concentration, 25 µl of gas-phase was pulled out from the vial and injected into the Gas chromatography (GC-2014, Shimadzu) equipped with (5%-phenyl)-methylpolysiloxane phase (HP-5ms column), nitrogen as the carrier gas with a flow rate at 30 ml min^{-1} and detected with a Flame Ionization Detector (FID). The injection port temperature was set to 180ºC, oven temperatures at 90ºC, and detector temperature at 230ºC (Fig. 2.13). The amount of ethylene generated was calculated using ethylene gas as standard (Sigma Aldrich, USA). The results were expressed in nmol of ethylene (C_2H_2) formed h^{-1} mg^{-1} protein.

2.14 Gene expression studies

Genes are responsible for synthesizing the functional gene product. Determining the changes in the expression level of the genes will provide information about the molecular responses influenced by different growth temperatures and nutrient treatment in both the *Nostoc* species. These changes can further relate to the biochemical parameters to determine the response to different growth temperatures and nutrient treatments. Gene expression analysis was done using RT PCR, and the purity of the genes was determined using the Tm curve of the primers.

RNA Extraction and quantification- 0.05 g lyophilized tissue was ground using liquid nitrogen and extracted using the RNA purification reagent (Invitrogen). The tissues were homogenized in 0.5 ml of RNA extraction buffer (Invitrogen) and collected in an eppendorf tube. The content was mixed by flicking the tube gently, and incubated at RT for 10 min, and spun at 10,000 *g* for 4 min. The supernatant was transferred carefully into a fresh eppendorf tube, and 100 µl of 5 M NaCl and 300 µl of chloroform were added and mixed thoroughly by inversion. The content was centrifuged at 10,000 *g* for 10 min at 4ºC. The top aqueous phase was transferred in a fresh eppendorf tube and added with an equal volume of isopropanol, mixed and incubated for 15 min at RT, and centrifuged at 10,000 *g* for 15 min at 4ºC. Pellet was washed with 1 ml of 70% ethanol, followed by centrifugation at 10,000 *g* for 3 min at 4ºC and the pellet was dissolved in 50 µl of mili Q water and quantified at 260 nm using a UV-Visible nanodrop spectrophotometer (Shimadzu, Japan). RNA quantification was done and expressed as ng of RNA μl^{-1}.

Preparation of cDNA and Real-time analysis: cDNA synthesis was performed using a PCR kit (Invitrogen Superscript III Reverse Transcriptase, Catalogue number 18080/093/044/085) following the given manufacturer's protocol. 5 µg of equivalent RNA, 1 µl of random primer, made up to 12 µl with sterile H_2O in PCR vials. Further, the content was heated for 5 min at 70ºC, cooled, and spun for 30 s. The content was further added with 5 µl of 5 X reaction buffer, 1.5 µl of 10 mM dNTPs, and 1 µl of superscript III RT, and the final volume was made up to 25 µl using sterile water. The reaction was performed at three different sequential temperatures of 25ºC for 10 min, 42ºC for 1 h, and 75ºC for 15 min using the MJ Miniopticon Real-time PCR detection system, Bio-Rad (Fig. 2.14). Gene expression using primers of SOD, CAT, HSP, and *nif H* was performed. The reaction was carried out in 25 µl total volume using the procedure given with the Bio-Rad kit (SsoAdvanced SYBR Green Supermix, Cat no: 172-5260). Briefly, cDNA prepared in the above step (5 µl), primer (2 µl), 2X master mix (12.5 µl), and sterile water (5.5 µl) was added in white optically flat-capped PCR tubes. Amplification was performed for 40 cycles. Data were analyzed using Bio-Rad CFX manager software. The primers (oligonucleotide, Table. 2.14) were bought from the Reprocell brand, bioserve.

Fig. 2.14 Real time PCR

Table. 2.14 List of primers (Reverse and Forward) used to study gene expression by Real-time PCR analysis acquired from Reprocell brand, Bioserve. Here, SOD = Superoxide dismutase, CAT = Catalase, HSP = Heat shock protein, and *nif* H = nitrogen fixing H gene

Sr. No	Primer names		Sequence (5'-3')
1	SOD	Forward	TGGTAGTGGTTGGGTTTGGT
		Reverse	ACGACGGTTGCGATATCTCA
2	CAT	Forward	TATGCTCCAGGACATTGCGA
		Reverse	ACATCCCCGCCCTCATTTAA
3	HSP	Forward	AGCCCTTCTCTTCTCTACGC
		Reverse	CCCGTTCCCTATTCCCTCTC
4	*nif H*	Forward	CCT GAT CGT TGG TTG TGA CCC T
		Reverse	AAG AAG TTG ATG GCG GTG AT

2.15 Statistical analysis

All experiments were conducted as a minimum in three biological repetitions, and data were represented in the mean value ± standard deviation (S.D.). All statistical analyses were carried out using Microsoft excel XLSTAT (version 2021.5.1.1241). A one-way ANOVA test was followed by a Dunnett (two-sided) Multiple Range Test where $p \leq 0.01$.

3.1 Growth curve

Nostoc spongiaeforme and *N. calcicola* cultures experienced a 43 days growth at 25ºC and 30ºC under a constant light intensity of 80µmol m^{-2} s^{-1}. During the 43 days of the growth at 25ºC and 30ºC, the highest biochemical activity (exponential phase) for *N. spongiaeforme* was observed on the 13th day, and for *N. calcicola* it was on the 25th day (Fig. 3.1). The growth of both the cyanobacteria was slightly higher at 30ºC than at 25ºC, measured based on O.D at 680 nm (Fig. 3.1.A) and Fv/Fm ratio (Fig. 3.1.B). Therefore, in this study, we have taken 30ºC as an optimum temperature to compare our data obtained during our experiment with temperatures and nutrients. For *N. spongiaeforme* all the analysis were done with 13 days old culture while for *N. calcilola* all analysis were done with 25 days old culture, their respective exponential phase (Fig. 3.1).

The results are divided into two sub-chapters; **sub-chapter 3.2,** deals with the effect of temperatures, and **sub-chapter 3.3**, deals with the effect of nutrients (starvation and excess of nitrogen and phosphorus) on *Nostoc spongiaeforme* a freshwater and *Nostoc calcicola* a marine cyanobacteria.

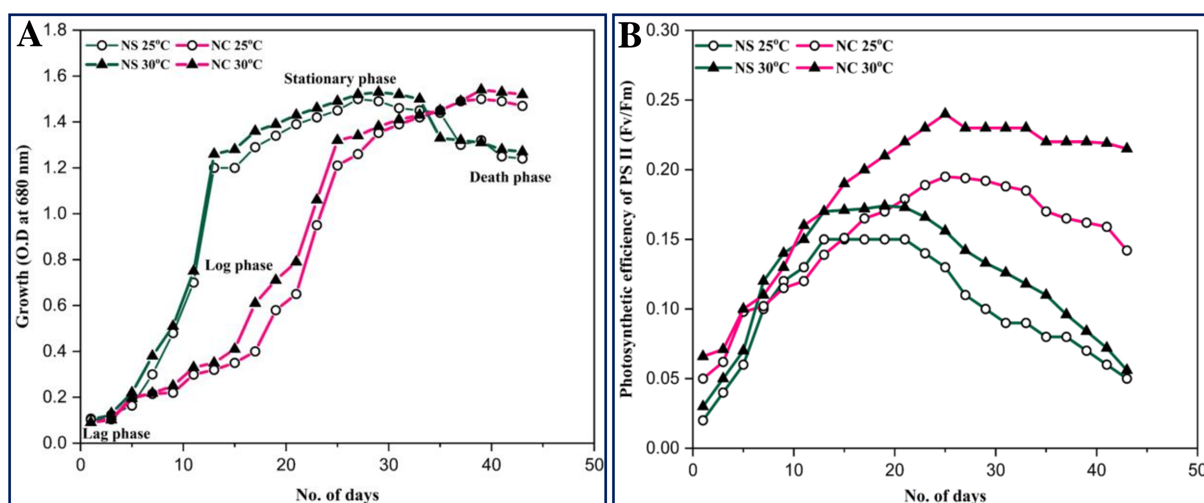


Fig. 3.1 The growth curve of *N. spongiaeforme* and *N. calcicola* grown at 25ºC and 30ºC, to determine the acceleration phase. The growth curve represents the lag, log, stationary, and death phase of both the *Nostoc* species.

Sub-chapter 3.2: Effect of temperature on various parameters

The current sub-chapter aims to understand the morphological, physiological, biochemical, and molecular responses of *N. spongiaeforme* (freshwater) and *N. calcicola* (marine) to different growth temperatures (10-40ºC).

3.2.1 Effect of temperature on *Nostoc* cell morphology

3.2.1.1 External morphology

External morphological changes in *N. spongiaeforme* and *N. calcicola* were studied in response to different growth temperatures. The effect of temperature on external morphology showed an adverse effect on the integrity of cell filament in both the *Nostoc* species (Fig. 3.2.1A & B). The culture of both the *Nostoc* species grown at the optimal temperature of 30ºC appeared healthy with a perfectly beaded shape and smooth texture; however, the cells of both the *Nostoc* species appeared desiccated, deformed, and paler at 10, 15, 20, and 35ºC. While at 40ºC, most of the *N. spongiaeforme* cells experienced severe distortion and shrinkage, leaving the cells with an aberrant shape, however, cells of *N. calcicola* when grown at 40ºC, were swelled, collapsed, filaments were severely broken, and cells were separated and turned unicellular.

3.2.1.2 Internal morphology

Internal morphological changes in *N. spongiaeforme* and *N. calcicola* due to different temperatures were also studied. Light microscope images of *N. spongiaeforme* and *N. calcicola* cells appeared normal and healthy when grown at 25-30ºC. Whereas suboptimal growth temperatures of 10-20ºC and supra-optimal temperatures of 35-40ºC resulted in size, shape, and color alterations. Cells of *N. spongiaeforme* at 10 and 15ºC were depigmented with no change in shape and size compared to the control. However, at 40ºC, the cells of *N. spongiaeforme* suffered swelling, aberrant breaking of filaments, and slight accumulation of blue pigment in the cells compared to cells grown at the optimum temperature of 30ºC (Fig. 3.2.1C). *Nostoc calcicola* when grown at 10ºC exhibited an alteration in cell shape, either elongated or bean-shaped, withered, and depigmented. Similarly, at 15ºC and 20ºC, the cells of *N. calcicola* were depigmented and swollen compared to the cells grown at the optimum

temperature. At a supra-optimal temperature of 40ºC, the *N. calcicola* cells were damaged, showing uneven size and breakage in the filament (Fig. 3.2.1D).

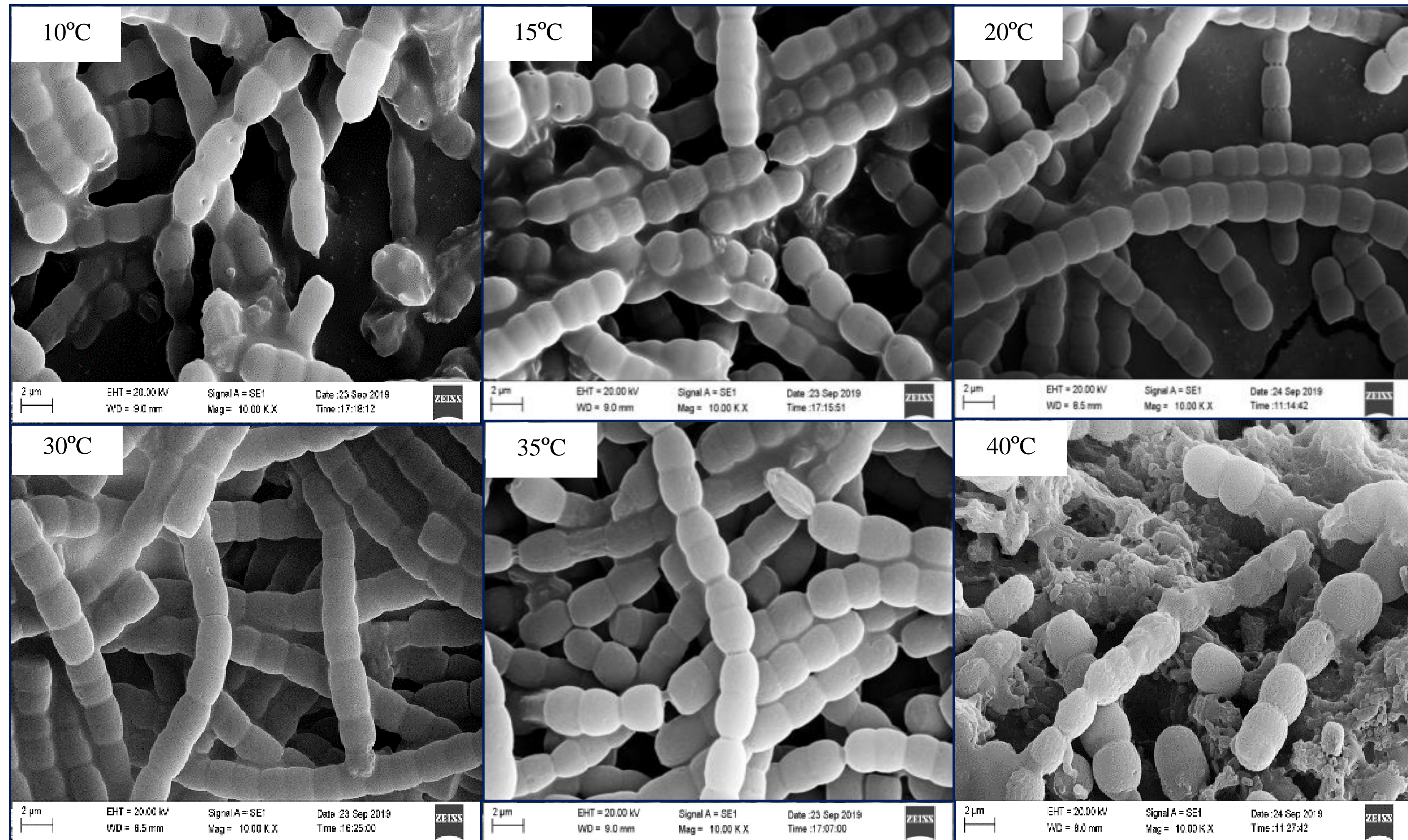


Fig. 3.2.1A Scanning electron microscopy (SEM) images of the *N. spongiaeforme* showing morphological variations at growth temperatures (10-40°C).

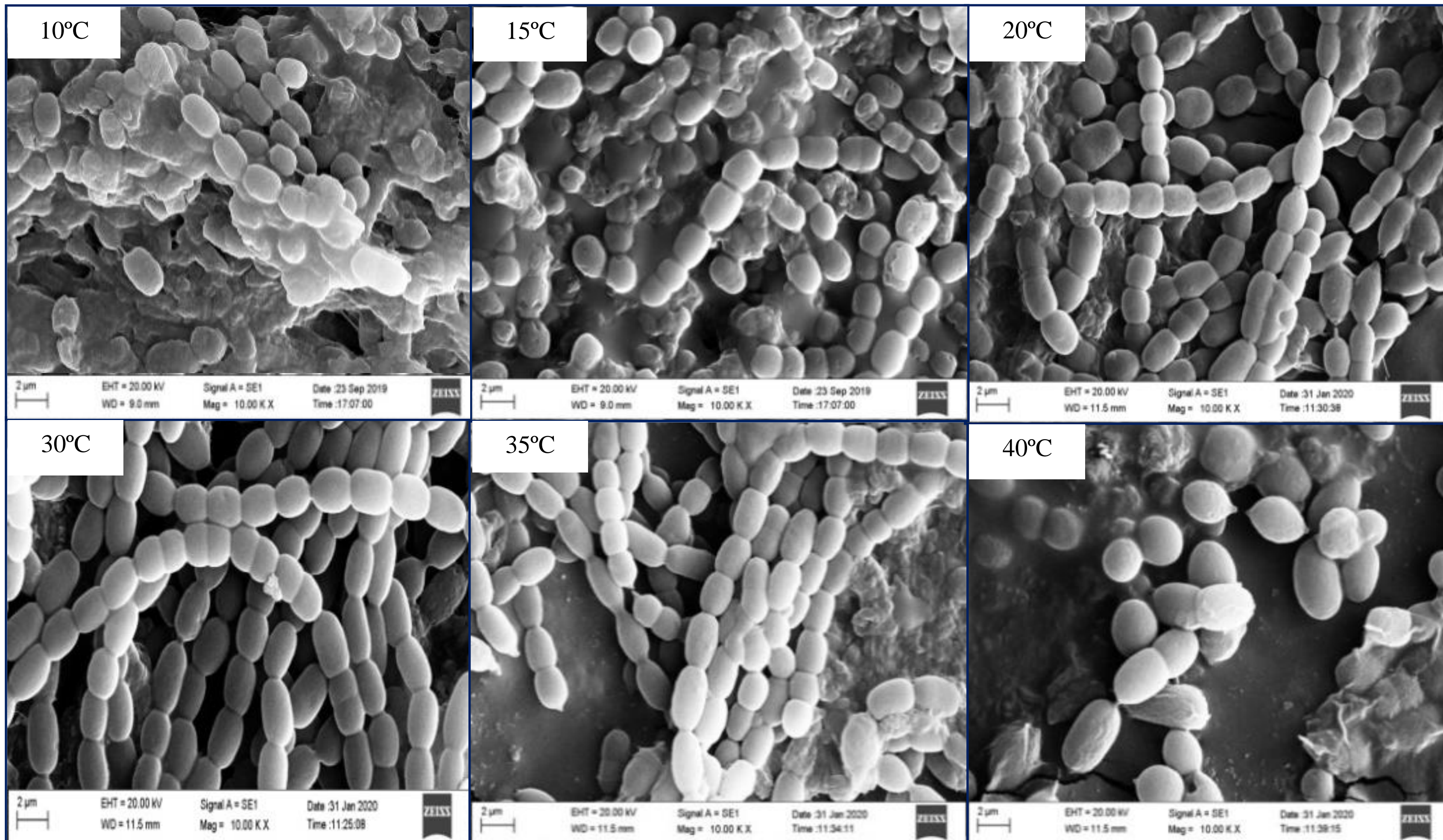


Fig. 3.2.1B Scanning electron microscopy (SEM) images of the *N. calcicola* showing morphological variations at growth temperatures (10-40°C).

3.2.2 Effect of temperature on growth and biomass

The growth rate of cyanobacteria was variable under different growth temperatures. In our study, *N. spongiaeforme* displayed the highest growth rate at 30ºC, but with no significant change recorded between 25 and 30ºC. However, the growth rate was decreased at suboptimal and supra-optimal temperatures. At suboptimal temperatures of 10, 15, and 20ºC, the growth rate of *N. spongiaeforme* showed a 343, 222, and 58% decline, respectively compared to growth seen at optimal temperature of 30ºC, whereas, under supra-optimal temperatures of 35 and 40ºC, growth rate decline by 44 and 668%, respectively as compared to 30ºC (Table 3.2.2).

Likewise, *N. calcicola* also showed the maximum growth rate at 30ºC, and no significant change was recorded between 25 and 30ºC. As observed in *N. spongiaeforme*, the growth rate of *N. calcicola* also decreased at suboptimal and supra-optimal temperature. *Nostoc calcicola* grown at suboptimal temperatures of 10, 15, and 20ºC, showed a decline in growth rate by 384, 231and 30%, respectively, whereas, under supra-optimal temperatures of 35 and 40ºC the drop in the growth rate was of 38 and 180% compared to 30ºC (Table 3.2.2).

Among the two species investigated, *N. spongiaeforme* revealed a greater growth rate than *N. calcicola* at 30ºC as the acceleration phase in *N. spongiaeforme* was achieved in 13 days compared to 25 days for *N. calcicola*. *N. calcicola* displayed a better growth rate at 40ºC than observed for *N. spongiaeforme.* Suboptimal temperature, however, did not show a significant difference in both the *Nostoc* species.

Biomass (measured on dry weight basis) and biomass productivity (determine as growth rate x biomass) are shown in Table. 3.2.2. Results showed that *N. spongiaeforme* displayed the highest biomass and biomass productivity at 30ºC. Like growth rate, biomass and biomass productivity of *N. spongiaeforme* also decreased due to the suboptimal and supra-optimal temperatures (Fig. 3.2.2A & Table 3.2.2). The suboptimal temperature of 10 and 15ºC showed a decline in biomass by 73 and 55% and biomass productivity by 166 and 155%, respectively, compared to 30ºC. Similarly, under a supra-optimal temperature of 35 and 40ºC, the biomass decreased by 15 and 94%, while biomass productivity declined by 53 and 132%, respectively, compared to 30ºC in *N. spongiaeforme*.

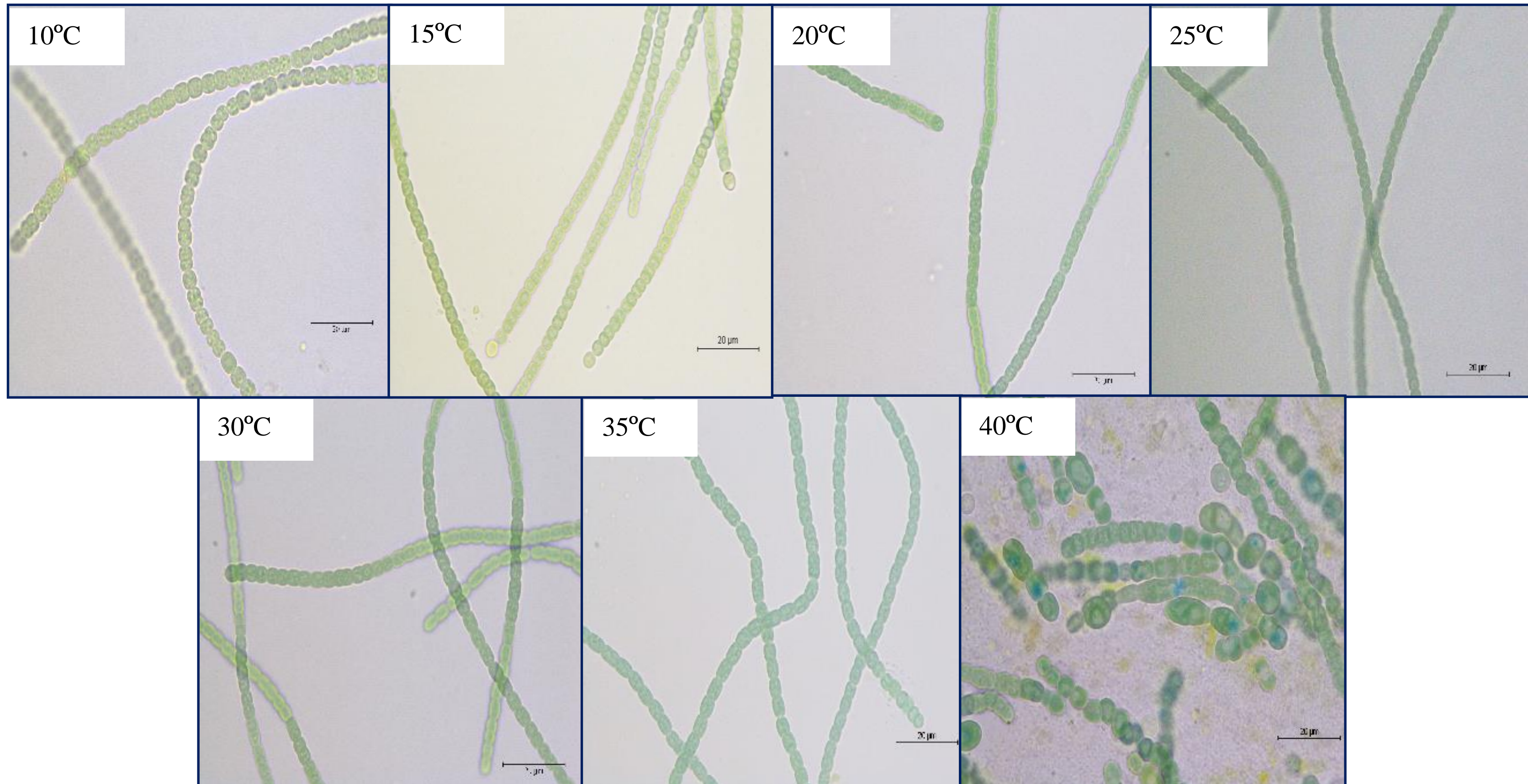


Fig. 3.2.1C Light microscopy images of the *N. spongiaeforme* showing morphological variations at growth temperatures (10-40°C).

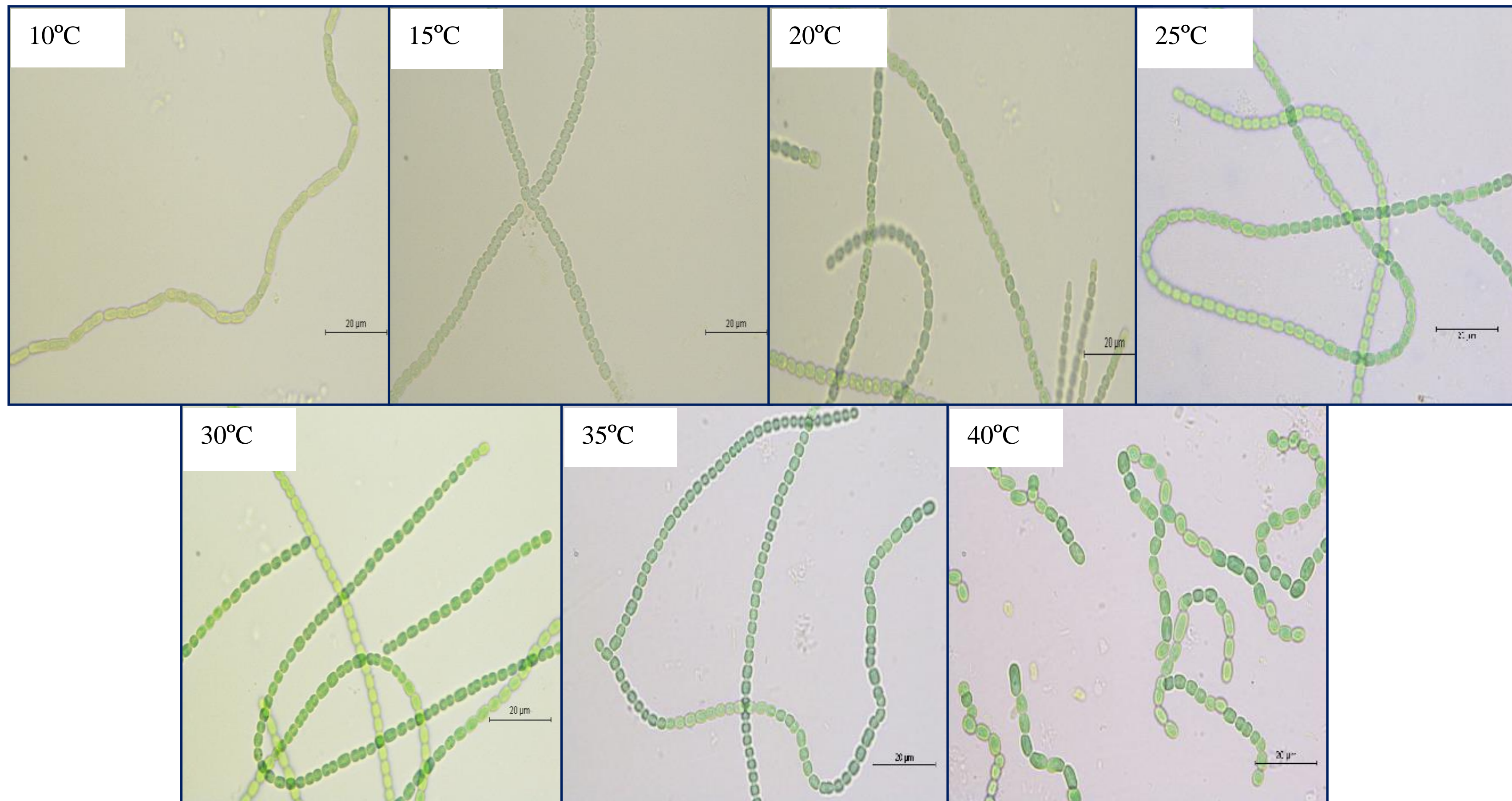


Fig. 3.2.1D Light microscopy images of the *N. calcicola* showing morphological variations at growth temperatures (10-40°C).

N. calcicola also showed a similar decrease in biomass and biomass productivity with regard to suboptimal and supra-optimal temperature (Fig. 3.2.2B and Table 3.2.2). Both species displayed more or less similar amounts of biomass under different growth temperatures; however, at a higher growth temperature of 40ºC, *N. spongiaeforme* showed a 90% decrease in biomass as compared to the decrease observed in *N. calcicola* when grown under the same growth temperature.

Table 3.2.2: Effect of growth temperatures on the maximum growth measured as O.D at 680 nm, growth rate (µ) on per day basis, biomass (g) on a dry weight basis, and biomass productivity (g L^{-1} day^{-1}) measured as growth rate x biomass. Data represent mean values ± SD (*n*=3). Same letter indicates insignificant differences at p≤0.01.

***Nostoc* spp.**	**Temperature (ºC)**	**Growth (O.D 680 nm)**	**Growth rate (µ)**	**Biomass (g L^{-1})**	**Biomass productivity (g L^{-1} day^{-1})**
N. spongiaeforme	10	$0.31{\pm}0.004^{e}$	-1.00±0.013	$0.14{\pm}0.015^{e}$	-0.15±0.015
	15	$0.51{\pm}0.006^{d}$	-0.50±0.012	$0.24{\pm}0.030^{f}$	-0.12±0.056
	20	$1.00{\pm}0.015^{c}$	0.17±0.015	$0.422{\pm}0.92^{c}$	0.073±0.412
	25	$1.19{\pm}0.029^{a}$	0.35±0.058	$0.511{\pm}0.015^{b}$	0.210±0.321
	30	$1.27{\pm}0.008^{a}$	0.41±0.01	$0.534{\pm}0.062^{a}$	0.220±0.01
	35	$1.05{\pm}0.013^{b}$	0.23±0.012	$0.451{\pm}0.014^{d}$	0.104±0.032
	40	$0.08{\pm}0.001^{f}$	-2.34±0.12	$0.030{\pm}0.002^{g}$	-0.070±0.032
N. calcicola	10	$0.35{\pm}0.03^{f}$	-0.957±0.080	$0.13{\pm}0.062^{e}$	-0.12±0.231
	15	$0.59{\pm}0.01^{e}$	-0.442±0.021	$0.25{\pm}0.025^{d}$	-0.11±0.021
	20	$1.15{\pm}0.01^{c}$	0.236±0.011	$0.439{\pm}0.051^{b}$	0.104±0.625
	25	$1.21{\pm}0.01^{b}$	0.286±0.004	$0.520{\pm}0.10^{c}$	0.149±0.014
	30	$1.28{\pm}0.01^{a}$	0.337±0.052	$0.547{\pm}0.015^{a}$	0.184±0.012
	35	$1.12{\pm}0.01^{c}$	0.209±0.081	$0.476{\pm}0.013^{d}$	0.100±0.231
	40	$0.70{\pm}0.02^{d}$	-0.269±0.025	$0.313{\pm}0.023^{f}$	-0.084±0.01

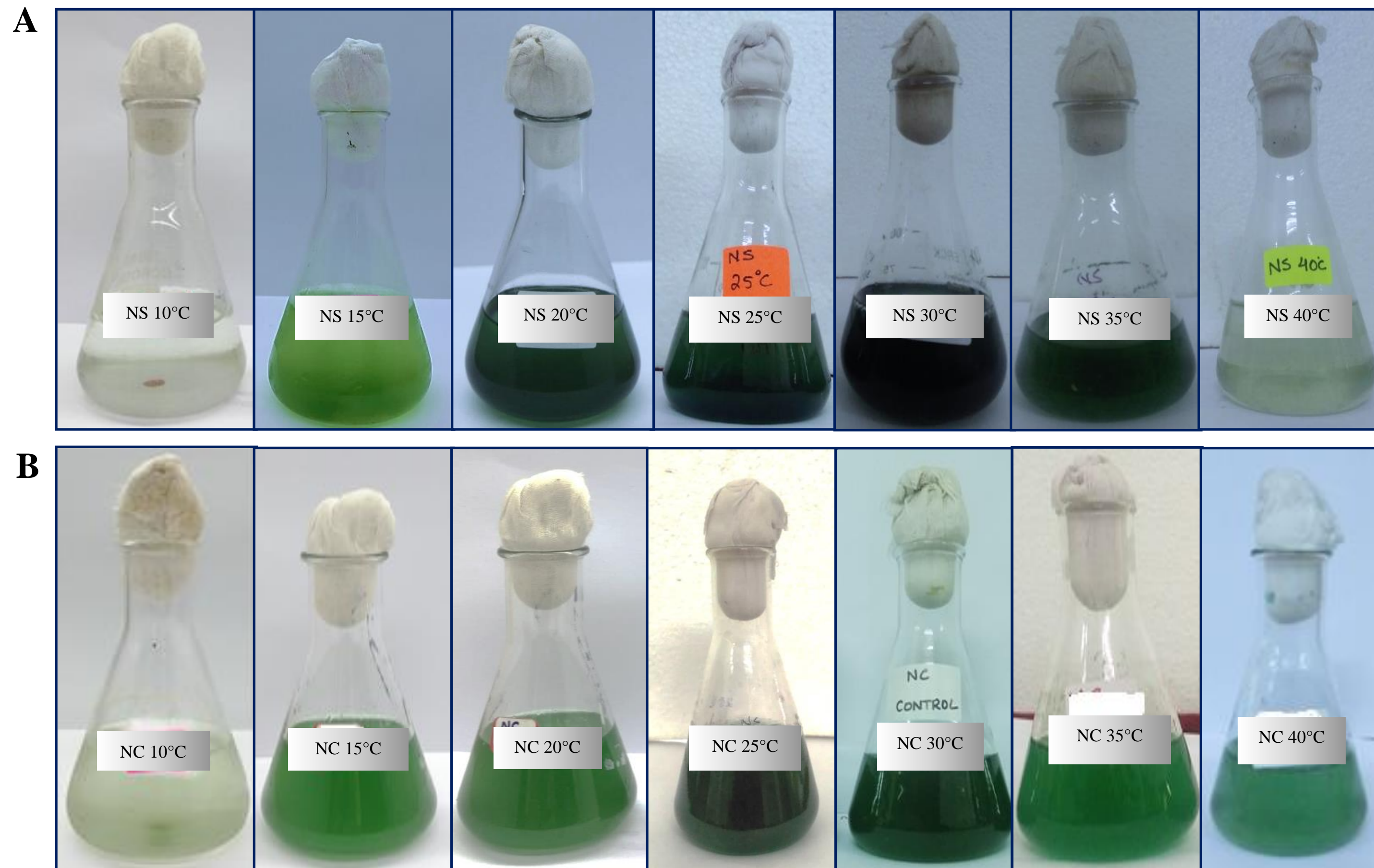


Fig. 3.2.2 Pictorial representation *N. spongiaeforme* (A) and *N. calcicola* (B) culture grown at different growth temperatures.

3.2.3 Effect of temperature on photosynthesis

Photosynthesis is directly related to growth and is susceptible to temperature. In our study, photosynthesis was studied using chlorophyll fluorescence, oxygen evolution, and pigment analysis.

3.2.3.1 Photosynthetic efficiency of PS II (Fv/Fm ratio)

The photosynthetic efficiency of PS II measured as the Fv/Fm ratio in both *Nostoc* species was affected by their growth temperatures. Among the two species studied, *N. calcicola* exhibited higher Fv/Fm than *N. spongiaeforme* at all the studied growth temperatures (Fig. 3.2.3A). *Nostoc spongiaeforme* and *N. calcicola* exhibited a maximum Fv/Fm ratio of 0.17 and 0.32, respectively at the optimal temperature of 30ºC. Compared to the optimum temperature, the suboptimal temperature of 20 and 10ºC caused a gradual decline in Fv/Fm ratio of 61 and 74% in *N. spongiaeforme* and 45 and 67% in *N. calcicola*. Similarly, the supra-optimal temperature of 35 and 40ºC also caused a decrease in the Fv/Fm ratio by 42 and 77% in *N. spongiaeforme* and 49 and 57% in *N. calcicola* as compared to the optimum temperature.

3.2.3.2 Photosynthetic light reaction (oxygen evolution)

Photosynthetic rate, measured as oxygen evolution was also affected due to growth temperature. The maximum photosynthetic rate was observed at the optimum temperature of 30ºC in both *Nostoc* species. The culture of *N. spongiaeforme* grown at 10 and 15ºC registered a 79 and 59% decline, respectively. The culture of *N. calcicola* grown at the same temperatures showed a decrease in their photosynthetic rate by 69 and 49%, respectively, compared to 30ºC. The supra-optimal temperature of 40ºC caused a 95% decrease in photosynthetic rate in *N. spongiaeforme* as compared to 87% in *N. calcicola* as compared to the optimal temperature (Fig. 3.2.3B).

3.2.3.3 Photosynthetic pigment content (chlorophyll and carotenoids)

Our results displayed that different growth temperatures caused changes in the photosynthetic pigments of both the *Nosotc* species (Fig. 3.2.3C & Table 3.2.3). The analysis showed the presence of chl *a* and carotenoids (echineone, β carotene and unidentified carotenoids) in all the samples of both *Nostoc* species. Chl *a* content of *N. spongiaeforme* grown at suboptimal temperatures of 10 and 15ºC showed a decrease of 83 and 62%, respectively, while cultures of *N. calcicola* exhibited a decrease of 61 and 49%, respectively, in comparison to cultures grown at optimum growth temperature of 30ºC. The supra-optimal temperatures of 40ºC caused a decrease of 86 and 77% in the chl *a* content in *N. spongiaeforme* and *N. calcicola,* respectively, compared to their optimal temperature (Table 3.2.3).

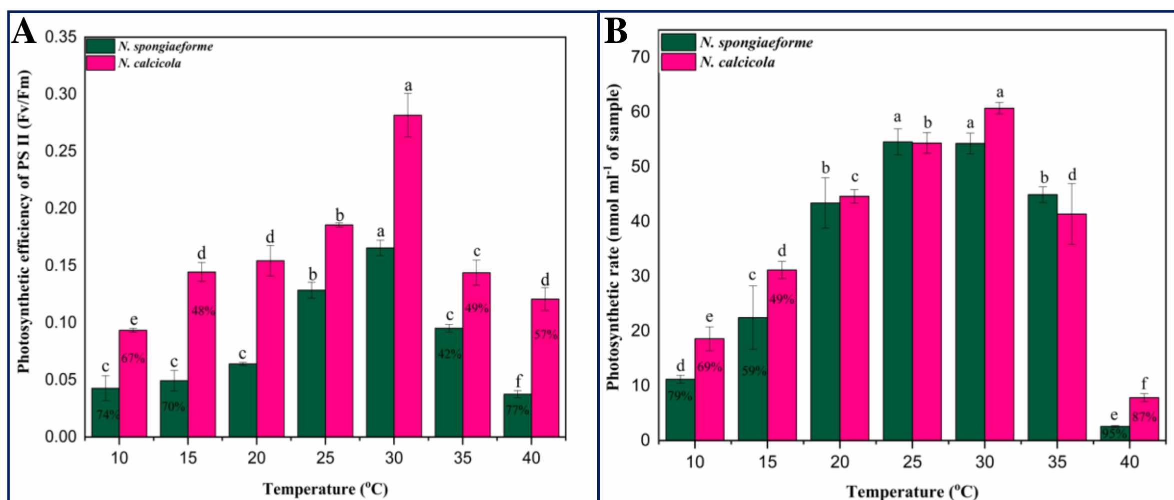


Fig. 3.2.3 Effect of growth temperatures on Fv/Fm ratio (A) and photosynthetic rate (B) of *N. spongiaeforme* and *N. calcicola*. Standard deviation is represented by vertical bars (±SD, *n* = 3). Bars denoted by the same letter indicates insignificant difference at p≤0.01.

The total carotenoid in the *Nostoc* species was also affected due to different growth temperatures. The total carotenoid content in *N. spongiaeforme* decreased by 61 and 44% due to the suboptimal temperature of 10 and 15ºC respectively, while the same was

decreased by 63 and 56%, respectively in *N. calcicola* as compared to the optimum temperature of 30ºC. Similarly, supra-optimal growth temperatures of 35 and 40ºC also caused a decrease in the total carotenoid up to 73% in *N. spongiaeforme* and up to 70% in *N. calcicola* as compared to 30ºC.

3.2.3.4 Phycobilin pigment content (phycocyanin and allophycocyanin)

Growth temperatures also caused a change in the phycocyanin and allophycocyanin contents of *Nostoc* species. When *N. spongiaeforme* grown at the suboptimal temperature of 10 and 15ºC showed a decrease of 75 and 60%, respectively in phycocyanin, while *N. calcicola* showed a decrease of up to 86% for the same suboptimal temperature in comparison to 30ºC. Cultures were grown at 40ºC resulted in a decline of 55% in phycocyanin content in *N. spongiaeforme* and 70% in *N. calcicola* in comparison to 30ºC.

Table 3.3.3: Effect of temperature on the photosynthetic pigments; chlorophyll *a*, total carotenoid, phycocyanin, allophycocyanin of *N. spongiaeforme* and *N. calcicola*

***Nostoc* spp.**	**Temperature (ºC)**	**Chl *a* (µg mL^{-1} cell suspension)**	**Total carotenoid (µg mL^{-1} cell suspension)**	**Phycocyanin (µg mL^{-1} cell suspension)**	**Allophycocyanin (µg mL^{-1} cell suspension)**
N. spongiaeforme	10	0.52±0.01^{g}	0.47±0.04^{e}	0.23±0.02^{e}	0.12±0.01^{d}
	15	1.17±0.01^{f}	0.68±0.02^{d}	0.36±0.01^{d}	0.21±0.01^{c}
	20	2.03±0.02^{c}	0.84±0.01^{c}	0.36±0.01^{d}	0.20±0.02^{c}
	25	2.66±0.03^{b}	1.09±0.02^{b}	0.88±0.02^{a}	0.50±0.06^{a}
	30	3.07±0.01^{a}	1.21±0.04^{a}	0.92±0.05^{a}	0.54±0.03^{a}
	35	1.73±0.02^{d}	0.33±0.02^{f}	0.52±0.01^{b}	0.26±0.02^{b}
	40	0.43±0.02^{h}	0.34±0.01^{f}	0.41±0.02^{cd}	0.25±0.01^{b}
N. calcicola	10	2.81±0.01^{d}	0.790±0.02^{e}	0.24±0.06^{f}	0.15±0.05^{g}
	15	3.71±0.01^{c}	0.936±0.01^{d}	0.26±0.09^{e}	0.26±0.03^{e}
	20	3.70±0.01^{c}	1.038±0.04^{c}	1.10±0.01^{c}	0.66±0.01^{c}
	25	6.82±0.05^{b}	2.133±0.06^{b}	1.66±0.12^{a}	0.87±0.10^{b}
	30	7.22±0.01^{a}	2.140±0.01^{a}	1.68±0.16^{a}	0.98±0.09^{a}
	35	2.25±0.01^{e}	0.745±0.01^{f}	0.58±0.04^{d}	0.30±0.02^{d}
	40	1.69±0.01^{f}	0.634±0.01^{g}	0.51±0.05^{e}	0.21±0.02^{f}

Allophycocyanin content decreased by 77 and 61% in *N. spongiaeforme* when grown at the suboptimal temperature of 10 and 15ºC, respectively, while *N. calcicola* showed a

decrease of up to 85% for the same suboptimal temperature in comparison to 30ºC. Culture grown at 40ºC resulted in a decrease of 53% in allophycocyanin content in *Nostocspongiaeforme* and 78% in *N. calcicola* in comparison to the optimum temperature of 30ºC (Table. 3.2.3).

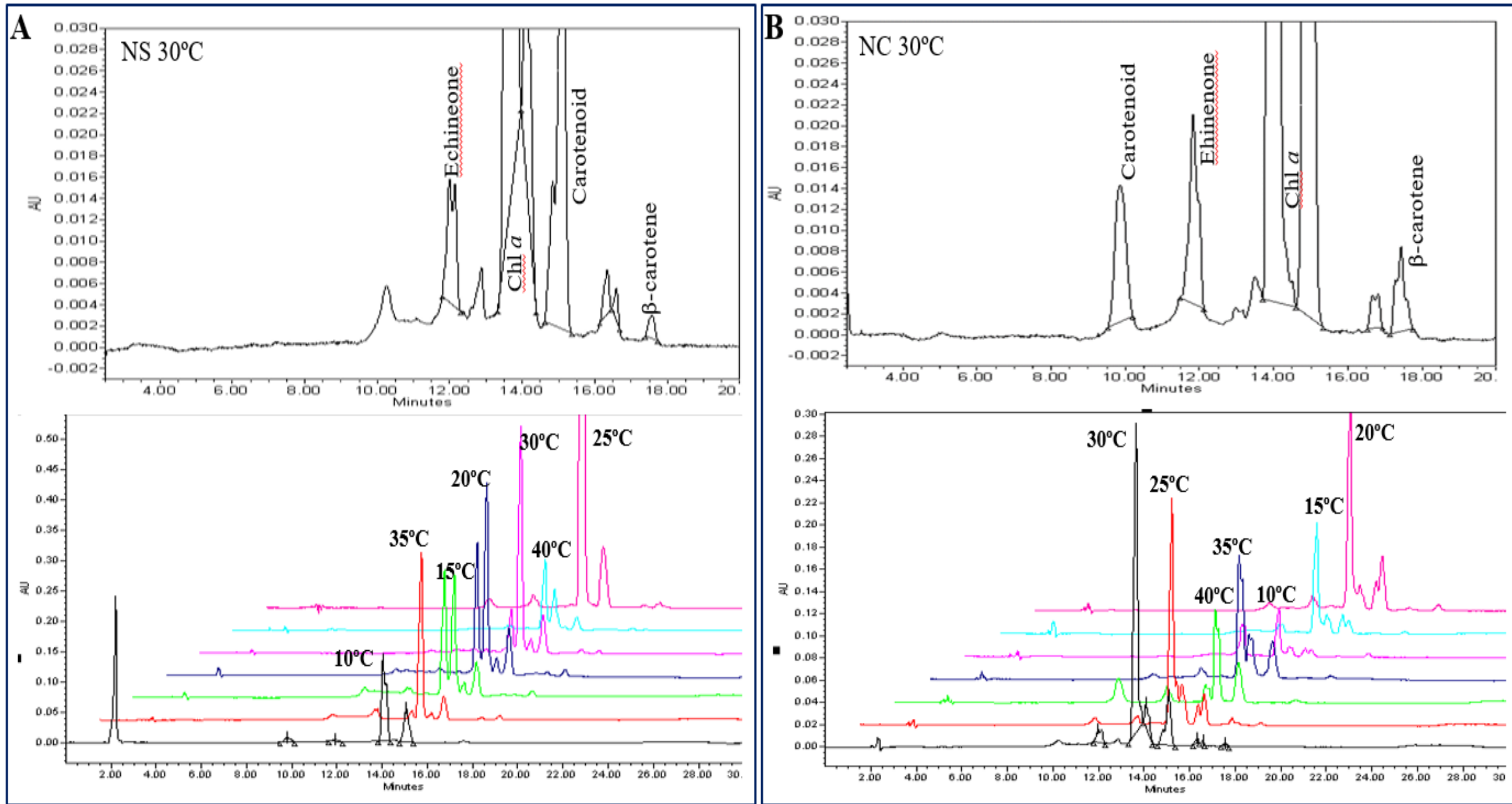


Fig. 3.2.3C. HPLC pigment profile extracted at 445 nm and comparative HPLC spectrum of pigment of *N. spongiaeforme* (A) and *N. calcicola* (B) at different temperatures.

3.2.4 Effect of temperature on respiratory rate (oxygen consumption)

Respiratory rate, measured as oxygen consumption was also affected due to growth temperatures. The highest respiratory rate (O_2 consumption) observed for both the *Nostoc* species was at 30ºC (Fig. 3.2.4). The respiratory rate in both species declined when grown at suboptimal or supra-optimal temperatures. It was observed that the respiratory rate in *N. spongiaeforme* decreased by 86 and 67% when grown at 10 and 15ºC, respectively, compared to 78 and 61% seen in the marine cyanobacterium, *N. calcicola* compared to their optimum temperature. The growth at a supra-optimal temperature of 40ºC decreased the respiratory rate by 88% in *N. spongiaeforme* and 53% in *N. calcicola* (Fig. 3.2.4).

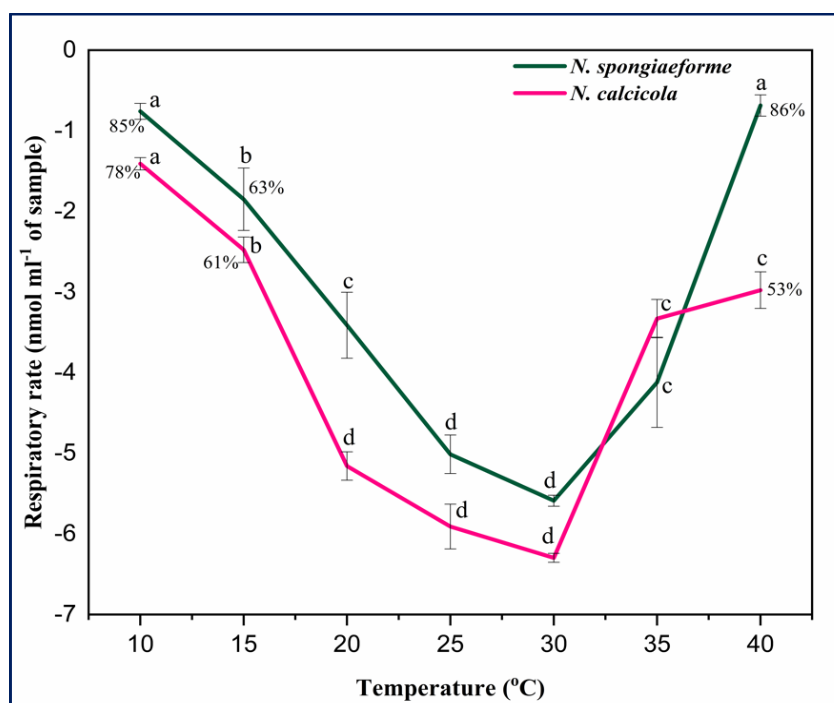


Fig. 3.2.4 Effect of growth temperatures on respiratory rate of *N. spongiaeforme* and *N. calcicola*. Standard deviation is represented by vertical bars (±SD, n = 3). Bars denoted by the same letter indicates insignificant difference at $p \leq 0.01$.

3.2.5 Effect of temperature on phenolic compounds

Our results displayed that different growth temperatures caused changes in the total phenolic content (TPC) and total flavonoid contents (TFC) of *Nostoc* species (Table. 3.2.5).

The TPC of both the *Nostoc* species grown at suboptimal temperatures in the range of 10-20ºC showed an increase of 67-100% in comparison to 30ºC. While the TPC of both the

Nostoc species grown at a supra-optimal temperature of 35ºC increased to 100% as compared to optimal temperature. An increase in the growth temperature to 40ºC resulted in an increase in TPC by 133% in *N. calcicola* but a decrease of 67% in *N. spongiaeforme*, which were still 33% higher than observed at the optimal temperature.

Table. 3.2.5: Effect of growth temperatures on phenolic compounds of *N. spongiaeforme* and *N. calcicola.*

***Nostoc* spp.**	**Temperature (ºC)**	**Phenols (µmol gm^{-1} of tissue)**	**Flavonoids (µmol gm^{-1} of tissue)**
N. spongiaeforme	10	0.05±0.01[d]	0.20±0.01[cd]
	15	005±0.01[d]	0.23±0.03[cd]
	20	0.06±0.02[c]	0.25±0.01[d]
	25	0.04±0.03[a]	0.13±0.01[a]
	30	0.03±0.05[a]	0.12±0.01[a]
	35	0.06±0.01[c]	0.20±0.01[cd]
	40	0.04±0.01[d]	0.14±0.01[b]
N. calcicola	10	0.05±0.01[b]	0.23±0.01[c]
	15	0.06±0.02[b]	0.28±0.02[d]
	20	0.06±0.05[b]	0.31±0.03[d]
	25	0.04±0.06[a]	0.16±0.08[b]
	30	0.03±0.04[a]	0.14±0.02[a]
	35	0.06±0.09[b]	0.33±0.02[e]
	40	0.07±0.01[c]	0.35±0.05[e]

Data represent mean values ± SD (*n*=3). Same letter indicates insignificant differences at $p \leq 0.01$.

Total flavonoid content (TFC) resulted in an increase of 66-108% in *N. spongiaeforme* and 64-121% in *N. calcicola* due to a suboptimal temperature of 10-20ºC compared to 30ºC. Cultures grown at 35ºC resulted in an increase of 67% in TFC in *N. spongiaeforme* and 136% in *N. calcicola* while at 40ºC, the TFC increased by 17% in

N. spongiaeforme and 150% in *N. calcicola* in comparison to the optimum temperature of 30ºC (Table 3.2.5).

3.2.6 Effect of temperature on ROS production: Hydrogen peroxide (H_2O_2); Hydroxyl radicals (OH•)

The study showed the generation of H_2O_2 and OH• in the *Nostoc* species due to various growth temperatures. It was observed that cultures grown at 30ºC had the lowest level of H_2O_2 content in both species as compared to suboptimal and supra-optimal growth temperatures. A decrease in the growth temperature from 30ºC to 10ºC increased the H_2O_2 production in the *Nostoc* species. *N. spongiaeforme* grown at suboptimal temperatures of 10 and 15ºC increased the H_2O_2 production by 186 and 132%, respectively, while cultures of *N. calcicola* exhibited an increase of 239 and 129%, respectively, in comparison to the optimum temperature of 30ºC. The supra-optimal temperatures of 40ºC caused a much greater increase of 357% of H_2O_2 production in *N. spongiaeforme* than 178% H_2O_2 production seen in *N. calcicola* in comparison to the optimum temperature of 30ºC (Fig. 3.2.6A).

The generation of OH• due to various growth temperatures also showed a similar pattern as seen for H_2O_2 generation except to a lesser extent. Both the *Nostoc* species grown at 10ºC showed an increase in OH• content up to 97% compared to their optimum growth temperature. The supra-optimal temperature of 40ºC, however, increased the OH• content by 120% in *N. spongiaeforme* and 64% in *N. calcicola* compared to the growth observed at optimal temperature for both the species (Fig. 3.2.6B).

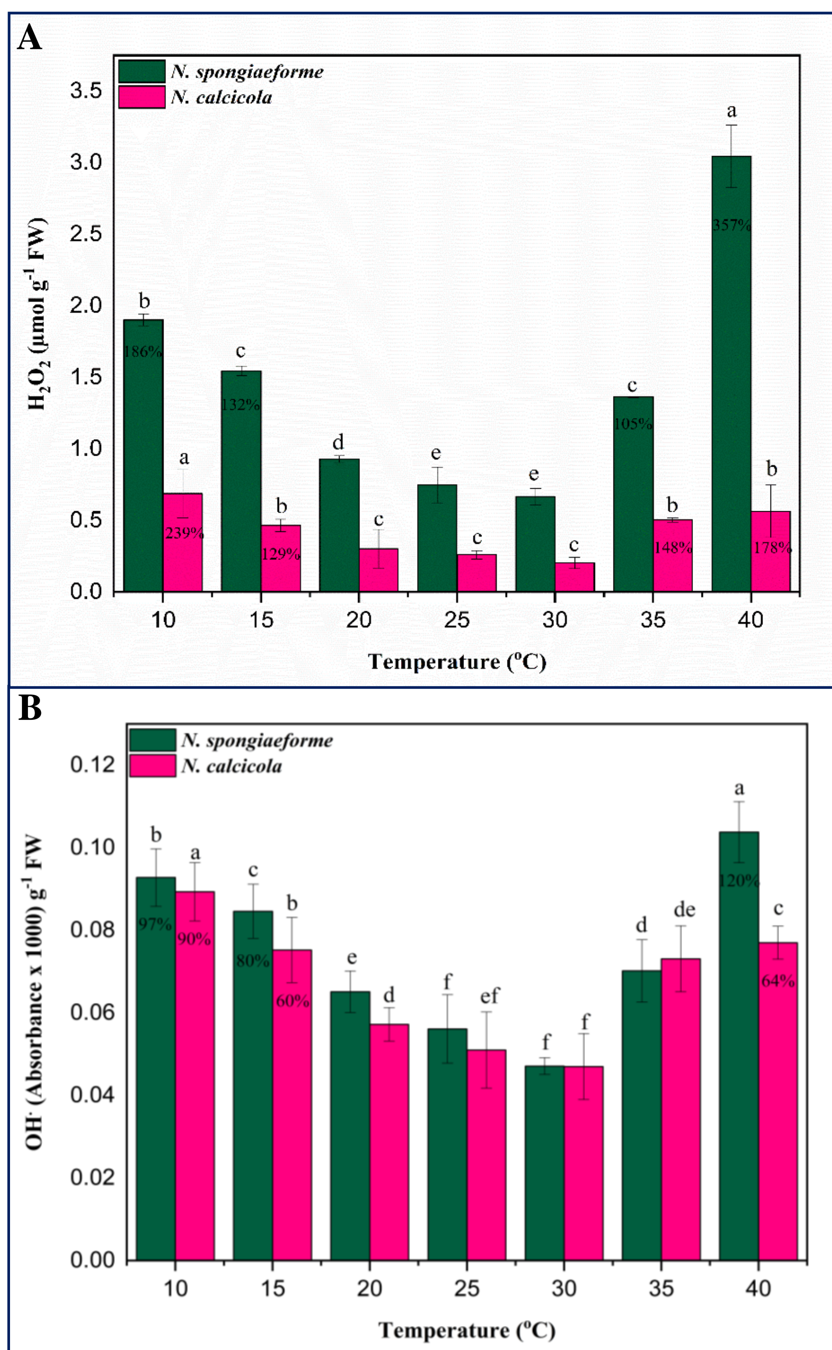


Fig. 3.2.6 Effect of growth temperature on production of hydrogen peroxide (A) and hydroxyl radicals (B) in *N. spongiaeforme* and *N. calcicola*. Standard deviation is represented by vertical bars (±SD, $n = 3$). Bars denoted by the same letter indicates insignificant difference at $p \leq 0.01$.

3.2.7 Effect of temperature on lipid peroxidation and protein oxidation

Our results displayed that different growth temperatures caused oxidative damage to lipid (observed as lipid peroxidation) and protein oxidation (observed as protein carbonyl content) in both the *Nostoc* species (Fig. 3.2.7). It was observed that cultures of *Nostoc* species grown at 30ºC had the lowest level of MDA content as compared to suboptimal and supra-optimal growth temperatures. The suboptimal temperature of 10ºC showed a 2-fold increase in MDA content in *N. spongiaeforme* and a 3-fold increase in *N. calcicola* compared to the optimum temperature of 30ºC. Due to the supra-optimal temperature of 40ºC, MDA content increased by 4-fold in *N. spongiaeforme* and 3-fold in *N. calcicola* compared to the optimum temperature of 30ºC (Fig. 3.2.7A). The result indicates relatively greater oxidative damage to *N. spongiaeforme* at higher temperature of 40ºC than *N. calcicola* which showed relatively higher oxidation of lipids at suboptimal temperature of 10-15ºC.

The study also revealed a more or less similar pattern for protein carbonyl formation as a result of the sub and supra-optimal temperature in both the *Nostoc* species. The least amount of protein carbonyl content in both the *Nostoc* species was observed at 30ºC. The protein carbonyl content increased up to 3-fold as a result of the growth of *N. spongiaeforme* at a suboptimal temperature of 10ºC while *N. calcicola* displayed up to a 4-fold increase under the same growth temperature compared to 30ºC. At a supra-optimal temperature of 40ºC, the protein carbonyl content increased by 4-fold in *N. spongiaeforme* and 3-fold in *N. calciocla*, as compared to the optimal temperature of 30ºC (Fig. 3.2.7B).

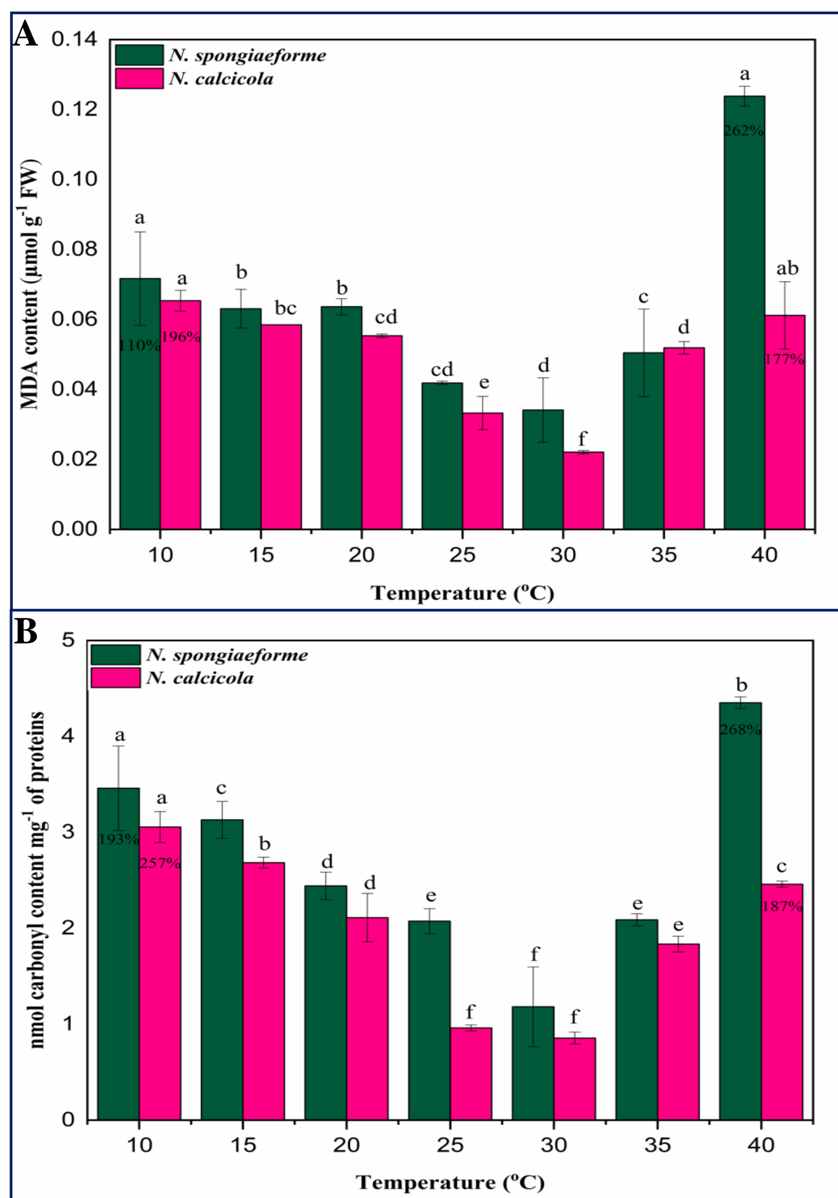


Fig. 3.2.7 Effect of different growth temperatures on lipid peroxidation (A) and protein oxidation (B) in *N. spongiaeforme* and *N. calcicola*. Standard deviation is represented by vertical bars (±SD, $n = 3$). Bars denoted by the same letter indicates insignificant difference at $p \leq 0.01$.

3.2.8 Effect of temperature on enzymatic and non-enzymatic antioxidants

3.2.8.1 Enzymatic antioxidants (SOD, APX, and CAT)

Our results displayed a change in the activity of SOD in both the *Nostoc* species due to different growth temperatures (Fig. 3.2.8.1A). Both suboptimal and supra-optimal temperatures increased the SOD activity in both the species of *Nostoc*. Cultures grown at

a suboptimal temperature of 10ºC, the SOD activity increased by 106% in *N. spongiaeforme* and 64% in *N. calcicola* compared to the optimum temperature of 30ºC (Fig. 3.2.8.1A). At a supra-optimal temperature of 40ºC, the SOD activity increased to only 69% in *N. spongiaeforme* and 50% in *N. calcicola* as compared to 30ºC (Fig. 3.2.8.1A).

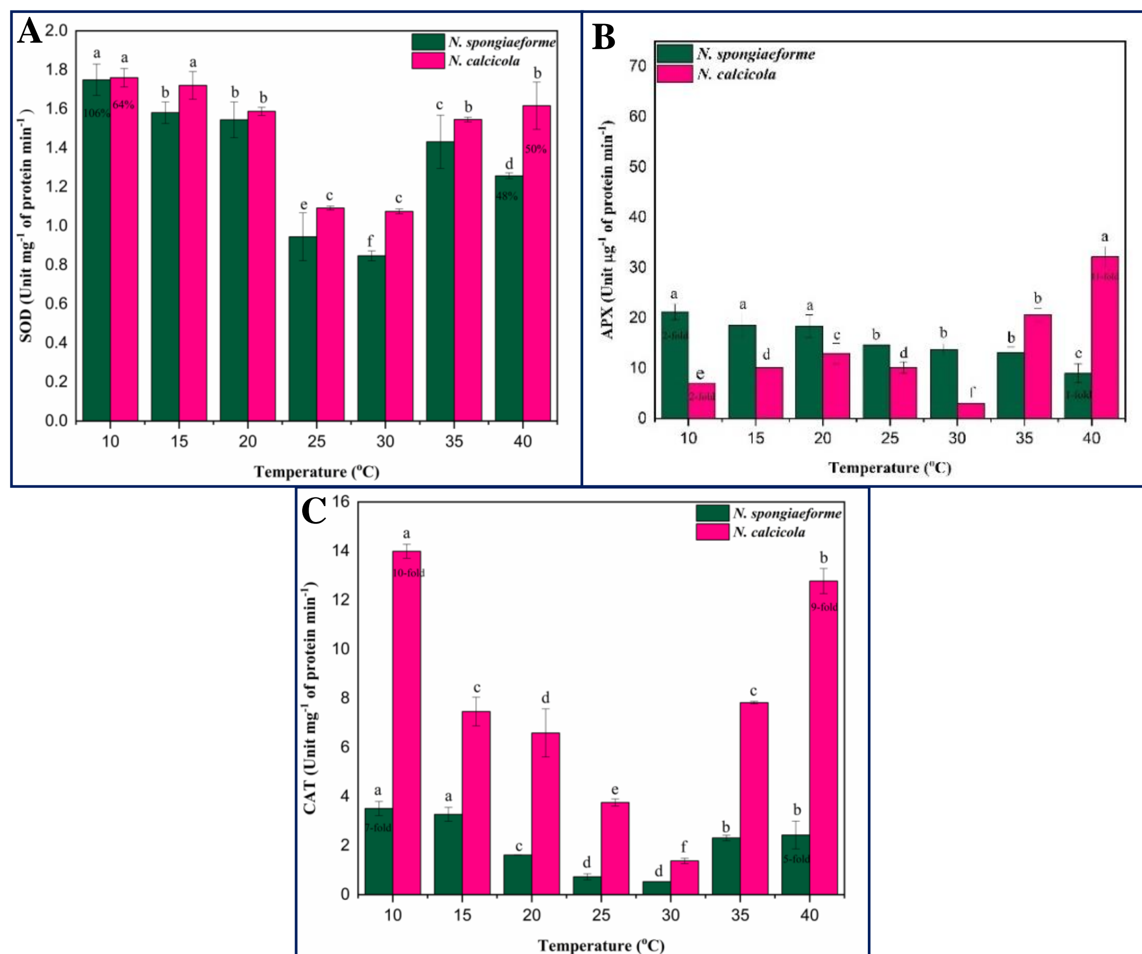


Fig. 3.2.8.1 Effect of growth temperatures on antioxidants enzymes activities of SOD (A); APX (B), and CAT (C) of *N. spongiaeforme* and *N. calcicola*. Standard deviation is represented by vertical bars (±SD, *n* = 3). Bars denoted by the same letter indicates insignificant difference at p≤0.01.

Our results showed that suboptimal temperatures caused an increase in the APX activity in both the *Nostoc* species, while supra-optimal temperature showed an increase in APX activity in *N. calcicola* but a decrease in *N. spongiaeforme* compared to 30ºC. At

the suboptimal temperature of 10ºC, both the species showed a 2-fold higher activity of APX which was increased to 3-4-fold in *N. calcicola* but remain unchanged in *N. spongiaeforme* when grown at 15-20ºC in comparison to 30ºC. *N. spongiaefrome* grown at a supra-optimal temperature of 40ºC also did not show any increase compared to the optimal temperature of 30ºC. However, *N. calcicola,* showed an increase of 11-fold when grown at 40ºC compared to optimal temperature (Fig. 3.2.8.1B).

Growth temperatures also affected CAT activity in both the *Nostoc* species. The suboptimal temperature of 10ºC increased the CAT activity by 7-fold in *N. spongiaeforme*, and 10-fold in *N. calcicola* as compared to the optimum temperature of 30ºC. On the other hand, the supra-optimal temperature of 40ºC showed only a 5-fold increase in CAT activity in *N. spongiaeforme,* and 9-fold in *N. calcicola* compared to the optimum temperature of 30ºC (Fig. 3.2.8.1C).

3.2.8.2 Non-enzymatic antioxidants (ascorbate and proline)

Ascorbate content also increased significantly in the *Nostoc* species when grown at different growth temperatures.

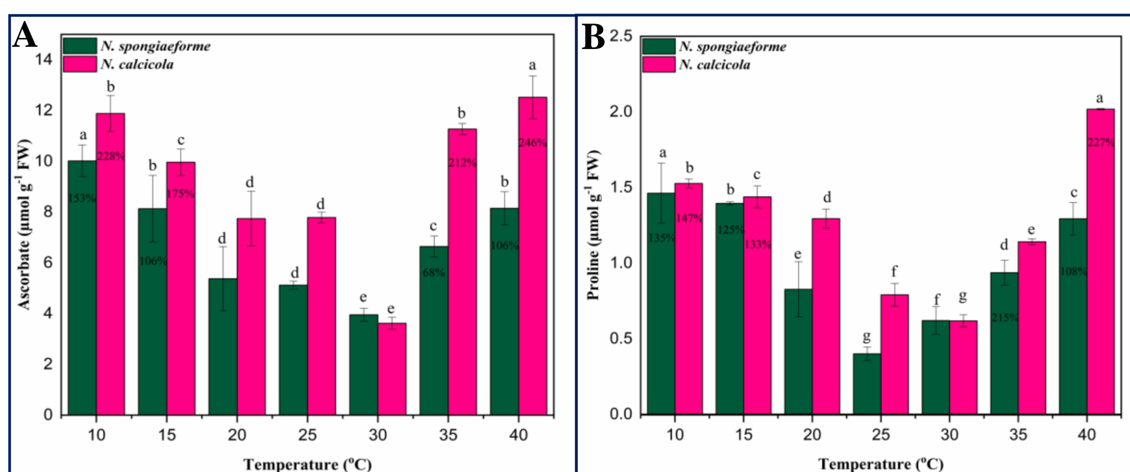


Fig. 3.2.8.2 Effect of growth temperatures on ascorbate content (A) and proline (B) in *N. spongiaeforme* and *N. calcicola*. Standard deviation is represented by vertical bars (±SD, *n* = 3). Bars denoted by the same letter indicates insignificant difference at p≤0.01.

The AsA content under suboptimal temperatures of 15 and 10ºC increased by 106 and 153% respectively in *N. spongiaeforme* and 175 and 228% respectively in *N. calciocla* in comparison to 30ºC. At the supra-optimal temperature of 40ºC, the AsA content increased

to 246% in *N. calcicola* compared to 106% increase seen in *N. spongiaeforme* as compared to 30ºC (Fig. 3.2.8.2A).

Our result also revealed a significant increase in proline content in the *Nostoc* species as a result of the different growth temperatures (Fig. 3.2.8.2B). The proline content increased by 2-fold when grown at a suboptimal temperature of 10ºC in both the *Nostoc* species compared to the optimal temperature of 30ºC. The supra-optimal temperature of 40ºC also increased the proline content to a 2-fold in *N. spongiaeforme* and 3-fold in *Nostoc calcicola* in comparison to the optimum temperature of 30ºC (Fig. 3.2.8.2B).

3.2.9 Effect of temperature on nitrogenase activity

In the present study, both the *Nostoc* species showed a decrease in nitrogenase activity at suboptimal and supra-optimal temperatures compared to the optimum temperature of 30ºC.

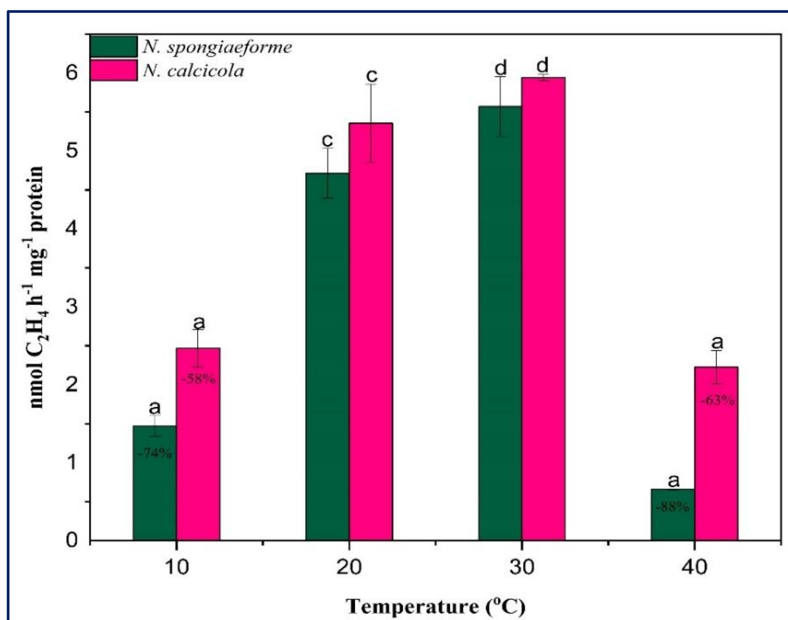


Fig. 3.2.9 Effect of growth temperatures on nitrogenase activity of *N. spongiaeforme* and *N. calcicola.* Standard deviation is represented by vertical bars (±SD, *n* = 3). Bars denoted by the same letter indicates insignificant difference at p≤0.01.

It was observed that *N. spongiaeforme* showed a greater decrease in nitrogenase activity when grown at a suboptimal temperature of 10ºC and supra-optimal temperature of 40ºC

than *N. calcicola.* The nitrogenase activity decreased by 74% in *N. spongiaeforme* and 58% in *N. calcicola*, as a result of the suboptimal growth temperature of 10ºC compared to the optimum temperature of 30ºC (Fig. 3.2.9). While, at a supra-optimal temperature of 40ºC, the nitrogenase activity decreased to 88% in *N. spongiaeforme* and 63% in *N. calcicola* compared to the optimum temperature of 30ºC (Fig. 3.2.9).

3.2.10 Gene expression studies of SOD, CAT, HSP, and *nif H*

3.2.10.1 Effect of temperatures on gene expression of SOD, CAT, and HSP

SOD, CAT, and HSP gene expression were higher in *N. calcicola* than in *N. spongiaeforme* under different growth temperatures. At suboptimal temperature of 10ºC

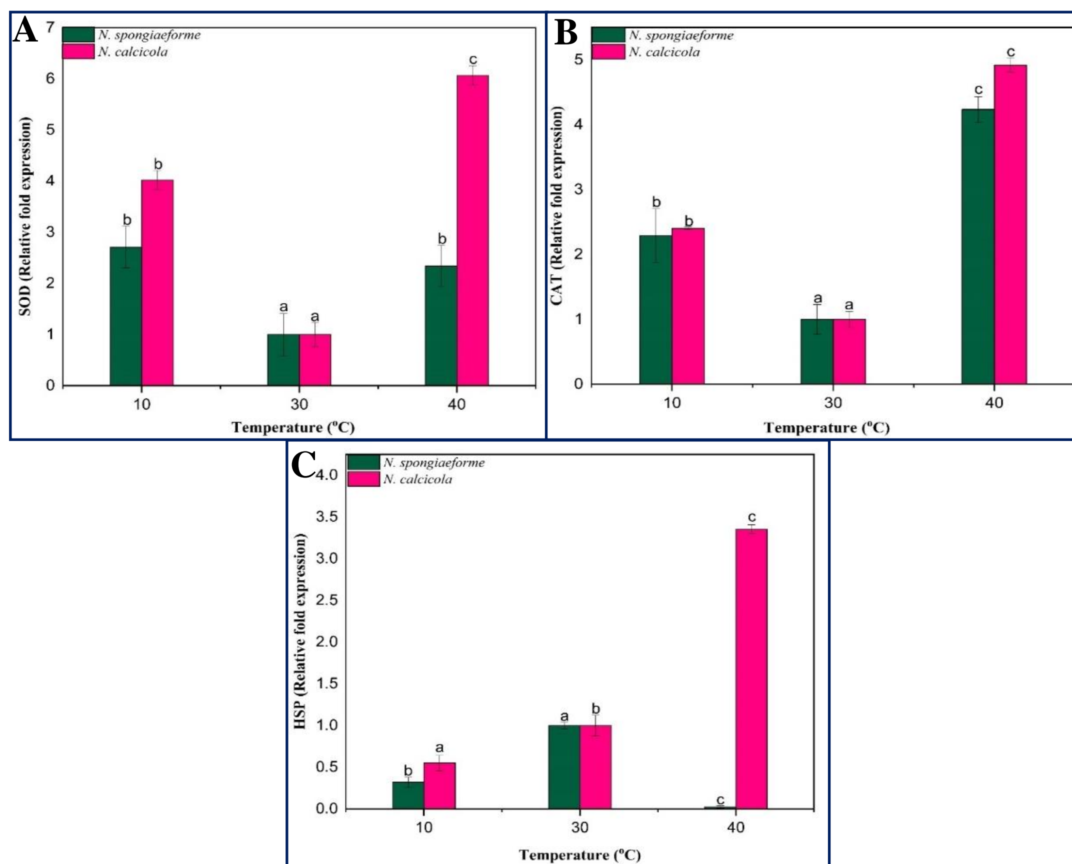


Fig. 3.2.10.1 Effect of growth temperatures on relative gene expression of SOD, CAT and HSP in *N. spongiaeforme* and *N. calcicola.* Standard deviation is represented by vertical bars (±SD, $n = 3$). Bars denoted by the same letter indicates insignificant difference at $p \leq 0.01$.

displayed approximately 3-fold increase in the expression level of SOD in *N. spongiaeforme* compared to 4-fold increase in *N. calcicola*. While the expression of SOD at a supra-optimal temperature of 40ºC resulted in a 2-fold increase in *N. spongiaeforme* and a 6-fold increase in *N. calcicola* as compared to their optimum temperature (Fig. 3.2.10.1A).

The expression level of CAT also increased by 2-fold due to the suboptimal temperature of 10ºC in both the *Nostoc* species. While the expression of CAT at a supra-optimal temperature of 40ºC resulted in a 4-fold increase in *N. spongiaeforme* and a 5-fold increase in *N. calcicola* than optimum temperature (Fig. 3.2.10.1B).

Our study also showed an increase in the HSP expression due to growth temperature. A 3-fold increase in HSP expression was observed in *N. calcicola* at a supra-optimal temperature of 40ºC. No increase in the expression of HSP was seen at 40°C in *N. spongiaeforme* in comparison to 30ºC (Fig. 3.2.10.1C).

3.2.10.2 Effects of varying growth temperatures on *nif H* genes

Cultures grown with sub and supra-optimal temperatures showed a significant decrease in the expression of *nif H* gene compared to the optimum temperature of 30ºC (Fig. 3.2.10.2).

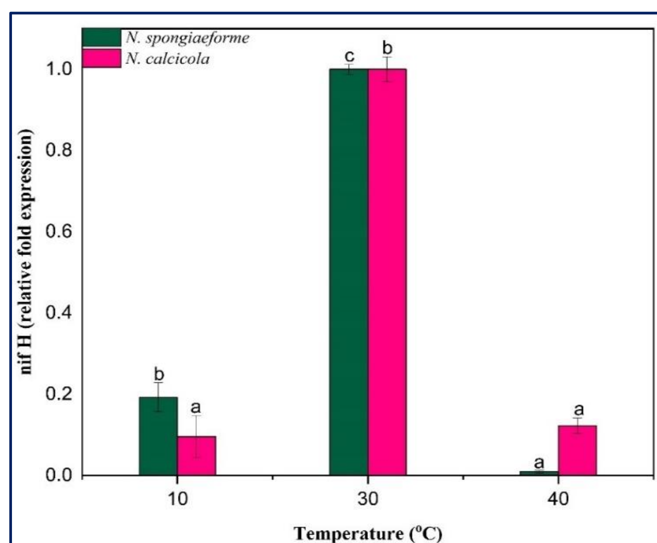


Fig. 3.2.10.2 Effect of growth temperatures on *nif H* gene expression in *N. spongiaeforme* and *N. calcicola.* Standard deviation is represented by vertical bars (±SD, n = 3). Bars denoted by the same letter indicates insignificant difference at p≤0.01.

Both the cultures grown at a suboptimal temperature of 10ºC decreases the expression level of *nif H* gene to 81% in *N. spongiaeforme* and 90% in *N. calcicola* compared to the optimum temperature of 30ºC (Fig. 3.2.10.2). The supra-optimal temperatures of 40ºC resulted in complete repression of *nif H* gene in *N. spongiaeforme,* while decreased to 88% in *N. calcicola* as compared to the optimum temperature of 30ºC (Fig. 3.2.10.2).

3.2.11 Effect of different temperatures on fatty acid profile

The different growth temperatures caused qualitative and quantitative changes in the fatty acid composition of both the *Nostoc* species (Table 3.2.11A, B). GC-MS chromatogram peaks of *N. spongiaeforme* were identified as saturated fatty acids viz; myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), pentadecanoic acid (C15:0) and unsaturated fatty acids as methyl palmitoleate (C16:1), oleic acid (C18:1), linoleic acid (C18:2), methyl linolenate (C20:3), and arachidonic acid (C20:4). Our data showed qualitative changes in the fatty acid profile as a result of the growth temperature. A suboptimal temperature of 10ºC resulted in the absence of myristic acid, a saturated fatty acid, and arachidonic acid, an unsaturated fatty acid, while a supra-optimal temperature of 40ºC resulted in loss of methyl linolenate, another unsaturated fatty acid in addition to the absence of the above-mentioned myristic acid and arachidonic acid.

Quantitative changes in the fatty acid composition showed that the cultures of *N. spongiaeforme* grown at 30ºC showed 27.62% identifiable SFA and 62.06% of identifiable UFA. *N. spongiaeforme* grown at 10ºC showed a slight decrease of 5% in the SFA but a much greater decrease of 41% in UFA content in comparison to 30ºC. The supra-optimal temperature of 40ºC, however, resulted in an increase of 18% in SFA but a decrease of 37% in UFA.

GC-MS chromatogram of marine cyanobacterium *N. calcicola* showed saturated fatty acids viz; myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), and nonadecanoic acid (C19:0), and unsaturated fatty acids viz; methyl palmitoleate (C16:1), oleic acid (C18:1), linoleic acid (C18:2), eicosenoic acid (C20:1) and methyl linolenate (C20:3). The growth temperatures affected the qualitative changes of the fatty acid profile. A suboptimal temperature of 10ºC resulted in the absence of saturated fatty acids, myristic acid, and nonadecanoic acid, and eicosenoic acid, an unsaturated fatty acid while

the supra- optimal temperature of 40ºC showed an absence of myristic and nonadecanoic saturated fatty acid, and methyl linolenate, an unsaturated fatty acid.

Result also recorded quantitative changes in the fatty acid composition of *N. calcicola.* It was observed that the cultures of *N. calcicola* grown at 30ºC showed 32.21% of identifiable SFA and 51.69% of identifiable UFA. A decrease in the growth temperature to 10ºC showed a slight decrease of 9.4% in identifiable SFA content and an increase of 12% in identifiable UFA content in comparison to 30ºC. The supra-optimal temperature of 40ºC resulted in a 53% increase in SFA while a decrease of 41% in UFA. The fatty acids data showed the presence of pentadecanoic acid and arachidonic acid fatty acids only in *N. spongiaeforme,* while the presence of nonadecanoic acid and eicosenoic acid in *N. calcicola.*

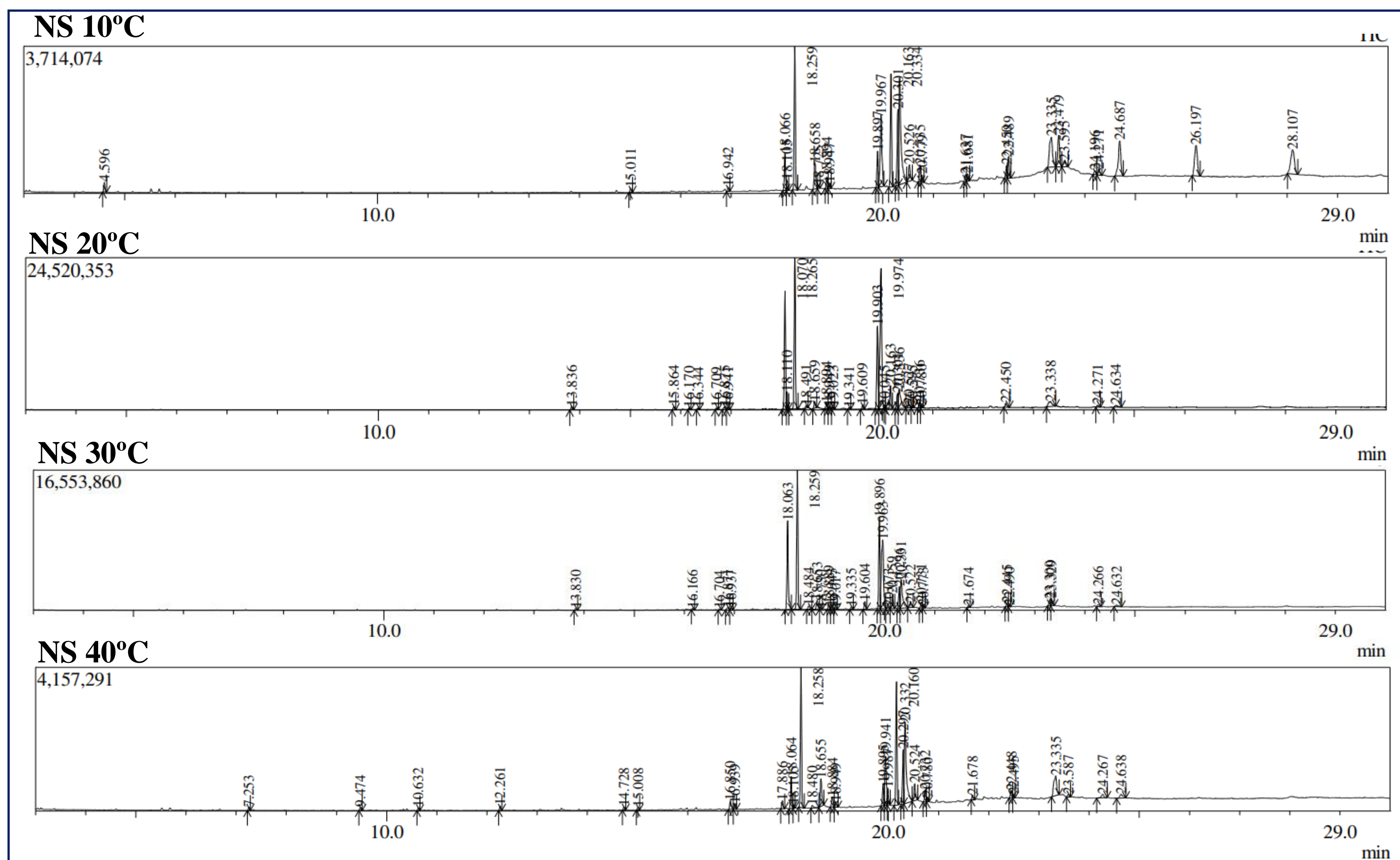


Fig.3.2.11A GC-MS chromatogram of fatty acid methyl esters (FAME) in *N. spongiaeforme* at different growth temperature.

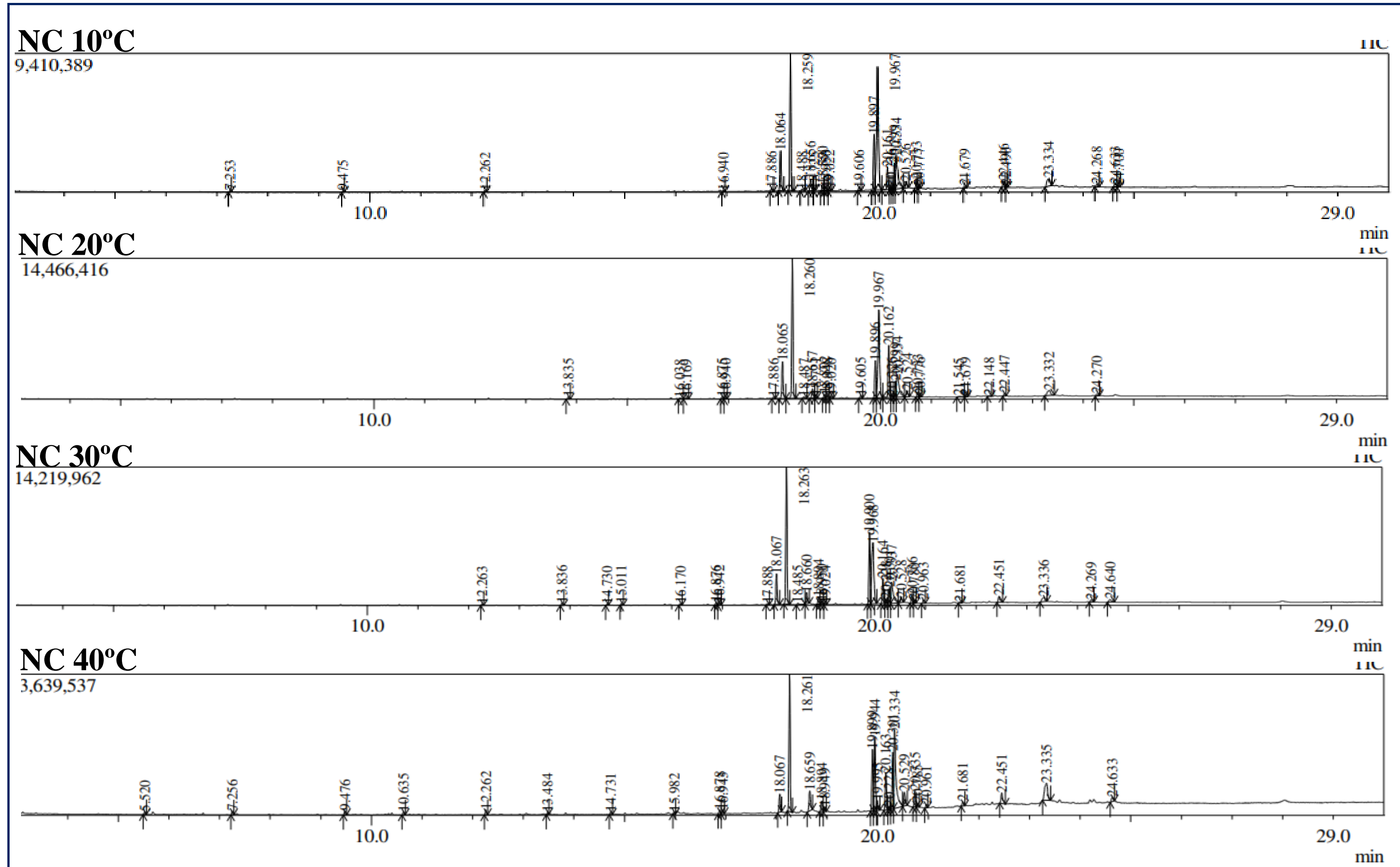


Fig.3.2.11B GC-MS chromatogram of fatty acid methyl esters (FAME) in *N. calcicola* at different growth temperature.

Table 3.2.11A Effect of growth temperatures on fatty acid profile of *N. spongiaeforme* (A) and *N. calcicola* (B) based on the methyl ester of fatty acids, area% of fatty acids). Data represent mean values ± SD (*n*=3). Same letter indicates insignificant differences at p≤0.01.

A

Percent of fatty acids (area% of total fatty acids)					
Sr. No	Fatty acids	10°C	20°C	30°C	40°C
1	Methyl palmitoleate (C16:1)	3.02±0.0145[b]	ND	15.18±0.002[d]	3.97±0.001[c]
2	Palmitic acid (C16:0)	15.44±0.478[a]	21.55±0.004[c]	24.23±0.005[d]	18±0.0025[b]
3	Linoleic acid (C18:2)	10.99±0.001[b]	16.55±0.254[c]	18.98±0.154[d]	2.86±0.0365[a]
4	Methyl linolenate (C20:3)	8.34±0.235[b]	27.16±0.001[d]	18.93±0.0006[c]	ND
5	Oleic acid (C18:1)	14.36±0.254[c]	5.01±0.005[a]	7.62±0.0014[b]	32.27±0.004[d]
6	Stearic acid (C 18:0)	10.81±0.004[c]	3.99±0.004[b]	3.16±0.001[a]	14.69±0.004[d]
7	Myristic acid (C14:0)	ND	0.20±0.01[b]	0.23±0.114[b]	ND
8	Arachidonic acid (C20:4)	ND	ND	1.35±0.0152[b]	ND
9	Pentadecanoic (C15:0)	ND	ND	ND	1.35±0142[b]
	Saturated fatty acids	26.25	25.74	27.62	32.69
	Unsaturated fatty acids	36.71	48.72	62.06	39.1
ND: Not detected					

B

Percent of fatty acids (area% of total fatty acids)					
Sr. No	Fatty acids	10°C	20°C	30°C	40°C
1	Methyl palmitoleate (C16:1)	6.19±0.5421[c]	7.05±0.325[d]	5.95±0.001[b]	1.84±0.124[a]
2	Palmitic acid (C16:0)	23.99±0.021[b]	29.22±0.003[d]	26.55±0.003[c]	20.31±0.325[a]
3	Linoleic acid (C18:2)	13.62±0.3652[c]	10.62±0.0005[a]	17.63±0.005[b]	17.91±0.142[c]
4	Methyl linolenate (C20:3)	27.51±0.0002[d]	22.4±0.365[c]	20.73±0.125[b]	ND
5	Oleic acid (C18:1)	10.68±0.001[c]	2.53±0.4215[a]	7.38±0.364[b]	10±0.251[d]
6	Stearic acid (C 18:0)	5.21±0.365[b]	11.88±0.521[d]	4.7±2.102[a]	28.84±0.112[c]
7	Myristic acid (C14:0)	ND	0.34±0.0001[c]	0.27±2.251[b]	ND
8	Nonadecenoic (C19:0)	ND	ND	0.69±0.214[a]	ND
9	Eicosenoic Acid (C20:1)	ND	ND	ND	0.51±0.121[b]
	Saturated fatty acids	29.2	41.44	32.21	49.15
	Unsaturated fatty acids	58	42.6	51.69	30.26
Nd: not detected					

3.2.12 Effect of growth temperatures on lipid phase transitions

Our result showed changes in lipid phase transition in both the *Nostoc* species due to different growth temperatures (10, 30, and 40ºC). It was observed that *N. spongiaeforme* when grown at optimum temperature of 30ºC showed maximum fluorescence intensity of 91 a.u at lemda max of 682 nm. With decrease in the growth temperature to 10ºC a shift in the peak toward lower wavelength from 682 nm to 671 nm and decline in the fluorescence
intensity to 71 a.u was observed. An increase in the temperature above 30ºC also caused a shift in the peak to lower wavelength from 680 nm to 677 nm and fluorescence intensity decline to 9.4 a.u (Fig. 3.3.12A).

N. calcicola also experienced a shift in the peak and a change in the fluorescence intensity at different growth temperatures (Fig. 3.3.12B). It was observed that at 30ºC, *N. calcicola* displayed maximum fluorescence intensity of 85 a.u at lemda max of 664 nm. A decrease in the growth temperature to 10ºC caused a shift in the peak to lower wavelength from 664 nm to 659 nm and a decline in the fluorescence intensity to 4.9 a.u. The supra-optimal temperature of 40ºC also caused a shift in the peak to lower wavelength from 664 nm to 651 nm with 71 a.u (Fig. 3.3.12B).

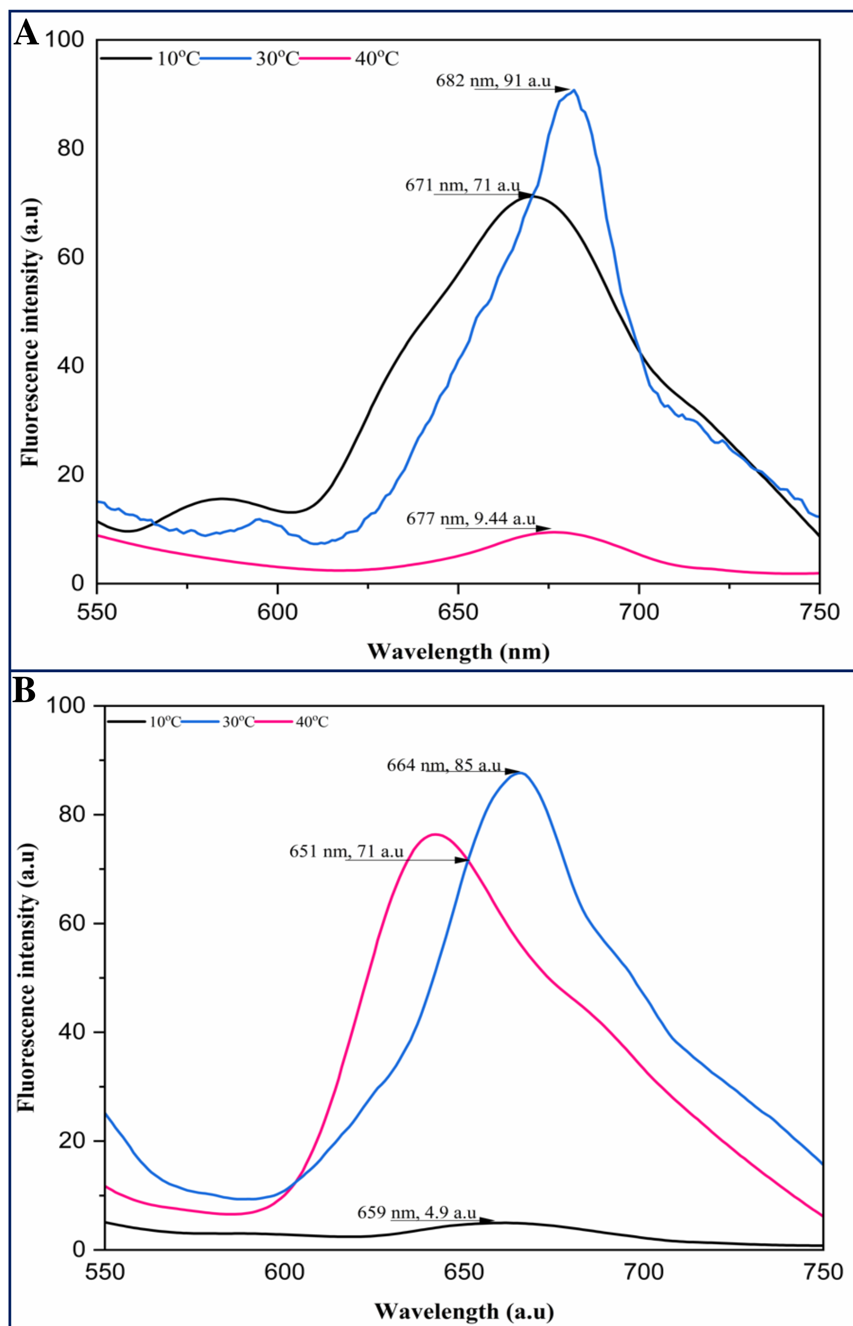


Fig. 3.2.12 Effect of growth temperatures on phase transition of membrane lipid of *N. spongiaeforme* (A) and *N. calcicola* (B).

Sub-chapter 3.3: Effect of nutrient on various parameters

The current sub-chapter aims to present the data with regard to the morphological, physiological, biochemical, and molecular responses of *N. spongiaeforme* (freshwater) and *N. calcicola* (marine) to different concentrations of nitrogen and phosphorus (double the concentration of phosphorus, DP; double the concentration of nitrogen, DN; nitrogen starvation, (-)N; phosphorus starvation, (-)P and combine starvation of nitrogen and phosphorus (-)NP).

3.3.1 Effect of nutrients on *Nostoc* cell morphology

3.3.1.1 External morphology

Cultures of *N. spongiaeforme* and *N. calciocla* grown with different concentrations of P and N showed an adverse effect on external morphology. Cells appeared healthy with an oval shape and smooth surface under control (with respective normal growth medium grown at 30°C), double the concentration of nitrogen (DN) or double the concentration of phosphorus (DP), and nitrogen starvation (-N). Whereas cultures grown under phosphorus starvation (-P) and combined starvation of nitrogen and phosphorus (-NP) cells appeared slightly deformed and shriveled, suggesting morphological damage in the cells of both the *Nostoc* species (Fig. 3.3.1A & B).

3.3.1.2 Internal morphology

Under nutrient stress, i.e., control, DP or DN, and (-)N, the *Nostoc* species remained healthy, and heterocysts production was observed only in the cultures grown under (-)N. On the contrary, cultures grown under (-)P, and (-)NP, displayed a decrease in the loss of photosynthetic pigments and led to the akinete formation in both *Nostoc* spp. Cells of both the *Nostoc* spp., grown under (-)P revealed complete absence of heterocyst while cultures grown under (-)NP showed a few heterocysts formation in the filaments (Fig. 3.3.1C & D).

3.3.2 Effect of nutrients on growth and biomass

Our result with nutrient treatment (different concentrations of nitrogen and phosphorus) showed a difference in the growth rates based on O.D at 680 nm of *Nostoc* spp., (Table 3.3.2A). *Nostoc spongiaeforme* grown under DP, DN, and (-)N exhibited a growth rate with no significant change in comparison to control; however, it experienced a 260% and 321% decline in the growth rate under (-)P and (-)NP respectively as compared to control. *Nostoc calcicola* also experienced the highest growth rate under control conditions, with insignificant changes observed in growth rate under DP, DN, and (-)N. However, under (-)P and (-)NP, the *N. calcicola* registered a decline of 284% and 378% respectively in growth rate as compared to the control.

Biomass and biomass productivity of *N. spongiaeforme* under different concentrations of N and P are shown in Fig. 3.3.2a (A) and Table 3.3.2A. Like growth rate, *N. spongiaeforme* displayed the highest biomass and biomass productivity under control conditions, with no significant change recorded under DP or DN and (-)N. While, under (-)P and (-)NP the biomass was reduced by 48 and 51%, respectively, and biomass productivity by 184% and 209%, respectively, compared to the control. *Nostoc calcicola* also showed similar changes in biomass and biomass productivity with regards to nutrient conditions (Fig. 3.3.2a (B) and Table 3.3.2A).

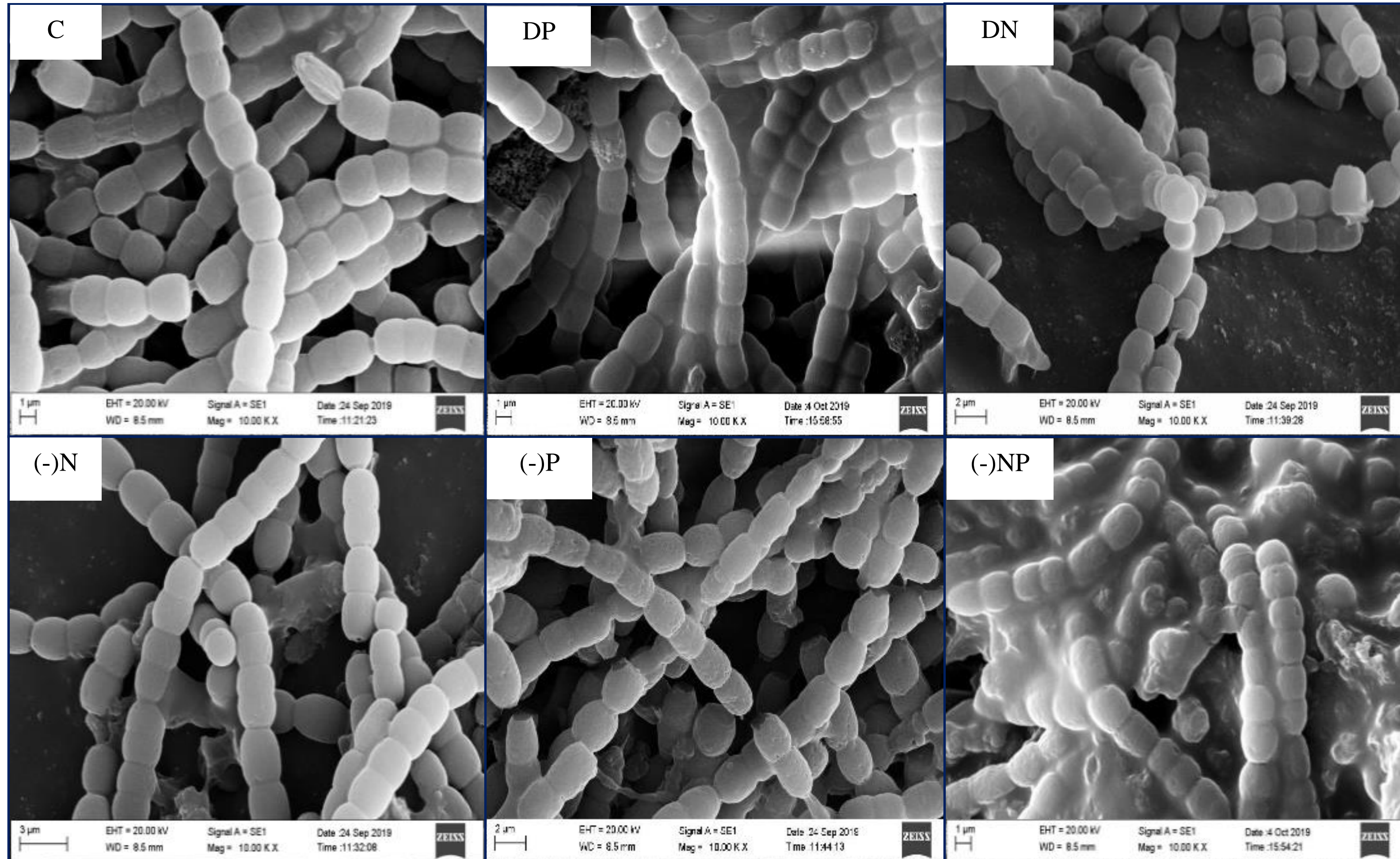


Fig. 3.3.1A Scanning electron microscopy (SEM) images of the *Nostoc spongiaeforme* showing morphological variations at varying nitrogen and phosphorus concentrations. Abbreviations for treatments: C= control, DP= double phosphorus, DN= double nitrogen, (-)N= nitrogen starvation, (-)P= phosphorus starvation, (-)NP = combine starvation of nitrogen and phosphorus.

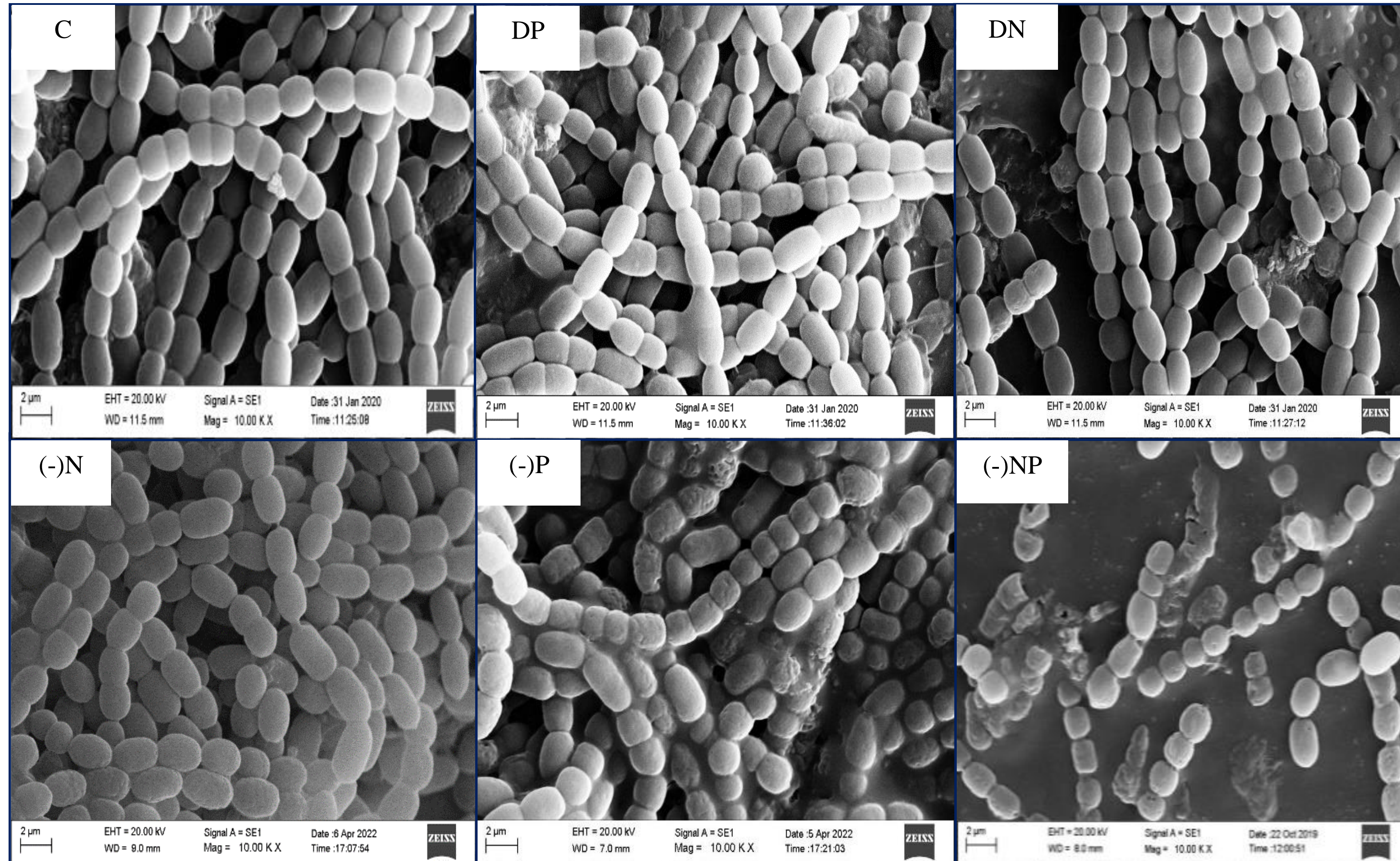


Fig. 3.3.1B Scanning electron microscopy (SEM) images of the *Nostoc calciocla* showing morphological variations at varying nitrogen and phosphorus concentrations. Abbreviations for treatments: C= control, DP= double phosphorus, DN= double nitrogen, (-)N= nitrogen starvation, (-)P= phosphorus starvation, (-)NP = combine starvation of nitrogen and phosphorus.

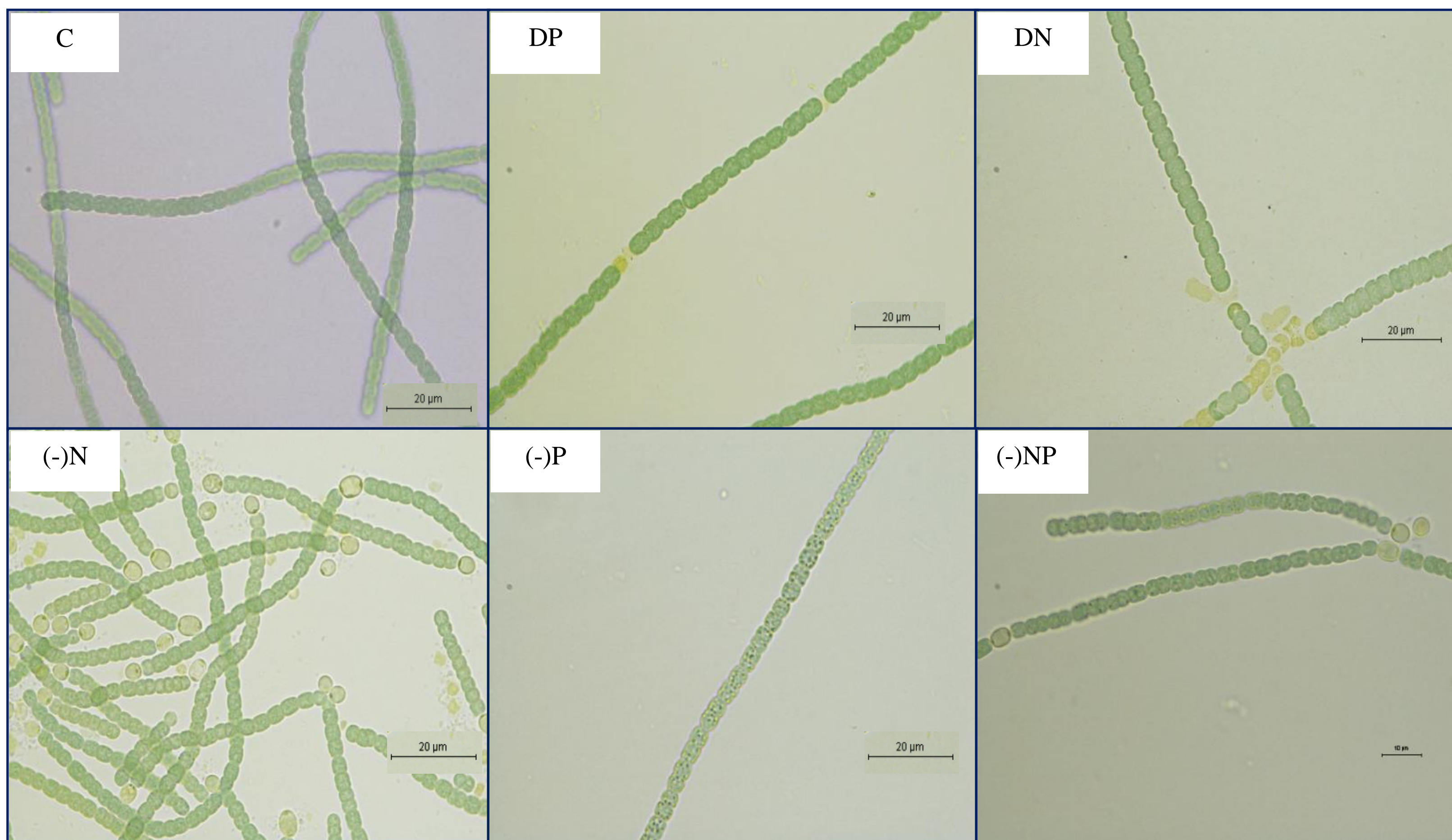


Fig. 3.3.1C Light microscopy images of the *Nostoc spongiaeforme* showing morphological variations at varying nitrogen and phosphorus concentration. Abbreviations for treatments: C= control, DP= double phosphorus, DN= double nitrogen, (-)N= nitrogen starvation, (-)P= phosphorus starvation, (-)NP = combine starvation of nitrogen and phosphorus.

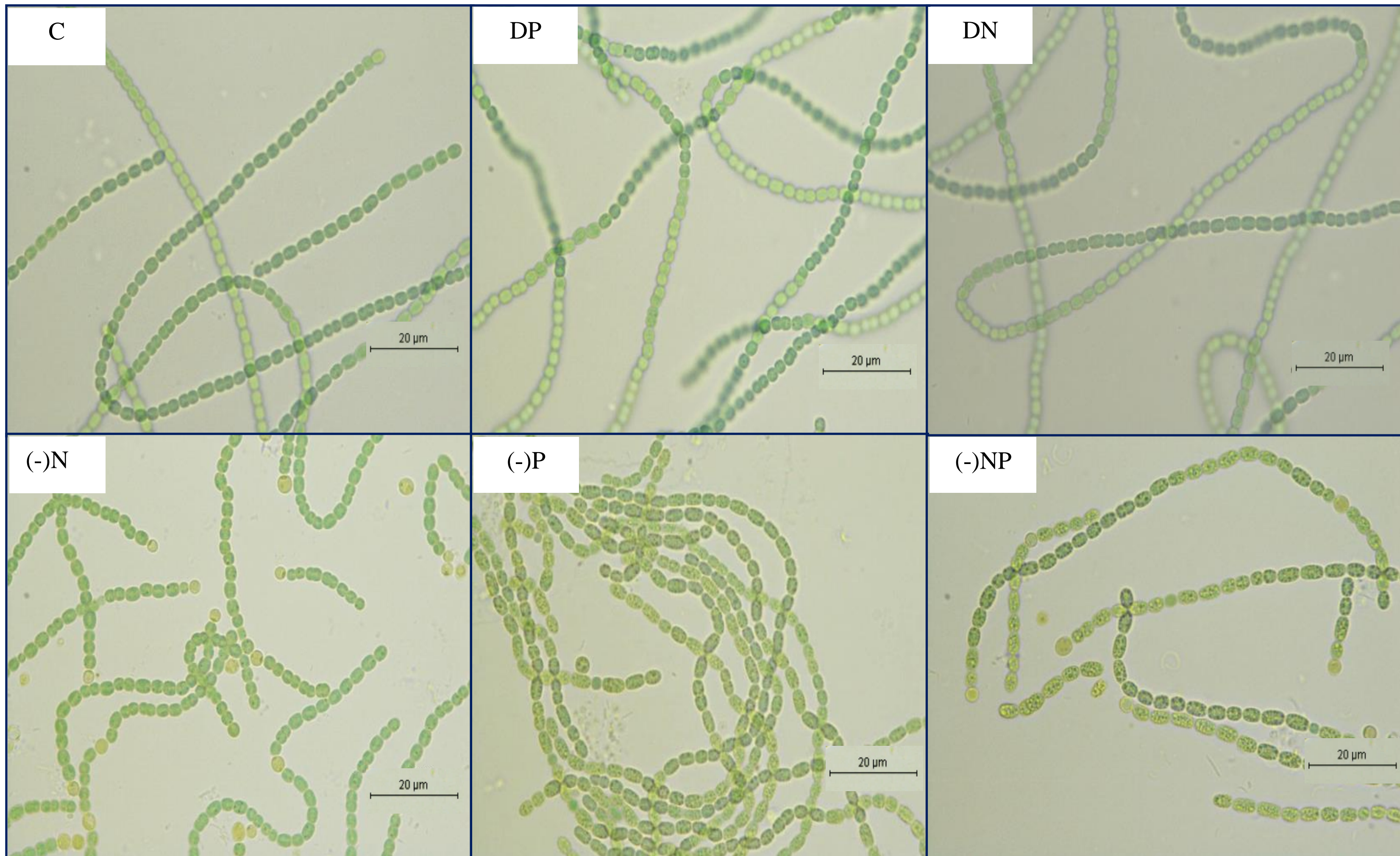


Fig. 3.3.1D Light microscopy images of the *Nostoc calcicola* showing morphological variations at varying nitrogen and phosphorus concentration. Abbreviations for treatments: C= control, DP= double phosphorus, DN= double nitrogen, (-)N= nitrogen starvation, (-)P= phosphorus starvation, (-)NP = combine starvation of nitrogen and phosphorus.

Table 3.3.2A Effect of nutrient on the growth measured at O.D 680 nm, growth rate (μ), biomass (g), and biomass productivity (g L^{-1} day^{-1}) of *N. spongiaeforme* and *N. calcicola*

***Nostoc* spp.**	**Nutrients**	**Growth (O.D 680 nm)**	**Growth rate (μ)**	**Biomass (g L^{-1})**	**Biomass productivity (g L^{-1} day^{-1})**
N. spongiaeforme	Control	1.27±0.01[a]	0.41±0.01	0.534±0.06[a]	0.220±0.01
	DP	1.22±0.01[a]	0.38±0.012	0.587±0.05[b]	0.221±0.032
	DN	1.22±0.01[a]	0.38±0.01	0.540±0.04[b]	0.203±0.041
	(-) N	1.13±0.03[b]	0.30±0.023	0.437±0.08[b]	0.130±0.052
	(-) P	0.43±0.01[c]	-0.66±0.016	0.280±0.02[a]	-0.185±0.014
	(-) NP	0.34±0.01[d]	-0.91±0.025	0.26±0.02[c]	-0.24±0.451
N. calcicola	Control	1.28±0.01[b]	0.337±0.005	0.547±0.02[a]	0.184±0.012
	DP	1.33±0.00[a]	0.348±0.054	0.633±0.02[d]	0.220±0.041
	DN	1.34±0.01[a]	0.354±0.056	0.60±0.01[d]	0.212±0.012
	(-) N	1.23±0.02[c]	0.27±0.042	0.473±0.02[c]	0.13±0.02
	(-) P	0.51±0.00[d]	-0.619±0.057	0.280±0.04[b]	-0.173±0.04
	(-) NP	0.37±0.00[e]	-0.936±0.058	0.22±0.02[a]	-0.211±0.06

In our study, both the *Nostoc* spp., were also grown with three and four-time concentrations of nitrogen or phosphorus individually and also with combined double concentrations of nitrogen and phosphorus (DNP). The cultures were also grown using ammonium chloride in place of sodium nitrate to understand the role of ammonium ions on the growth of *Nostoc* spp. The growth of *N. spongiaeforme* showed a 57% decline under DNP, whereas it did not affect the growth of *N. calcicola*. When grown under three and four-time concentrations of phosphorus, *N. spongiaeforme*, displayed a decrease of 28% and 20%, however, *N. calcicola* displayed only a 2 and 8% decrease in growth (Fig. 3.3.2b (D, E) & Table. 3.3.2B). When grown under three and four-time concentrations of nitrogen *N. spongiaeforme* displayed a decrease of 75 and 90%, while *N. calcicola* displayed only a 20% decline in growth (Fig. 3.3.2b (D, E) & Table 3.3.2B).

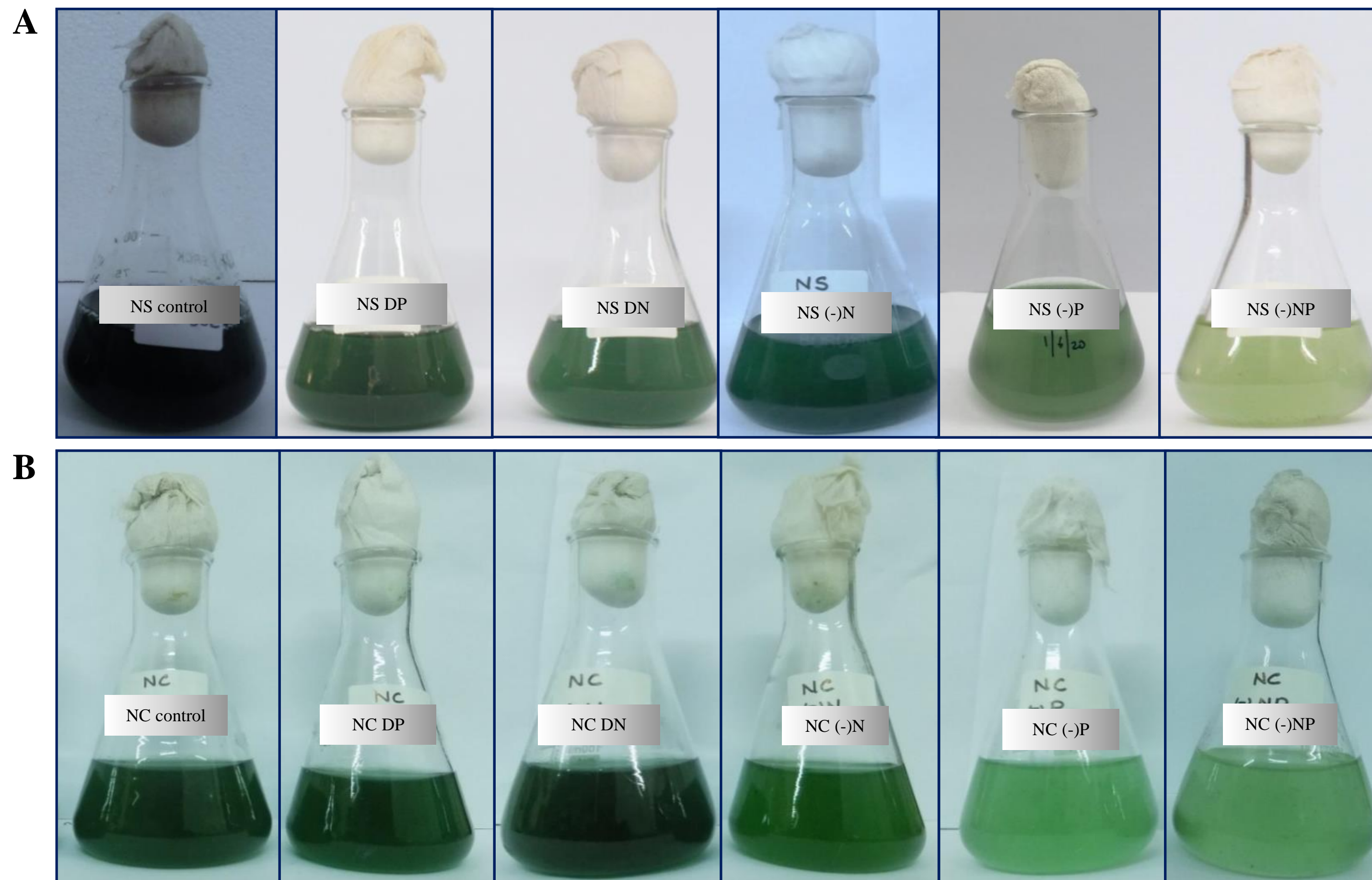


Fig. 3.3.2a Pictorial representation of growth (biomass) of *Nostoc spongiaeforme* (A) and *Nostoc calcicola* (B) grown under nutrient treatment. Abbreviations for treatments: C= control, DP= double phosphorus, DN= double nitrogen, (-)N= nitrogen starvation, (-)P= phosphorus starvation, (-)NP = combine starvation of nitrogen and phosphorus.

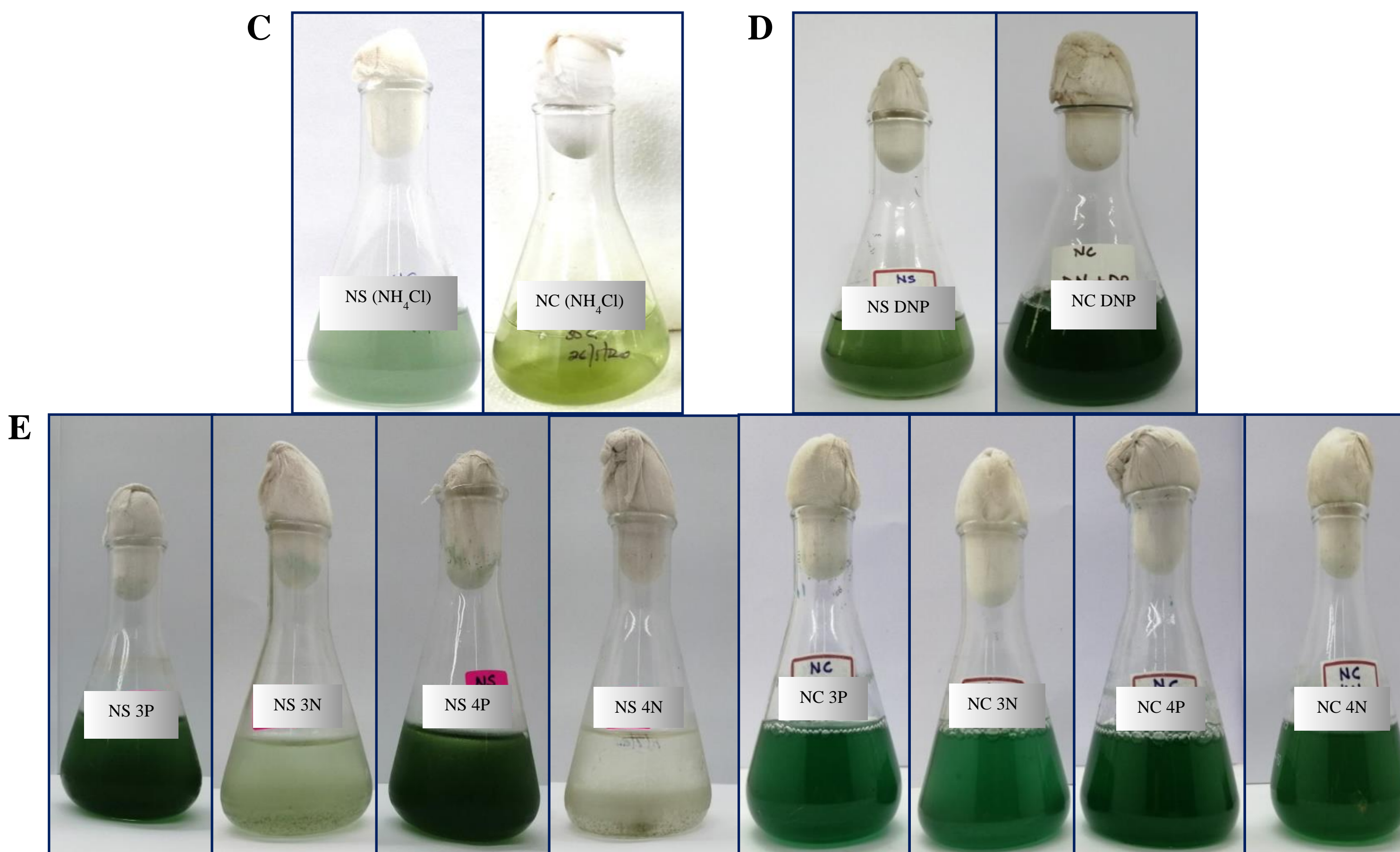


Fig. 3.3.2b Pictorial representation of growth of *N. spongiaeforme* and *N. calcicola* when grown under ammonium (C); double nitrogen and phosphorus concentration, DNP (D); triple concentration of phosphorus, 3P; triple concentration of nitrogen, 3N; four time concentration of phosphorus, 4P and four time concentration of nitrogen, 4N (E).

Table 3.3.2.B Effect of nutrient on the growth of *Nostoc* spp., measured at O.D 680 nm

Concentration of N & P	*N. spongiaeforme*	*N. calcicola*
Control (C)	1.27±0.01	1.28±0.01
Double con. of phosphorus (DP)	1.22±0.01	1.33±0.00
Double con. of nitrogen (DN)	1.22±0.01	1.34±0.01
Double nitrogen phosphorus (DNP)	0.53±0.012	1.264±0.03
Triple con. of phosphorus (3P)	0.91±0.03	1.26±0.01
Triple con. of nitrogen (3N)	0.32±0.01	1.03±0.06
Four-time con. of phosphorus (4P)	1.02±0.00	1.18±0.00
Four-time con. of nitrogen (4N)	0.13±0.01	1.03±0.03

3.3.3 Effect of nutrient on photosynthesis

3.3.3.1 Photosynthetic efficiency of PS II (Fv/Fm ratio)

The photochemical efficiency of PS II measured as the Fv/Fm ratio of both *Nostoc* species under different N and P concentrations is displayed in Fig. 3.3.3A. The Fv/Fm ratio did not change significantly in the *Nostoc* species when grown under DP, DN, and (-)N culture medium. P starvation, however, caused up to 61% decline in the Fv/Fm ratio, whereas (-)NP resulted in up to 66% decline in the Fv/Fm ratio of both the studied *Nostoc* species as compared to their controls.

3.3.3.2 Photosynthetic rate (oxygen evolution)

Photosynthetic rate measured as oxygen evolution in *N. spongiaeforme* and *N. calcicola* was not significantly affected under DP or DN and (-)N compared to their controls (Fig. 3.3.3B). However, the photosynthetic rate of both the *Nostoc* species declined up to 87% under (-)P condition and up to 92% under (-)NP as compared to their controls (Fig. 3.3.3B).

3.3.3.3 Photosynthetic pigment content (chlorophyll and carotenoids) of *Nostoc* species

Our results displayed that different concentrations of N and P also caused changes in the photosynthetic pigments of both the *Nosotc* species (Table 3.3.3). Result showed that the chl *a* content of both the *Nostoc* species did not change significantly under DP, DN and (-)N the growth of *Nostoc* spp. The growth of *N. spongiaeforme* showed a 57% decline under DNP, whereas it did not affect the growth of *N. calcicola*. When grown under three and

four-time concentrations of phosphorus, *N. spongiaeforme*, displayed a decrease of 28% and 20%, however, *N. calcicola* displayed only a 2 and 8% decrease in growth (Fig. 3.3.2b (D, E) & Table. 3.3.2B). When grown under three and four-time concentrations of nitrogen *N. spongiaeforme* displayed a decrease of 75 and 90%, while *N. calcicola* displayed only a 20% decline in growth (Fig. 3.3.2b (D, E) & Table 3.3.2B).

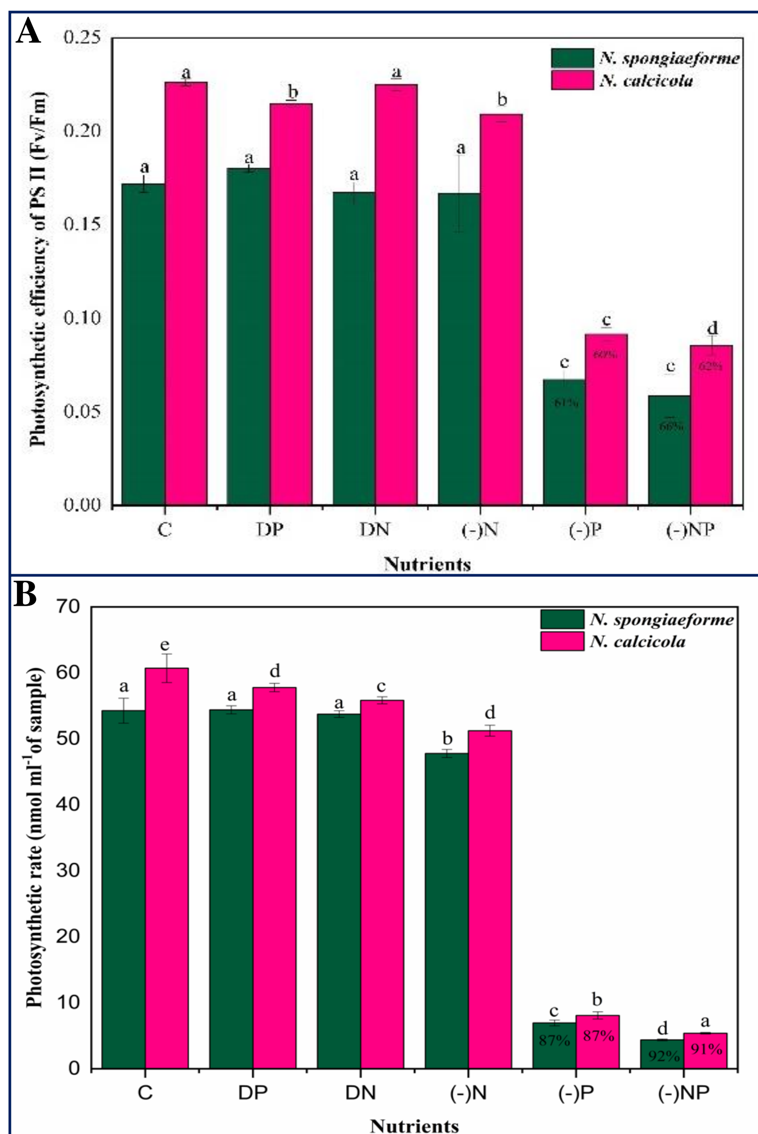


Fig. 3.3.3 Effect of nutrient stress on the Fv/Fm ratio (A) and photosynthetic rate (B) of *N. spongiaeforme* and *N. calcicola*. Standard deviation is represented by vertical bars (±SD, *n* = 3). Bars denoted by the same letter indicates insignificant difference at $p \leq 0.01$. C= control, DP= double phosphorus, DN= double nitrogen, (-)N= nitrogen starvation, (-)P= phosphorus starvation, (-)NP = combine starvation of nitrogen and phosphorus.

Total carotenoid also showed similar pattern of changes as seen for chlorophyll due to nutrients concentration (Table 3.3.3). The total carotenoid content did not show any significant change under DP, DN and (-)N in both the *Nostoc* species, however, (-)P and (-)NP growth conditions resulted in a decline of 29 and 52%, respectively, in *N. spongiaeforme,* and a decrease of 57 and 70% respectively in *N. calcicola* in their total carotenoid content in comparison to its control (Table 3.3.3).

3.3.3.4 Phycobilins pigment content (phycocyanin and allophycocyanin) of *Nostoc* species

The phycocyanin content of both the *Nostoc* species did not change significantly under DP, DN, and (-)N, but P starvation and combined starvation of nitrogen and phosphorus resulted in a decline of 67 and 77%, respectively in phycocyanin content in *N. spongiaeforme*. *Nostoc calcicola*, however showed a greater decrease of 71 and 82% in phycocyanin content when grown under the (-)P and (-)NP nutrient starvation in comparison to its control (Table 3.3.3).

Table 3.3.3: Effect of nutrients on the photosynthetic pigments; chlorophyll *a*, total carotenoid, phycocyanin (PC), allophycocyanin (APC) of *N. spongiaeforme* and *N. calcicola*

***Nostoc* spp.**	**Nutrients**	**Chl *a* (µg mL^{-1} cell suspension)**	**Total carotenoid (µg mL^{-1} cell suspension)**	**Phycocyanin (µg mL^{-1} cell suspension)**	**Allophycocyanin (µg mL^{-1} cell suspension)**
N. spongiaeforme	Control	3.07±0.02^{a}	1.21±0.04^{a}	0.9±0.05^{a}	0.54±0.03^{a}
	DP	2.84±0.01^{b}	1.11±0.05^{b}	0.8±0.06^{a}	0.46±0.04^{b}
	DN	2.68±0.27^{b}	1.16±0.12^{b}	0.8±0.04^{a}	0.43±0.04^{b}
	(-) N	2.54±0.42^{b}	1.13±0.10^{b}	0.8±0.01^{a}	0.45±0.01^{b}
	(-) P	0.50±0.02^{c}	0.86±0.01^{c}	0.3±0.03^{b}	0.17±0.01^{c}
	(-) NP	0.48±0.01^{c}	0.58±0.03^{d}	0.2±0.02^{c}	0.14±0.02^{c}
N. calcicola	Control	7.22±0.01^{a}	2.14±0.01^{a}	1.7±0.02^{a}	0.98±0.09^{a}
	DP	7.26±0.54^{a}	2.00±0.14^{a}	1.6±0.02^{a}	0.95±0.06^{a}
	DN	7.12±0.49^{a}	2.04±0.18^{a}	1.4±0.05^{b}	0.89±0.01^{b}
	(-) N	7.10±0.07^{a}	1.76±0.21^{b}	1.4±0.01^{b}	0.86±0.02^{b}
	(-) P	1.78±0.01^{b}	0.92±0.06^{c}	0.5±0.1^{c}	0.16±0.03^{c}
	(-) NP	1.21±0.11^{c}	0.64±0.08^{d}	0.3±0.1^{c}	0.17±0.01^{c}

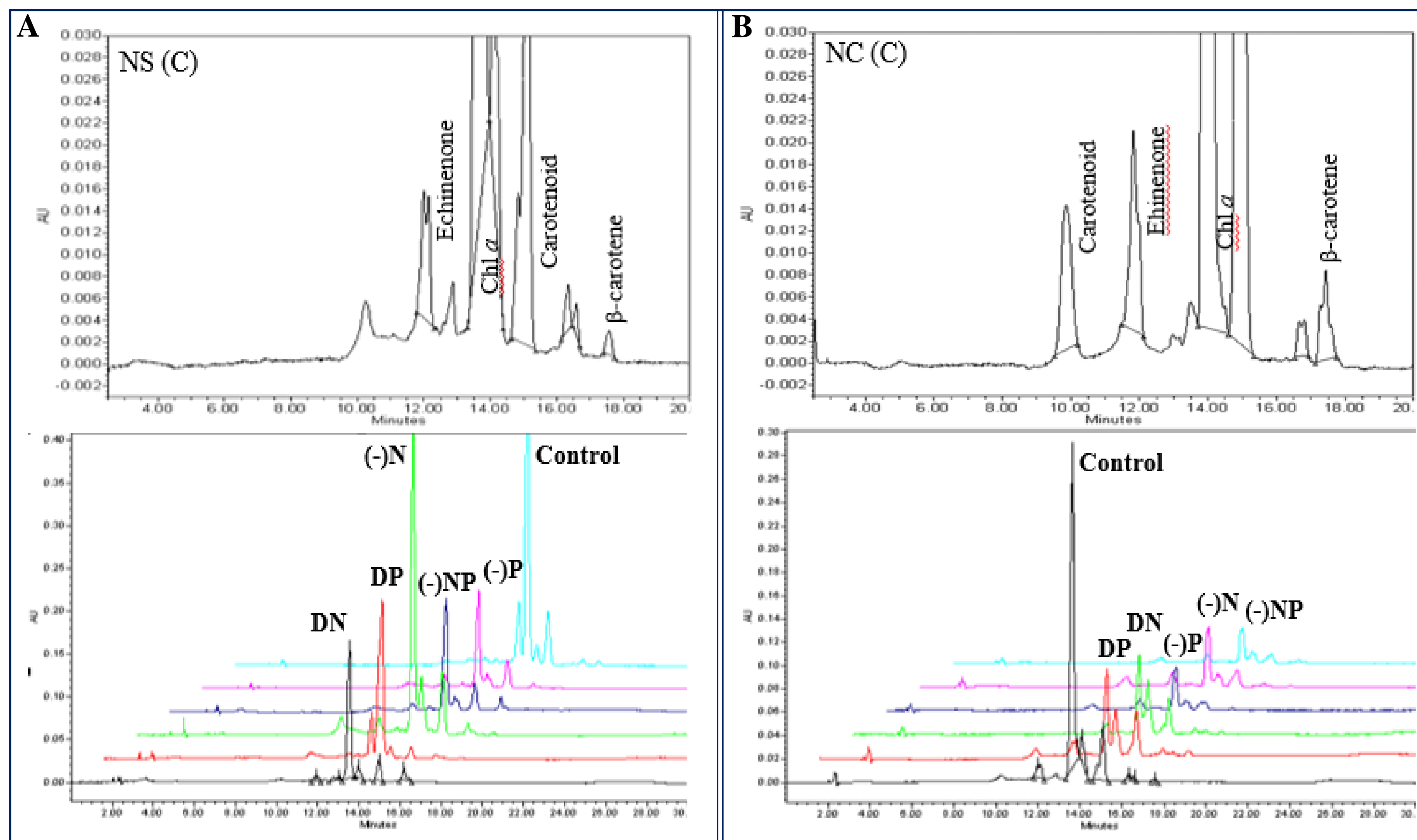


Fig. 3.3.3C HPLC pigment profile extracted at 445 nm and comparative HPLC spectrum of pigment of *N. spongiaeforme* (A) and *N. calcicola* (B) under different nitrogen and phosphorus concentration. C= control, DP= double phosphorus, DN= double nitrogen, (-)N= nitrogen starvation, (-)P= phosphorus starvation, (-)NP = combine starvation of nitrogen and phosphorus.

The allophycocyanin pigment of both the *Nostoc* species also showed similar result. Allophycocyanin pigment did not change significantly in both the *Nostoc* species under DP, DN, and (-)N as compared to their controls. P starvation and combined starvation of nitrogen and phosphorus growth conditions, however, resulted in a decrease up to 74% in *N. spongiaeforme* and up to 84% in *N. calcicola* in comparison to its control (Table. 3.3.3). Deficiency of N&P being slightly more than observed for only P deficiency.

3.3.4 Effect of nutrients on respiratory rate (oxygen consumption)

The effect of different concentrations of N and P on the respiratory rate of *N. spongiaeforme* and *N. calcicola* are presented in Fig. 3.3.4. Double the concentration of P or N and (-)N did not cause any significant change in the respiratory rate of *N. spongiaeforme* and *N. calcicola* compared to their controls. P starvation alone and combined starvation of nitrogen and phosphorus growth conditions resulted in a decline of 88 and 93%, respectively, in the respiratory rate of *N. spongiaeforme*, while the respiratory rate of *N. calcicola* decreased by 43 and 57% when grown under the same nutrient starvation respectively in comparison to its control (Fig. 3.3.4).

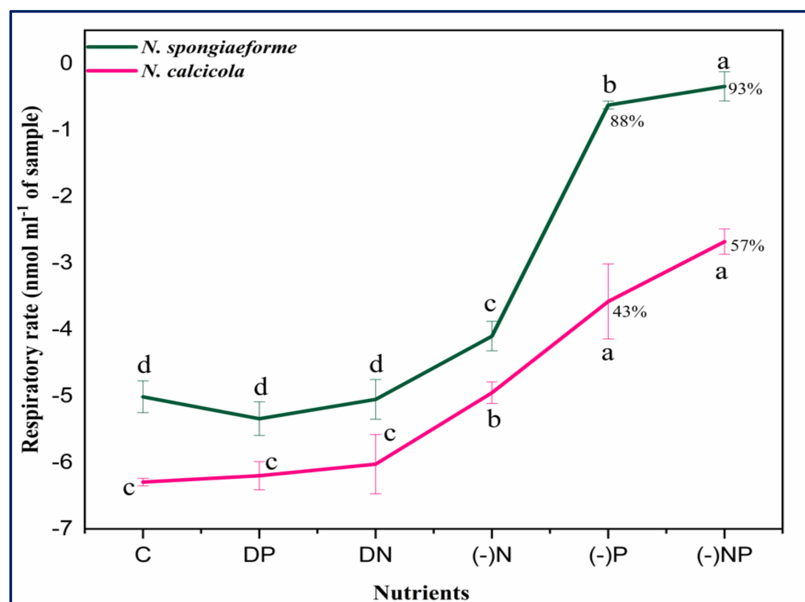


Fig. 3.3.4 Effect of nutrient stress on the respiratory rate of *N. spongiaeforme* and *N. calcicola*. Standard deviation is represented by vertical bars (±SD, $n = 3$). Bars denoted by the same letter indicate insignificant difference at $p \leq 0.01$. C= control, DP= double phosphorus, DN= double nitrogen, (-)N= nitrogen starvation, (-)P= phosphorus starvation, (-)NP = combine starvation of nitrogen and phosphorus.

3.3.5 Effect of nutrients on phenolic compounds of *Nostoc* species

Growth under excess and starvation of N and P also resulted a change in the production of TPC and TFC in both the *Nostoc* species (Table. 3.3.5). Double the concentration of P or N caused a 5-fold increase in the TPC in *N. spongiaeforme* and a 7-fold in *N. calcicola* than seen in their respective controls. Nitrogen deprivation (-N) resulted in a 2-fold increase in the TPC in *N. spongiaeforme* and a 3-fold in *N. calcicola* in comparison to their controls. Phosphorus starvation showed a 2-fold increase in both *Nostoc* species. Combined starvation of nitrogen and phosphorus (-)NP did not show any change in the TPC in both the *Nostoc* species.

The TFC was increased up to 3-fold in both the *Nostoc* species under double the concentration of N or P. However, under (-)P and (-)NP conditions, the TFC did not show any significant change in both the *Nostoc* species as compared to control.

Table 3.3.5 Effect of nutrients on the phenol and flavonoid content of *N. spongiaeforme* and *N. calcicola*

Nostoc spp.	Nutrients	Phenols (μmol gm^{-1} of tissue)	Flavonoids (μmol gm^{-1} of tissue)
N. spongiaeforme	Control	0.03±0.05[a]	0.12±0.01[a]
	DP	0.14±0.01[d]	0.36±0.03[e]
	DN	0.15±0.01[d]	0.34±0.09[e]
	(-) N	0.07±0.01[c]	0.19±0.02[b]
	(-) P	0.05±0.01[b]	0.07±0.09[c]
	(-) NP	0.03±0.04[a]	0.02±0.02[d]
N. calcicola	Control	0.03±0.04[a]	0.14±0.02[a]
	DP	0.21±0.04[d]	0.40±0.07[f]
	DN	0.22±0.01[d]	0.39±0.01[e]
	(-) N	0.10±0.01[c]	0.20±0.06[d]
	(-) P	0.05±0.01[b]	0.11±0.05[b]
	(-) NP	0.03±0.01[a]	0.08±0.12[c]

3.3.6 Effect of nutrient on ROS production (H_2O_2 and OH•)

The growth of *Nostoc* species under excess and starvation of N and P also showed a change in generation of ROS (H_2O_2 and OH•). It was observed that culture media containing double 88 and 93%, respectively, in the respiratory rate of *N. spongiaeforme*, while the respiratory rate of *N. calcicola* decreased by 43 and 57% when grown under the same nutrient starvation respectively in comparison to its control (Fig. 3.3.4).

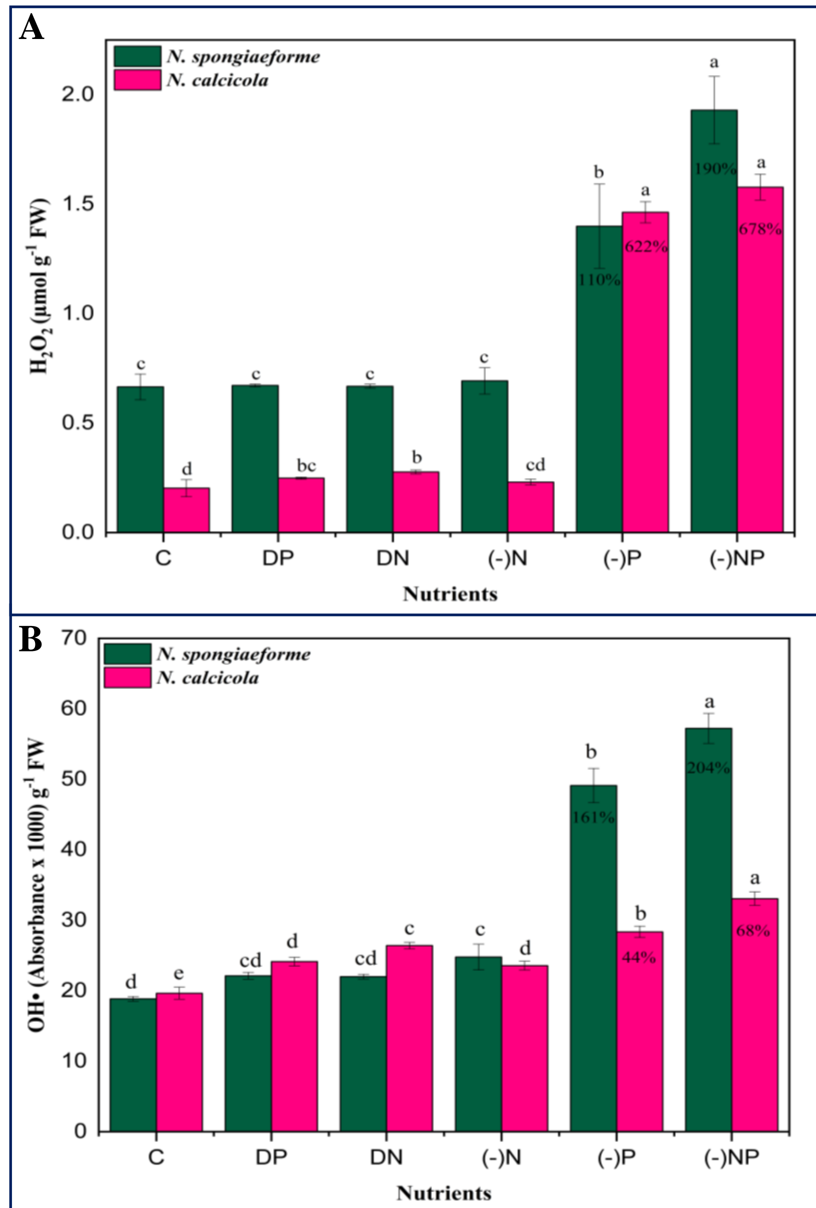


Fig. 3.3.6 Effect of nutrient stress on hydrogen peroxide (A) and hydroxyl radicals (B) of *Nostoc spongiaeforme* and *Nostoc calcicola*. Standard deviation is represented by vertical bars (±SD, n = 3). Bars denoted by the same letter indicate insignificant difference at $p \leq 0.01$. C= control, DP= double phosphorus, DN= double nitrogen, (-)N= nitrogen starvation, (-)P= phosphorus starvation, (-)NP = combine starvation of nitrogen and phosphorus.

The OH• content in both the *Nostoc* species did not change significantly under DP, DN, and (-)N compared to their controls. However, due to (-)P, an increase of 161% and 44% production in OH• was observed in *N. spongiaeforme* and *N. calcicola,* respectively (Fig. 3.3.6B). Likewise, (-)NP, also exhibited a rise of 204 and 68% production in OH• in *N. spongiaeforme* and *N. calcicola,* respectively, compared to their respective controls (Fig. 3.3.6B).

3.3.7 Effect of nutrient on lipid peroxidation and protein oxidation

Our data showed that excess and starvation of N and P also caused lipid peroxidation and protein oxidation in both the *Nostoc* species (Fig. 3.3.7). It was observed that double the concentration of P or N and (-)N did not cause any significant increase in the MDA content in both the *Nostoc* species compared to their controls.

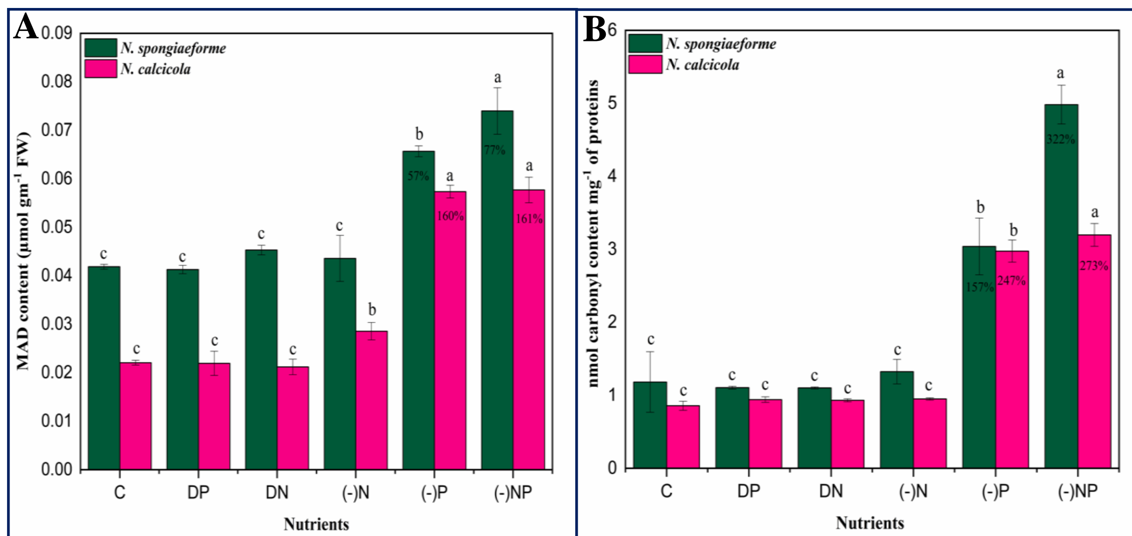


Fig. 3.3.7 Effect of nutrient stress on lipid peroxidation (A) and protein oxidation (B) of *N. spongiaeforme* and *N. calcicola*. Standard deviation is represented by vertical bars (±SD, *n* = 3). Bars denoted by the same letter indicate insignificant difference at p≤0.01. C= control, DP= double phosphorus, DN= double nitrogen, (-)N= nitrogen starvation, (-)P= phosphorus starvation, (-)NP = combine starvation of nitrogen and phosphorus.

However, (-)P and (-)NP displayed up to 77% and 161% increase in MDA content in *N. spongiaeforme* and *N. calcicola,* respectively in comparison to control (Fig. 3.3.7A).

Likewise, the protein carbonyl content in both the *Nostoc* species also did not vary significantly when grown under DP or DN and (-)N compared to their controls but when grown under (-)P and (-)NP the protein carbonyl content increased by 157 and 322% respectively in *N. spongiaeforme* and 247 and 273% respectively in *N. calcicola* in comparison to their respective control (Fig. 3.3.7B).

3.3.8 Effect of nutrients on enzymatic and non-enzymatic antioxidants

3.3.8.1 Enzymatic antioxidants (SOD, APX, and CAT)

SOD activity in both the *Nostoc* species did not increase significantly when grown under DP or DN compared to their respective controls. However, nutrient conditions of (-)N, (-)P and (-)NP increased the SOD activity up to 1.2-fold in *N. spongiaeforme* and in *N. calcicola* in comparison to their control (Fig. 3.3.8.1A).

The APX activity showed the similar pattern of changes as a result of the nutrients concentration. The APX activity did not change significantly under DP or DN in both the *Nostoc* spp., compared to their respective controls. While the APX activity was decreased under (-)N in *N. spongiaeforme* compared to its control. However, phosphorus starvation and combined starvation of N and P increased the APX activity by 2-fold in *N. spongiaeforme* and 2.6 and 3.5-fold, respectively, in *N. calcicola* compared to their respective controls (Fig. 3.3.8.1B).

The catalase activity in both the *Nostoc* species under excess and starvation of N and P was studied and showed no markedly change under DP or DN and (-)N. However, the CAT activity increased by 6-fold and 14-fold, respectively, in *N. spongiaeforme*, under (-)P and (-)NP growth conditions respectively compared to its control. However, the same nutrient starvation in *N. calcicola* increased the CAT activity up to 2-fold compared to its control (Fig. 3.3.8.1C).

3.3.8.2 Non-enzymatic antioxidants (ascorbate and proline content)

Ascorbate content (AsA) varies significantly in *N. spongiaeforme* under excess and starvation of N and P. The AsA content in *N. calcicola* did not change significantly when

grown with DP or DN and (-)N. However, nutrient conditions of (-)P and (-)NP increased the AsA content by 178 and 200%, respectively, in *N. spongiaeforme* and 109 and 145%, respectively, in *N. calcicola* in comparison to their respective control (Fig. 3.3.8.2A).

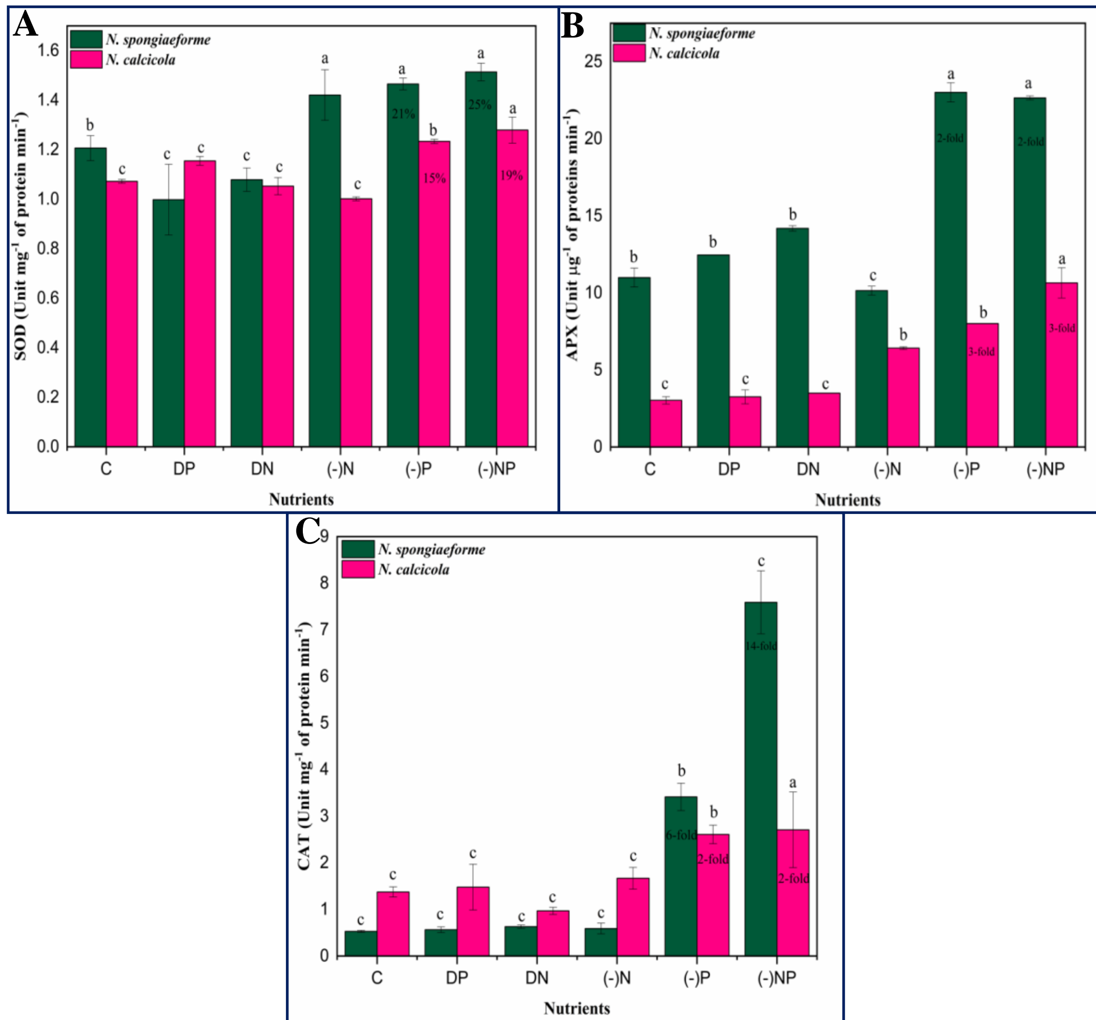


Fig. 3.3.8.1 Effect of nutrient on antioxidants enzymes activities of SOD (A); APX (B) and CAT (C) of *Nostoc spongiaeforme* and *Nostoc calcicola*. Standard deviation is represented by vertical bars (±SD, n = 3). Bars denoted by the same letter indicate insignificant difference at $p \leq 0.01$. C= control, DP= double phosphorus, DN= double nitrogen, (-)N= nitrogen starvation, (-)P= phosphorus starvation, (-)NP = combine starvation of nitrogen and phosphorus.

Our results showed that excess and starvation of N and P resulted changes in proline content in both the *Nostoc* species (Fig 3.3.8.2B). The proline content change significantly

due to DP, DN, and (-)N in *N. calcicola* while *N. spongiaeforme* did not show any significant change in proline content under DN and (-)N however there was a significant change observed under DP. Cultures grown with (-)P and (-)NP increased the proline content by 152 and 182%, respectively, in *N. spongiaeforme*, and 75 and 113%, respectively, in *N. calcicola* in comparison to their respective control (Fig. 3.3.8.2B).

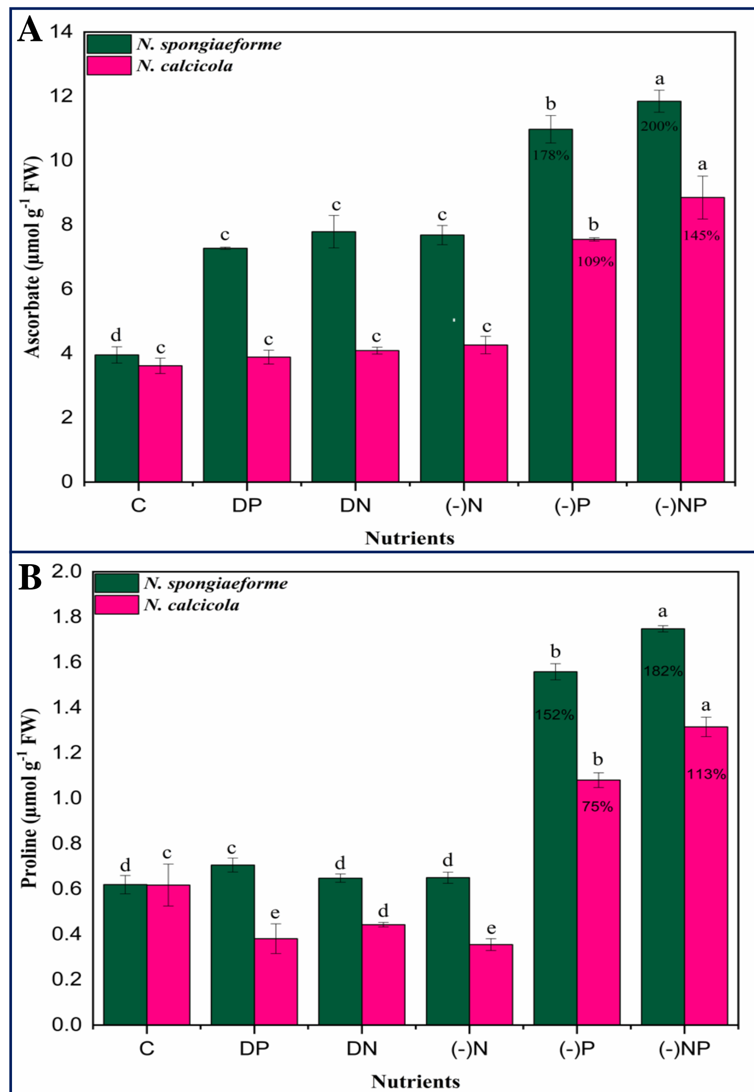


Fig. 3.3.8.2 Effect of nutrient on ascorbate (A) and proline (B) of *N. spongiaeforme* and *N. calcicola*. Standard deviation is represented by vertical bars (±SD, *n* = 3). Bars denoted by the same letter indicate insignificant difference at p≤0.01. C= control, DP= double phosphorus, DN= double nitrogen, (-)N= nitrogen starvation, (-)P= phosphorus starvation, (-)NP = combine starvation of nitrogen and phosphorus.

3.3.9 Effect of nutrient on nitrogenase activity

The nitrogenase activity in both the *Nostoc* species was affected due to excess and starvation of N and P (Fig. 3.3.9). Our results exhibited that nitrogen starvation significantly increased the nitrogenase activity. It was observed that N starvation to *N. spongiaeforme* resulted in an increase of 313% in nitrogenase activity in comparison to 270% increase seen in *N. calcicola* for some nutrient conditions as compared to their respective controls (optimum concentration of N and P). However, both the species of *Nostoc* grown under medium with (-)P and (-)NP resulted in a decrease in nitrogenase activity up to 96%. The *N. spongiaeforme* cultures grown with DP or DN caused a decrease of 8 and 26% in nitrogenase activity, comparison to a decrease of 19 and 32% seen in *N. calcicola* compared to the control (Fig. 3.3.9).

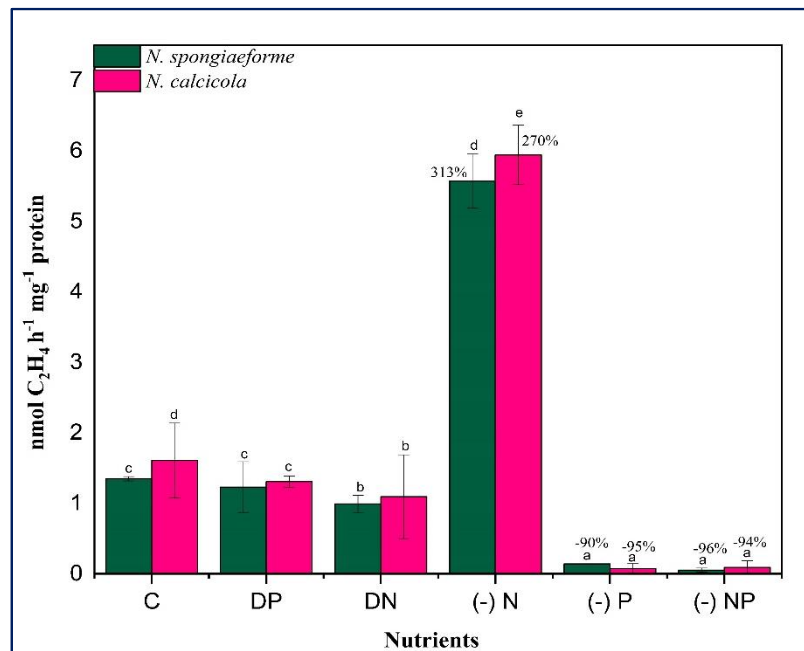


Fig. 3.3.9A Effect of nutrient on nitrogenase activity of *N. spongiaeforme* and *N. calcicola.* Standard deviation is represented by vertical bars (±SD, n = 3). Bars denoted by the same letter indicate insignificant difference at $p \leq 0.01$. C= control, DP= double phosphorus, DN= double nitrogen, (-)N= nitrogen starvation, (-)P= phosphorus starvation, (-)NP = combine starvation of nitrogen and phosphorus.

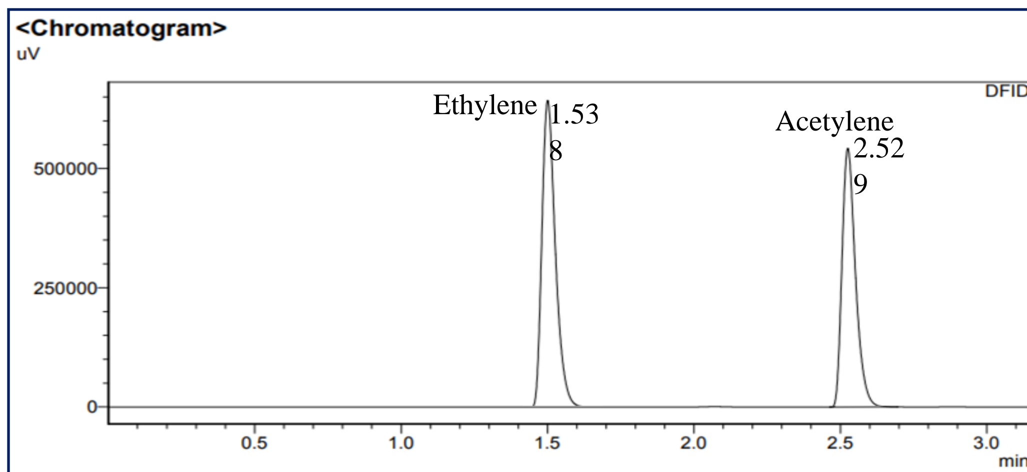


Fig. 3.3.9B GC-chromatogram of ethylene and acetylene gas used for estimating nitrogenase activity.

3.3.10 Gene expression studies of SOD, CAT and nif *H*

The results demonstrates that the SOD gene expression decreased under DN and (-)N as compared to the control in both the *Nostoc* species. Whereas the same was increased to 3-fold in *N. spongiaeforme* and 5-fold in *N. calcicola* under phosphorus starvation compared to their controls (Fig. 3.3.10A).

The expression of CAT was also altered due to excess and starvation of N and P in both the *Nostoc* species. The CAT expression showed a significant change under DN and (-)N in *N. spongiaeforme* and *N. calcicola* than their respective control. While phosphorus starvation resulted in a 1.9-fold increase in the CAT expression in *N. spongiaeforme* while a 4.5-fold increase in *N. calcicola* compared to their controls (Fig. 3.3.10B).

The expression of *nif H* genes was decreased in both the *Nostoc* species due to DN and (-)P. However, under (-)N, the expression of *nif H* gene was increased to 7-fold in *N. calcicola* and 6-fold in *N. spongiaeforme* compared to their controls (Fig. 3.3.10C).

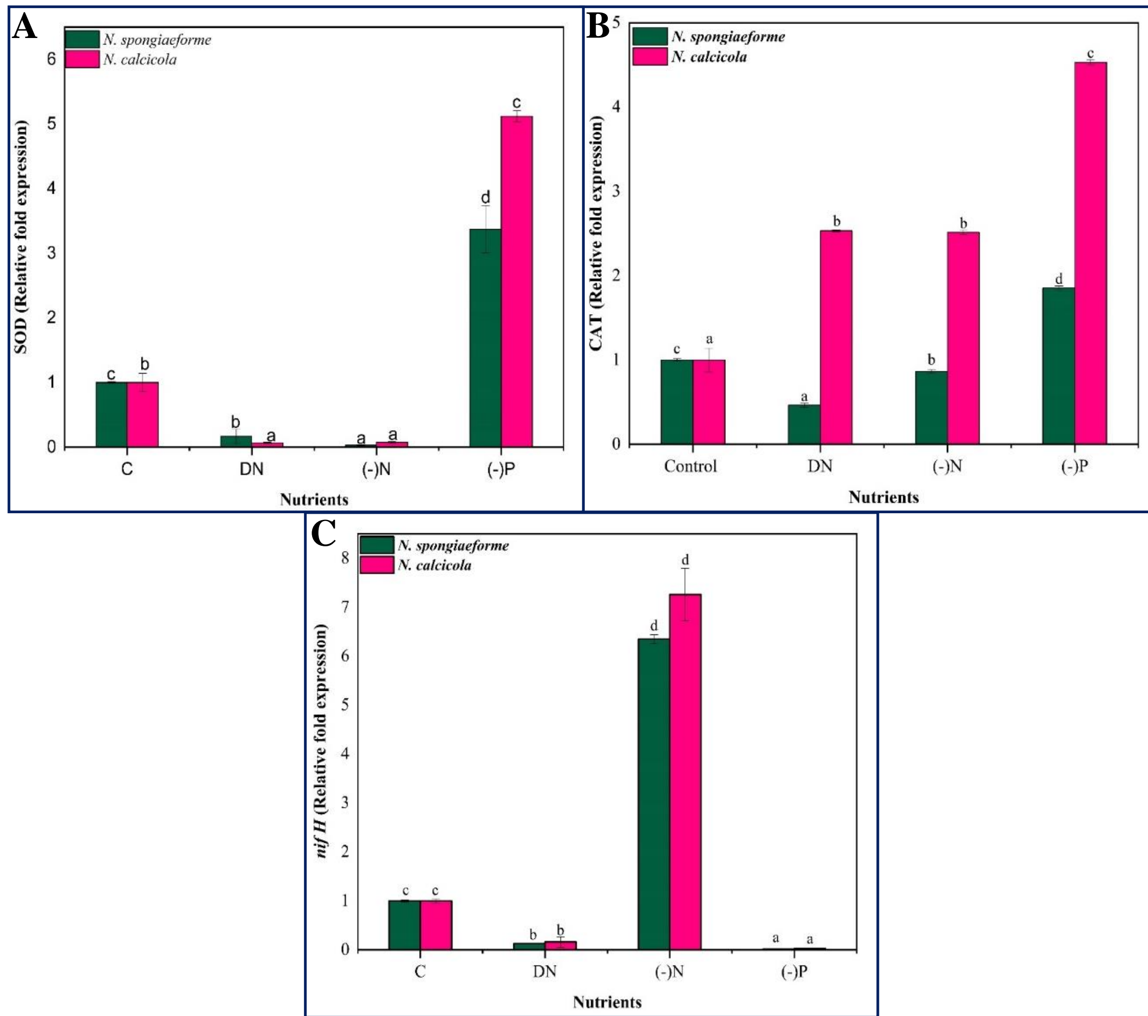


Fig. 3.3.10 Effect of nutrients on expression of SOD (A) and CAT (B) in *N. spongiaeforme* and *N. calcicola.* Standard deviation is represented by vertical bars (±SD, *n* = 3). Bars denoted by the same letter indicate insignificant difference at p≤0.01. C= control, DP= double phosphorus, DN= double nitrogen, (-)N= nitrogen starvation, (-)P= phosphorus starvation, (-)NP = combine starvation of nitrogen and phosphorus.

3.3.11 Effect of nutrient on fatty acids

GC-MS data also showed qualitative and quantitative variation in the fatty acid composition of both the *Nostoc* species when grown with excess and starvation of N and P (Table 3.3.11A, B). Freshwater cyanobacterium, *N. spongiaeforme* showed four saturated fatty acids viz; myristic acid (C14:0), palmitic acid (C16:0), stearic acid

(C18:0), pentadecanoic acid (C15:0) and five unsaturated fatty acids viz; methyl palmitoleate

(C16:1), oleic acid (C18:1), linoleic acid (C18:2), methyl linolenate (C20:3), and arachidonic acid (C20:4). The excess and starvation of N and P affected the qualitative changes of the fatty acid profile. *N. spongiaeforme* grown with DP and DN showed the absence of myristic acid, and (-)NP showed the presence of pentadecanoic fatty acid. Unsaturated fatty acids, methyl palmitoleate was absent under (-)P, while methyl linolenate and arachidonic acid were absent under all the nutrient treatment except for the control condition (Table. 3.3.11A). Quantitative changes in the fatty acid composition showed that the cultures of *N. spongiaeforme* grown with control conditions showed 27.62% of identifiable SFA and 62.06% of identifiable UFA. Double the concentration of phosphorus showed an increase of 12% in the SFA but a decrease of 20% in UFA content in comparison
to control. However, cultures of freshwater cyanobacterium grown with DN showed a much greater decline, up to 69% in the SFA and UFA compared to the control. The cultures grown with P starvation showed an increase of 8% in the SFA, while combined starvation of nitrogen and phosphorus did not show any change in the SFA content compared to the control. However, the UFA acid was decreased up to 35% under (-)P and (-)NP compared to the control.

The marine cyanobacterium, *N. calicola* when grown with absolute nutrient concentration at 30°C exhibited four saturated fatty acids viz; palmitic acid, stearic acid, nonadecanoic acid, and myristic acid, and four saturated fatty viz; methyl palmitoleate, linoleic acid, oleic acid, and methyl linolenate due to excess and starvation of N and P. The excess and starvation of N and P affected the qualitative changes of the fatty acid profile. *N. calcicola* grown with DP, (-)P and (-)NP showed the absence of myristic acid, while oleic acid was absent in cultures grown with DN, (-)P and (-)NP and methyl palmitoleate was absent under DN and (-)NP. Quantitative changes in the fatty acid composition showed that the cultures of *N. calcicola* grown with control conditions showed 32.21% of identifiable SFA and 51.69% of identifiable UFA. *N. calcicola* grown with DP or DN, (-)P, and (-)NP did not show any significant change in the SFA in

comparison to its control. Likewise, UFA content also did not change due to DP however, DN, (-)P and (-)NP led to 24, 8, and 15% decline, respectively in comparison to its control (Table 3.3.11B).

Table 3.3.11A Effect of nutrient on fatty acid profile of *N. spongiaeforme* (A) and *N. calcicola* (B) (based on the methyl ester of fatty acids, area% of fatty acids). Data represent mean values ± SD (*n*=3). Means in the column followed by the same letter indicate insignificant differences at $p \leq 0.01$.

A

Sr. No	Fatty acids	Percent of fatty acids (% of total fatty acids)				
		Control	DP	DN	(-) P	(-) NP
1	Methyl palmitoleate (C16:1)	15.18±0.02^{e}	8.37±0.032^{c}	4.38±1.32^{b}	ND	11.34±0.021^{d}
2	Palmitic acid (C16:0)	24.23±0.03^{e}	21.16±0.025^{c}	7.42±0.004^{a}	22.09±0.0254^{d}	19.24±0.451^{b}
3	Linoleic acid (C18:2)	18.98±0.01^{b}	20.16±3.1^{c}	14.97±2.354^{a}	23.22±0.0125^{d}	17.08±0.654^{a}
4	Methyl linolenate (C20:3)	18.93±0.04^{b}	ND	ND	ND	ND
5	Oleic acid (C18:1)	7.62±0.02^{b}	21.37±0.001^{e}	ND	17.01±0.0006^{d}	13.27±0.001^{c}
6	Stearic acid (C 18:0)	3.16±0.01^{a}	9.73±0.035^{c}	3.37±0.005^{a}	7.45±0.0124^{b}	7.77±0.15^{b}
7	Myristic acid (C14:0)	0.23±0.03^{b}	ND	ND	0.26±0.365^{b}	0.27±36^{b}
8	Pentadecanoic (C15:0)	ND	ND	ND	ND	0.41±0.254^{d}
9	Arachidonic acid (C20:4)	1.35±0.0152^{b}	ND	ND	ND	ND
	Saturated fatty acid	27.62	30.89	10.79	29.8	27.69
	Unsaturated fatty acids	62.06	49.9	19.35	40.23	41.69
ND: not detected						

B

Sr. No	Fatty acids	Percent of fatty acids (area% of total fatty acids)				
		Control	DP	DN	(-) P	(-) NP
1	Methyl palmitoleate (C16:1)	5.95±0.02^{c}	6.31±0.04^{d}	ND	6.5±0.01^{d}	ND
2	Palmitic acid (C16:0)	26.55±0.04^{a}	27.22±0.03^{b}	27.41±0.01^{c}	29.1±0.04^{d}	31.00±0.01^{e}
3	Linoleic acid (C18:2)	17.63±0.03^{a}	18.63±0.05^{b}	16.95±0.06^{c}	17.81±0.01^{d}	19.64±0.04^{e}
4	Stearic acid (C 18:0)	4.7±0.06^{d}	4.05±0.01^{c}	3.08±0.01^{a}	3.26±0.01^{b}	2.93±0.05^{a}
5	Oleic acid (C18:1)	7.38±0.02^{c}	27.22±0.05^{d}	ND	ND	ND
6	Nonadecenoic acid (C19:0)	0.69±0.01^{d}	0.58±0.01^{c}	0.35±0.02^{a}	0.44±0.01^{b}	0.47±.0.01^{b}
7	methyl linolenate (C20:3)	20.73±0.05^{d}	ND	22.23±0.04^{b}	23.24±0.03^{c}	24.37±0.03^{d}
8	Myristic acid (C14:0)	0.27±0.01^{b}	ND	0.96±0.01c	ND	ND
	Saturated fatty acids	32.21±0.01	31.85±0.05	31.8±0.01	32.8±0.01	34.4±0.02
	Unsaturated fatty acids	51.69±0.03	52.16±0.06	39.18±0.02	47.55±0.04	44.01±0.04
ND: not detected						

3.3.12 Effect of nutrients on lipid phase transitions

The excess and starvation of N and P did not cause a significant shift in the peak and change in the fluorescence intensity in both the *Nostoc* species. *N. spongiaeforme*, grown with double the concentration of P or N, did not show any shift in the peak; however, the maximum fluorescence intensity was observed due to DP to 122 a.u, DN to 91 a.u and (-)N to 95 a.u as compared to the control which showed an a.u of 91. Phosphorus starvation and (-)NP, however, caused a shift in the peak to higher wavelength from 682 nm to 684 nm and 683 nm and a decline in the fluorescence intensity to 86 and 73 a.u as compared to control (Fig. 3.3.12A).

N. calciocla grown with excess and starvation of N and P also showed shift in the peak as well as a change in the fluorescence intensity (Fig. 3.3.12B). *N. calciocla* grown with DP or DN and (-)N showed a shift in the peak to lower wavelength from 662 nm to 657 and 655 nm respectively and change in the fluorescence intensity to 102 a.u, 85 a.u and 80 a.u, respectively, compared to the control which showed an a.u of 83 . *N. calcicola* grown with (-)P and (-)NP also caused a shift in the peak to lower wavelength from 662 nm to 654 nm and 660 nm and a decrease in the fluorescence intensity to 51 and 40 a.u respectively, in comparison to its control (Fig. 3.3.12B).

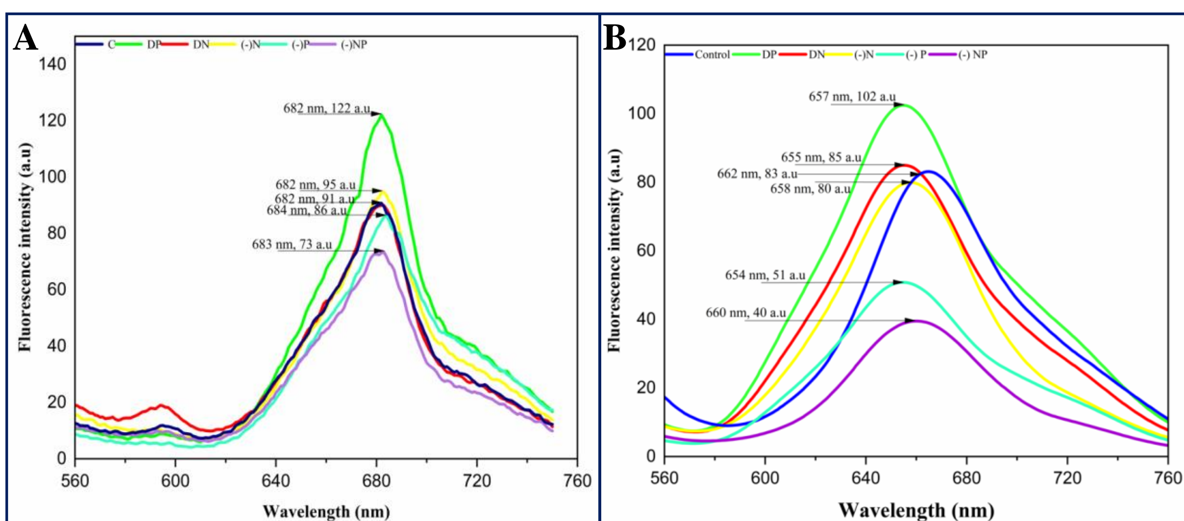


Fig. 3.3.12 Effect of nutrient stress on phase transition of membrane lipid of *N. spongiaeforme* (A) and *N. calcicola* (B). Abbreviations for treatments: C= control, DP= double phosphorus, DN= double nitrogen, (-)N= nitrogen starvation, (-)P= phosphorus starvation, (-)NP = combine starvation of nitrogen and phosphorus.

4.1 Temperature and Nutrients

Among various environmental factors, the temperature is one of the critical factors affecting cyanobacteria physiological and biochemical processes. However, any seasonal fluctuations in temperature cause them to adapt to low and high temperatures irrespective of their optimum growth temperature. It is established that while adapting to low temperatures, cyanobacteria may lower their enzyme activity, membrane fluidity, and nutrient uptake, whereas, in response to higher temperatures, these parameters increase gradually up to a threshold level, beyond which a reduction occurs, which affects the various physiological and biochemical activities. The mineral nutrients such as micronutrients and macronutrients are chemicals required by organisms in optimal concentration for normal growth and development. Nitrogen and phosphorus are two essential constituents of all structural and functional proteins in cyanobacteria cells; therefore, any alteration in their concentrations may influence the growth and development of cyanobacteria. Thus, in the present study, we discuss the implication of different range of temperatures (10-40ºC) and nutrients specifically nitrogen and phosphorus concentrations on the physiological and biochemical mechanisms of two *Nostoc* species *Nostoc spongiaeforme,* a freshwater and *Nostoc calcicola,* a marine cyanobacteria.

4.1.1 Temperature effect on photosynthesis and growth of *Nostoc* species

Our result with a suboptimal temperature of 10-20ºC and supra-optimal temperature of 35-40ºC exhibited a significant effect on the growth of both the *Nostoc* spp. The observed higher growth between 25-30ºC may be due to these temperatures being optimal in terms of growth and metabolic activities for both the *Nostoc* species, thus, maintaining the greater cell density and, thereby highest biomass in both the *Nostoc* species (Table. 3.2.2). However, the observed decline in the growth (Table 3.2.2) at a suboptimal temperature of 10-20ºC was probably due to loss of structural integrity (Fig. 3.2.1A&B), decreased number of cells (Fig. 3.1.2C&D), depigmentation (Fig. 3.2.1C&D; Table. 3.2.3), a decline in photosynthetic efficiency of PS II (Fig. 3.2.3A), and oxygen evolution (Fig. 3.2.3B). The observed decrease in the growth at suboptimal temperatures could also be due to a decline in the enzyme activity related to physiological processes in general and photosynthesis in particular (Ras et al. 2013). The observed decline in the

growth of both species at supra-optimal temperatures (Table 3.2.2) was due to shrinkage and breakage of filaments, causing negative alteration in the structural integrity of the membrane (Fig. 3.2.1A&B). The supra-optimal temperature may also lower the cell metabolic activities of enzymes, especially ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Wei et al. 2015), leading to an imbalance between energy demand and ATP production (Leggat et al. 2004), thus decline in growth, and productivity (Table. 3.2.2).

The observed decline in the phycocyanin and allophycocyanin pigment (Table 3.2.3) at suboptimal and supra-optimal temperatures in both the *Nostoc* species might have caused disruption of light energy and passing on to carotenoid, and chl *a.* Observed decrease in photosynthetic pigment content at suboptimal and supra-optimal temperature in both the *Nostoc* species may also be due to photo-oxidation due to disruption of energy transfer (Fig.3.2.3B) as a result of significant production of reactive oxygen species (Fig 3.2.6) (Sharma, 2002).

The observed increase in quantum efficiency of PS II, as indicated by Fv/Fm ratio at optimum temperature in our study (Fig. 3.2.3A), suggests optimum photosynthesis by maintaining a good energy balance between the photosynthetic energy absorption due to organization and orientation of photosynthetic pigments in light-harvesting complex, and consumption, due to electron carrier proteins. However, a decrease was observed in Fv/Fm ratio at suboptimal and supra-optimal temperatures (Fig. 3.2.3A), directed by decreased Fo, indicating a decrease in the excitation energy reaching the PS II reaction center, largely due to the loss and/or disorganization of pigments in the LHC II, while a decline in the Fm is an indicator of damage to the PS II reaction center itself affecting electron capture and transport to plastoquinone (Krause, 1988; Jansen et al. 1996). Alteration in Fo and Fm value could also be due to structural damage to the thylakoid membrane, which may also decrease antenna size and thereby decrease the photosynthetic pigments at suboptimal and supra-optimal temperatures (Table 3.2.3), thus, reducing energy flux into the photosystem of both the *Nostoc* species.

Our results with photosynthetic electron transport assay measured as oxygen evolution (Fig. 3.2.3B) also suggest damage to the light reaction of photosynthesis as an

overall decrease in the electron transport activity of PS II + PS I in response to suboptimal and supra-optimal temperature of both the *Nostoc* species. The data indicate a disruption in the photosynthetic electron transport chain which may have resulted in a drop in the production of NADPH and ATP for carbon fixation (Nash et al. 1985; Yamane et al. 1998).

The observed decrease in the respiration rate at a suboptimal temperature in both the *Nostoc* spp., (Fig. 3.2.4) could be due to the decrease in kinetic energy of respiratory electron transport enzymes as low temperature decreases the overall motion of groups of atoms within the enzyme and substrate, thus declines the collision between atoms which further reduces the affinity of the enzyme for the substrate (oxygen) which causes an imbalance in metabolism at suboptimal temperature. Also, the observed decline in the respiratory rate at a supra-optimal temperature in our study is suggested to be due to the denaturation of the respiratory enzyme, as supra-optimal temperature increases the kinetic energy and causes the molecules to vibrate more rapidly and violently, which can disturb or break the intra- and intermolecular bonds (hydrogen bond, hydrophobic interaction as well as Van der Waals forces) between the amino acid side which is responsible for the folding of the polypeptide chain and the specific shape of the protein leading to an alteration in its specific 3D structure and a decrease in the substrate-enzyme interaction. Also, in cyanobacteria, respiratory enzymes are integral components of the thylakoid membrane, and the conformation of the active site appears to be influenced by the fluidity and molecular order of the membrane lipids. Therefore, the structural changes that occurred in the membrane lipids of the thylakoid membrane at sub and supra-optimal may influence activation energy of the respiratory enzymes due to the alteration in membrane lipid structure (Raison et al. 1971). Thus, a decrease in the respiratory rate at sub and supra-optimal temperatures of both the *Nostoc* species are suggested to be due to the inactivation and denaturation of enzymes.

A few researchers have reported the effect of temperature on growth in algae. Renaud et al. (2002) studied the effect of temperature on algae *Cryptomonas* sp., *Rhodomonas* sp., *Prymnesiophyte* NT19 and *Chaetoceros* sp., and showed the variable effects of temperature depending on the species. They reported maximum growth rates

between 25-30ºC, with a sharp decline at a temperature above 30ºC in *Cryptomonas* sp., *prymnesiophyte* NT19, *Rhodomonas* sp., while *Chaetoceros* sp., exhibited tolerant behavior up to 35ºC and concluded that temperature higher than the optimum temperature conditions caused a disruption of metabolic activity followed by the death of the cells. Likewise, Kumar et al. (2011) also studied the effect of a range of temperatures from 20 to 40ºC on the growth and biomass of *Spirulina platensis* and exhibited maximum growth and biomass concentration at 35ºC but temperatures below and above 35ºC displayed the least growth rate and biomass concentration as 20 and 40ºC did not support the growth of *Spirulina platensis* due to decrease in the photosynthetic pigments. Chaisutyakorn et al. (2018) also reported a change in the growth rate of *Chaetoceros* sp and *Tetraselmis suecica,* and *Nannochloropsis* sp., (marine algae) when cultured at different growth temperatures from 25 to 40ºC. The study showed that the growth rate and biomass of all algae decreased with increasing temperature compared to 25-30ºC and concluded that the decrease in the growth rate was mainly due to change in cell metabolic activities and activity of photosynthetic enzymes and an increase in respiration with increasing temperature. Similarly, Van et al. (2012) studied the impact of temperature on the growth of *Nannochloropsis salina,* and showed that the growth of *Nannochloropsis salina* was maximum at 26ºC and a sharp decline in growth rates was observed with higher temperature of 35ºC which was reported to be due to loss of structural integrity of the membrane. Li et al. (2021) studied the combined effect of temperature (35, 45, or 55ºC) and nitrogen starvation on the growth rate and biomass of *Thermosynechococcus*, a cyanobacterium, and reported that temperature of 35ºC significantly suppressed, but nitrogen starvation did not affect the growth and biomass. However, combined treatment of temperature and nitrogen starvation led to the lowest growth rate and biomass productivity by decline in pigment content. El-Sheekh et al. (2017) in *Scenedesmus acutus*; Ranjitha & Kaushik, (2005) in *Nostoc muscorum* and Richmond, (1988) in different strains of *Spirulina* also reported the highest growth and biomass productivity at their optimal growth temperature while temperature below and above optimum resulted in a severe decline in the growth and productivity. However, they did not assign any specific reason for the decrease in growth.

A decrease in photosynthetic pigment and photosynthetic efficiency as a consequence of growth temperature has also been reported by many researchers. Kumar et al. (2020) reported a decline in the photosynthetic pigments in *Kappaphycus alvarezii* at a temperature above 28ºC, which they attributed to the interference in chlorophyll biosynthesis and inhibition of the electron transport chain. Other studies, by Kumar et al. (2020) and Araujo et al. (2014) study on *Kappaphycus alvarezii* at 28ºC, and Chaneva et al. (2007) on *Arthronema africanum* at 36ºC reported the highest amount of phycobiliproteins while further increase in temperature affected the concentration of phycobiliproteins in both the algae as the direction of energy transfer in phycobilisomes is from phycocyanin to allophycocyanin, which is then ultimately transferred to the reaction center of chl *a*. Therefore, the temperature adversely affected these energy transfers by reducing the pigment content. In the same study (Kumar et al. 2020) suggested a decline in the photosynthetic efficiency of PS II with an increase in temperature, which was attributed to the role of temperature in the oxygen-evolving complex (OEC), enzymatic reactions of carbon fixation, photophosphorylation and electron transport during photosynthesis. Borlongan et al. (2017) in *Eucheuma denticulatum* and *Kappaphycus alvarezii* (Rhodophyta), also reported similar results. Likewise, Zheng et al. (2020) in *Microcystis aeruginosa*; Roos & Vincent, (1998) in *Phormidium murrayi* showed the highest photosynthetic pigment content at optimum temperatures of 23ºC and 30ºC, while photosynthetic pigment content declined when grown at a low temperature of 15ºC and high temperature of 37ºC suggesting higher degradation in chlorophyll due to excessive production of ROS. Bojko et al. (2017) noted that *Thalassiosira pseudonana* a diatom, when grown at 12ºC, exhibited a decrease in chlorophyll content, in comparison to 20ºC, suggesting that the change in the photosynthetic pigment content was associated with the maintenance of the balance between the temperature independent process of light harvesting and temperature sensitive enzymatic steps of photosynthesis. The same author also reported a decline in the Fv/Fm ratio at low temperature, indicating a lower activity of the PS II reaction center caused by insufficient D1 turnover. Liao et al. (2006), study on *Synechococcus lividus* under different temperatures of 44-61ºC showed a decrease in the Fv/Fm ratio with increasing temperature due to the occurrence of photo inhibitory damage.

Likewise, Zhu et al. (2020) study on *Scytonema javanicum* under different temperatures of 25-40ºC showed a decline in the Fv/Fm ratio with increasing temperature, indicating that the photosynthetic activity was impeded under high-temperature stress. Schmidt et al. (2011) in *Amphistegina radiata*; Zhao et al. (2008) in *Heterostegina depressa*; Campbell et al. (2006) in *Calcarina hispida*; Mackey et al. (2013) in *Spirulina platensis* and Breitbarth et al. (2007) in seagrasses also indicated a decrease in the Fv/Fm ratio under high-temperature stress showing damage to PS II. Cerveny et al. (2015), conducted experiments with wild-type and histidine kinase 34 (ΔHik34) mutant of *Synechocystis* sp. PCC 6803 at 32ºC till they achieved their exponential phase and then subjected to heat stress at 44ºC for 24 h, exhibited a decrease in the photosynthetic activity suggesting a change in the membrane integrity which further causes damage to the site of photosynthetic apparatus, thus affecting the photosynthetic performance. Zhang & Liu, (2016) also showed a decline in the photosynthetic efficiency of PS II and photosynthetic oxygen evolution under high-temperature stress of 40ºC in *Arthrospira* sp., suggesting that heat stress resulted in damage at the donor side of PS II, the over-reduction of PS II acceptor side and a decrease in the energetic connectivity of the PS II units, thus causing a decrease in the performance index of PS II. Venkataramanaiah et al. (2003) also showed that high temperature of 35-45ºC caused inhibition of electron transport activity in *Spirulina platensis,* suggesting an alteration at the level of OEC polypeptide or to the loss of Mn ions and due to changes in the organization of thylakoid membrane structure. Jodłowska & Latala, (2013) reported that an increase in temperature above 25ºC resulted in a decline in the photosynthetic activity in *Geitlerinema amphibium,* indicating a decrease in light absorption by phycobilin pigments as their ratio to chl *a* declined considerably at high temperature.

While, Vona et al. (2004) reported that when *Koliella antarctica* and *Chlorella saccharophila* were grown at a temperature between 5-35ºC, the respiratory rate in both the algae was high at low temperatures as these cryophilic algae were able to obtain a large quantity of metabolic energy to power cellular reaction at low temperature which was further decreased with increase in temperature to 35ºC. However, Vásquez-Elizondo & Enríquez (2016) found that respiration increased with temperature in *Neogoniolithon* sp., *Lithothamnion* sp., and *Amphiroa tribulus*, but after 10 days under high temperature and

high CO_2 conditions, algae decreased their respiration rates due to decline in the photosynthesis. On the contrary, Sordo et al. (2016) in *Phymatolithon lusitanicum* and Noisette et al. (2013) in *Lithothamnium corallioides* reported an increase in the respiration rates with increasing temperature, indicating that *Phymatolithon lusitanicum* and *Lithothamnium corallioides* used large quota of photosynthate as a respiratory substrate which caused limitation in carbon for biosynthetic activities and ultimately affected their growth.

4.1.2 Nutrient effect on photosynthesis and growth of *Nostoc* species

Our study, due to excess phosphorus or nitrogen concentration, observed no significant change in the growth and productivity of both the *Nostoc* species (Table 3.3.2A; Fig. 3.3.2a), suggesting that DP or DN have no detrimental effect rather provided favorable nutrient conditions for maintaining normal and healthy vegetative cells and promoting their productivity of both the *Nostoc* species by improved pigment content (Table 3.3.3), and photosynthetic efficiency (Fig. 3.3.3A). Our result also observed no heterocysts formation in both *Nostoc* species under DP or DN, which may be due to the presence of nitrogen source in the growth media; thus, no need of differentiation of vegetative cells into heterocysts. Nitrogen starvation on the other hand displayed a slight decline in the growth and productivity (Table 3.3.2A) in both the *Nostoc* species probably due to a decrease in the vegetative cell density at the cost of heterocysts development (Fig. 3.3.1C&D). The observed decrease in the growth and productivity under (-)P and (-)NP is suggested to be due to distorted, shriveled with breakage in the filaments (Fig.3.3.1A&B) and depigmentation in the cells (Fig. 3.3.1C&D) in both the *Nostoc* species. Phosphorus starvation did not cause differentiation of vegetative cells into heterocysts in both the *Nostoc* species as there was no need for nitrogen as the culture medium was supplemented with it, but deprivation of NP led to slight differentiation of vegetative cells to heterocysts (Fig. 3.3.1C&D) to meet the requirement of nitrogen fixation; therefore, growth was further compromised.

Observed insignificant change in Fv/Fm ratio under DP, DN, and (-)N (Fig. 3.3.3A) suggest that excess of phosphorus and nitrogen and starvation of nitrogen had no effect on growth, pigment, and photosynthetic efficiency in both the species, allowing optimum

photosynthetic efficiency of PS II by maintaining the optimum photosynthetic electron transport (Fig.3.3.3B) and pigment content (Table 3.3.3) in the cells of both the *Nostoc* species. However, under (-)P and (-)NP a decrease observed in Fv/Fm ratio (Fig. 3.3.3A) indicated a decrease in Fo as well as Fm level. A decline in Fo is an indicator of a reduction in the excitation energy reaching the PS II reaction center, which was due to the loss of pigments. While a decline in Fm is an indicator of low efficiency of energy transfer to the reaction centre due to damage to the PS II apparatus itself and may represent direct damage to the key components i.e., D1 and D2 proteins of PS II, resulting in a decrease in the photosynthetic efficiency of PS II. Also, observed decrease in photosynthetic rate in both the *Nostoc* species due to P starvation and (-)NP could be due to decreased Fv/Fm ratio (Fig. 3.3.3A) and whole chain electron transport activities that generate NADPH, a reductant and ATP responsible for CO_2 fixation via carbon reduction cycle (Kumari et al. 2021; Qi et al. 2013) which ultimately caused a reduction in the productivity of both the *Nostoc* species. Observed higher content of PC and APC (Table 3.3.3) under DP or DN caused an increase in the light-harvesting capacity, thus funneling light energy effectively to carotenoid and Chl *a,* which improved the photosynthesis efficiency and, in turn, growth (Prasad & Gunjan, 2011). However, under P starvation and (-)NP, our study observed a decrease in photosynthetic pigment content in both the *Nostoc* species, which may be due to the oxidation of pigments by ROS (Fig. 3.3.6). Also, P starvation and (-)NP caused a decline in the pigment content which may result to the greater decrease in the Fv/Fm ratio and could be one reason for the decline in photosynthetic activity and growth of both *Nostoc* species. However, a reduction in the photosynthesis and productivity upon P deprivation and deprivation of NP observed in our study was mainly due to phosphorus deprivation, as nitrogen deprivation alone did not show any significant change in the photosynthesis and productivity of both the *Nostoc* species.

Our study, due to (-)P and (-)NP, also observed a decline in the respiratory rate (Fig. 3.3.4) which could be due to reduced photosynthesis, which reduced the supply of metabolic energy to power cellular reactions (Kumari et al. 2021) which may further caused a reduction in biomass productivity (Table 3.3.2A) of both the *Nostoc* species.

Though in our study we did not observe any deleterious or beneficial effect on growth, pigments and photosynthesis due to excess P and N in media, however there are reports which suggest that increase in N and P content led to improved growth through increase pigments. Costa, (2005) in *Gracilaria birdiae*; Martins et al. (2011) in brown strain of *Hypnea musciformis*; An et al. (2020) in *Scenedesmus obliquus*; Zhu et al. (2014) in *Chlorella zofingiensis*; Chen et al. (2011); Paerl et al. (2001); Schindler et al. (2008) in several cyanobacteria species and Davis et al. (2015) in phytoplankton reported an increased growth and pigment content in response to increasing nitrate and phosphorus availability. Figueroa et al. (2009) in *Ulva lactuca* showed an increased pigment content under high nutrient stress in the form of fishpond effluents. Peng et al. (2017) working with *Microcystis aeruginosa* FACHB-905, also reported higher content of Chl *a* and PC under 16:1 ratio of N:P in and a lower PC and chl *a* content at 1:4 ratio of N:P.

However, there are several reports on the effect of phosphorus and nitrogen deficiency on the growth, photosynthetic pigments and photosynthesis of several microalgae. Degerholm et al. (2006) reported a decline in the growth and chl *a* working with diazotrophic cyanobacteria *Nodularia* and *Aphanizomenon* under phosphorus limitation and suggested a decline may be due to slow down of the metabolic activity and cell division which ultimately led to low cellular concentrations of chl *a*. Kumari et al. (2021) working with non-diazotrophic *Chlorella vulgaris* under nitrogen or phosphorus starvation also observed a decrease in the growth and biomass, which was suggested to be due to reduce chlorophyll content as nitrogen limitation triggered the degradation of nitrogenous compounds that resulted in the downregulation of chl content, reduced cell division and negatively affected algal growth and biomass production. Downregulation of photosynthetic rate was suggested to be in order of N>P, however P deficiency resulted greater decline in lipid content. Yang et al. (2018) in *Chlamydomonas Reinhardtian*; Neha & Khan, (2016) in *Chlorella Vulgaris* and El-Kassas, (2013) in *Pichochlorum* sp., reported a decline in the growth under phosphorus or nitrogen deficiency, suggesting that as nitrogen is a major element for the formation of protein and nucleic acid and phosphorus is an essential part of the backbone of DNA and RNA thus deficiency of these critical nutrients inhibit the cell division causing a decline in the growth of various algae.

Our result contradicts the findings of An et al. (2020) in *Scenedesmus obliquus*; Zhu et al. (2014) in *Chlorella zofingiensis*; Ji et al. (2011) in *Tetrasemis subcordiformis* and Zarrinmehr et al. (2020) in *Isochrysis galbana* who reported a reduction in the growth and biomass under nitrogen starvation conditions as nitrogen is an essential macro-element involved in the composition of crucial molecules thus causes chlorosis, reduction of protein content and degreening of algal cells.

Our results on photosynthetic pigments and photosynthetic efficiency are in agreement with findings of Wu et al. (2012) who also observed a decline in the photosynthetic pigments and photosynthetic efficiency of PS II in *Cylindrospermopsis raciborskii* under phosphorus limitation due to inhibition of synthesis of the light-harvesting pigment (chl-*a*) and damage to PS II reaction center. Wang et al. (2010); He et al. (2015), and Ou et al. (2005) working with non-nitrogen fixing cyanobacterium *Microcystis aeruginosa,* also reported a decline in chl *a* content due to decreased biosynthesis under phosphorus limitation. Likewise, Ji et al. (2011) in *Tetraselmis subcordiformis*; Liu et al. (2011) in *Thalassirosira weissflogii* and Beardall et al. (2001) in *Nitzschia* species reported decreased photosynthetic efficiency as a result under phosphorus deficiency. Goiris et al. (2015) studied the effect of phosphorus and nitrogen deficiency on *Chlorella vulgaris*, *Phaeoodactylum tricornutum* and *Tetraselmis suecica* and showed a decline in the carotenoid content, Fv/Fm ratio and biomass, suggesting that the flow of electrons from the photosystems to the electron transport chain was impaired under nutrient deficiency thus, caused a decline in the photosynthesis and leading to lower cell density. Similarly, Wykoff et al. (1998) performed an intensive study on the effect of sulfur and phosphorus limitation on photosynthetic electron transport, including active PS II centers in *Chlamydomonas reinhardtii* cells and showed that photodamage and loss of functional PS II occurred during nutrient limitation, resulting in a loss of active PS II centers. Yang et al. (2014) studied the effect of phosphorus limitation and excess phosphorus along with UV-B radiation on the pigment content and photosynthetic activity of *Microcystis aeruginosa*. The study reported a decline in the photosynthetic pigments mainly due to the degradation of phycobiliproteins and decreased in photosynthetic activity

due to a reduction in the nucleotides ATP and GTP, required for various metabolic processes, under phosphorus limited condition.

However, our study contradicts the finding of Pancha et al. (2014) in *Scenedesmus* sp., and Zhang & Liu (2016) in *Isochrysis galbana,* who demonstrated a decrease in nitrogen concentrations decreased the pigment content suggesting that nitrogen stress might reduce light harvesting and efficiency of PS II activity. Also, Li et al. (2021) reported an expression of *nblA* gene in *Thermosynechococcus* under nitrogen starvation which is essential for degradation of PBS in cyanobacteria (Collier & Grossman, 1994) however no such degradation was observed in our study under N starvation. El-Sheek & Rady, (1995) in *Chlorella kessleri*; Negi et al. (2016) in *Chlorella sorokiniana* reported a decline in the respiratory rate under nitrogen or phosphorus starvation. The decrease in the respiration rate in *Chlorella* species was in agreement with the reduction in the biomass and metabolic activity of *Chlorella* species.

4.2.1 Temperature effect on phenolic compounds

We observed an increase in phenolic compounds when both the *Nostoc* sp., subjected to growth at 10-20ºC and 35ºC than optimum temperature (Table 3.2.5), indicating development of stress, as phenolic and flavonoid are associated with adaptability strategy against temperature stress (Trabelsi et al. 2016). Flavonoids and phenols are known to protect the protein/enzymes against oxidative damage (Ghosh & Xu, 2014). Our result also suggests a role for flavonoid in an adaptation, such as chemical defense, of both the *Nostoc* species against temperature as the lesser growth of *N. spongiaforme* at 40ºC than *N. calcicola* could be related to an accumulation of lesser phenols/ flavonoid in former species than later (Fig. 3.2.5).

Phenolic compound production in algae due to temperature stress has been reported by a few authors. Mannino et al. (2016) studied the effect of temperature (25 and 30ºC) on the phenolic content of a brown seaweed *Cystoseira amentacea* for different growth period of 0, 8, 24 and 48 h, and noted an increase in the phenolic content after 8 h of incubation at both the studied temperature, which they suggested to be due to an activation and mobilization of insoluble phlorotannins, bound to the cell walls in order to rapidly increase

the pool of chemical defenses as consequence of the increasing temperature. However, the same author observed reduction in the TPC after 48 h at both the studied temperature which was suggested to be due to a release through exudation, more than transport to other parts of the thallus, a slow and not very efficient process. Jiménez-Escrig et al. (2001) studied the effect of temperature on brown seaweed *Fucus* and reported a 98% reduction in the TPC content on drying at 50ºC for 48 h which they attributed to the binding of polyphenols with other compounds (proteins) and alterations in the chemical structure of polyphenols (Martín-Cabrejas et al. 2009). While, Gupta et al. (2011) in *Himanthalia elongate* reported a 49% reduction in TFC at 25ºC and 30% at 40ºC suggesting that low temperature did not inactivate the oxidative enzymes completely, which in turn resulted in oxidation of the phenolic substances and relatively lower phenolic content at 25ºC. Mancuso et al. (2019) studied the influence of seawater temperature on the phenolic content of *Cystoseira compressa* and reported that the TPC was affected by the long-term thermal seawater conditions, rather than short-term variations encountered during tidal cycles.

4.2.2 Nutrients effect on phenolic compounds

The observed increase in the phenolic content in our study under DN in both *Nostoc* species may suggest that excess nitrogen in the culture medium may be used to produce phenol as a storage source for the synthesis of aromatic amino acids (Abd El-Baky et al. 2009). However, the observed decrease in the TPC and TFC in both the *Nostoc* species under (-)P and (-)NP could be due to limitation of nitrogen as substrate and oxidative damage as a result of higher generation of ROS (Fig. 3.3.6). Interestingly, the total phenolic contents found in our study were lower than the total flavonoids content in both *Nostoc* species under excess nitrogen and phosphorus suggesting that theses nutrient conditions may facilitate aromatic phenolic amino acid such as tryptophane, phenylalanine and tyrosine to act as precursor for condensation of phenolic compounds with aliphatic amino acid to form flavonoid.

Phenolic compound production in algae under nutrient variation has been reported by a few authors. Hamouda & Abou-El-Souod, (2018) working with *Scenedesmus obliquus* under increasing concentrations of phosphorus in the range of 0, 0.0035- 0.014 g

L^{-1} showed an increase in the flavonoid content with higher concentration of phosphorus and indicated increase in protein content and antioxidant activity with increase in the phosphorus concentration. Abd El-Baky et al. (2009) working with *Spirulina maxima* under excess nitrate concentration showed an increase in the phenolic and flavonoid content, suggesting that nitrogen was used for the synthesis of aromatic amino acid, as a precursor for the biosynthesis of phenolic compounds.

While, Goiris et al. (2015) studied the impact of (nitrogen and phosphorus limitation) on *Phaeodactylum tricornutum*, *Tetraselmis suecica* and *Chlorella vulgaris* and reported a decline in the phenolic content under nitrogen limitation in all the studied algae while, under phosphorus limitation only *Phaeodactylum tricornutum* and *Chlorella vulgaris* displayed reduction in phenolic content. They indicated that such limiting nutrient condition leads to increase in antioxidant content in higher plants but microalgal response to nutrient deficiency may be different and need to be further studied. Arnold et al. (1995), however, reported an increase in the content of phenolics compounds in brown algae *Lobophora variegata* in response to low nitrogen concentration. There are reports of an increase in the content of phenolic compounds in microalgae due to other stress factors in nature mainly UV-B which may complicate the results of total phenolic content and total flavonoid content under combined stress in nature (Kovacik et al. 2010).

4.3.1 Temperature and oxidative damage

The observed increase in ROS content in our study at sub and supra-optimal temperature suggested oxidative damage in both the *Nostoc* species which was confirmed by determining the extent of lipid peroxidation (Fig. 3.2.7) and protein oxidation (Fig. 3.2.7). Temperature stress is known to generate ROS via Haber–Weiss reaction and Fenton's reaction leading to oxidative damage to membrane lipids, proteins and DNA (Barati et al. 2019). The observed depigmentation in both the *Nostoc* species at sub and supra-optimal temperatures could also be due to oxidative damage to chlorophyll molecules in LHC as ROS are largely produced at photosynthetic system as a consequence of over energization and reduction of light reaction and pigments are highly sensitive to such oxidative damage. In case of cyanobacteria membrane act site for both photosynthesis and respiration and both are the process leading to electron leakage to form ROS. This may

also explain lower biomass production (Table 3.2.2) at sub and supra-optimal temperatures in both the *Nostoc* species.

Our results on lipid peroxidation and carbonyl oxidation also confirm that sub and supra-optimal temperature resulted in ROS generation, thus, oxidative damage. Further, the same can also be concluded by our result on activity and expression (Fig. 3.2.10.1 A&B) of antioxidant enzymes at sub and supra-optimal temperature (Fig. 3.2.8.1), probably to protect the cell against ROS. However, *N. calcicola* experienced less oxidative damage at sub and supra-optimal temperatures, than *N. spongiaeforme*, which possibly could be due to the higher activity and expression of antioxidants in *N. calcicola.* Significant increase in the heat shock proteins in *N. calcicola* at supra-optimal temperature may suggest better adaptation of it to supra-optimal temperature than *N. spongiaeforme* (Fig. 3.2.10.1C). Our study also observed an increase in the unsaturated and saturated fatty acid content in *N. calcicola* than *N. spongiaeforme* upon temperature stress (Table 3.2.11B) which might have helped in maintaining the integrity of the cell membrane for photosynthesis and also provided protection against the oxidative damage.

A similar observation of oxidative damage to algae by temperature was also reported by other researchers. Ismaiel & Piercey-Normpore, (2021), studied the effect of low (23ºC) and high temperature (37ºC) on *Arthrospira platensis* and showed an increase in the MDA and protein oxidation at low and high temperature than 30ºC, due to excessive production of ROS led oxidative degradation of PUFA and amino acids. Shohael et al. (2006) reported an increased formation of H_2O_2 and lipid peroxidation in *Eleutherococcus senticosus* due to 12ºC and 30ºC than control temperature of 24°C and as H_2O_2 accelerated Haber-Weiss reaction caused increase in the OH• formation leading to higher lipid peroxidation. They also suggested that lipid peroxidation might be initiated by lipoxygenases, which convert fatty acids to hydroperoxides and causing lipid peroxidation.

Study done by Reddy et al. (2019) and Mishra et al. (2005) in *Anabaena doliolum*; Chalanika & Asaeda, (2017) in submerged macrophytes; Brutemark et al. (2015) in *Dolichospermum sp.*; Cruces et al. (2013) in *Durvillaea antarctica* and *Lessonia nigrescens* and Xing et al. (2022) in *Auxenochlorella protothecoides* grown under higher temperature also showed similar result of ROS production and peroxidative damage to the

membranes. Likewise, Han et al. (2015) working with *Microcystis aeruginosa*, showed an increase in the MDA content at 35ºC compared to 25ºC, indicating that antioxidant enzyme activities were not sufficient to cope with the increasing amount of free radicals content. Studies by Davidson & Schiestl (2001), Larkindale & Kinght (2002), Vacca et al. (2004) also reported impairment of functions and oxidative damage in mitochondria in several algae species due to temperature stress. However, de la Rosa et al. (2020), reported a low level of ROS generation and MDA-TRABS content in *Microcystis aeruginosa* at higher temperatures of 29ºC than 26ºC throughout the growth period due to an increase in antioxidant protection probably due to the insignificant difference in the growth temperature.

Activity of antioxidant enzymes corresponds to the ROS production causing oxidative damage due to temperature stress. Our result on antioxidants is in agreement with work carried out by Chien & Vonshak, (2011) who reported an increase in SOD, and CAT in *Arthrospira platensis* Kenya than *Arthrospira platensis* M2 at a low temperature of 15ºC than 33ºC, suggesting that SOD and CAT were produced to scavenge excess superoxide radicals and H_2O_2. Similarly, Perelman et al. (2006) studied on Antarctic plankton and temperate phytoplankton showed an increase in SOD activity under low temperatures, indicating a quick breakdown of superoxide radicals by SOD to keep their levels in control and allowed algae to combat oxidative stress. Choo et al. (2004) studied on the effect of low temperature on *Cladophora glomerata* showed high activities of SOD, APX, and CAT against intracellular ROS, whereas *Enteromorpha ahlneriana* had a high activity of SOD but extremely low activities of APX and CAT. This suggests that the enzymatic defense mechanisms to scavenge $O_2^{\cdot-}$ to H_2O_2 was functioning well in both the species but break down of H_2O_2 was strongly expressed in *Cladophora glomerata*, but at low levels in *Enteromorpha ahlneriana*. Gacheva et al. (2013) on *Gloeocapsa* sp., and Ismaiel & Piercey-Normpore, (2021) on *Arthrospira platensis* studied the effect of a low temperature of up to 23ºC and a high temperature of up to 40ºC showed higher SOD, CAT, and APX activity compared to the optimal temperature of 30 and 34ºC, which indicated that the antioxidant defense system was effective in helping the cells to overcome the temperature stress.

While, Babele et al. (2017) and Han et al. (2015) on *Microcystis aeruginosa*; Reddy et al. (2019) on *Anabaena doliolum*; Shohael et al. (2006) on *Eleutherococcus senticosus* reported an increase in the activity of SOD, CAT, and APX due to high temperature stress of 35ºC and 45ºC indicating that higher antioxidant activity may have allowed the cyanobacteria to grow under oxidative stress. However, Mishra et al. (2005) growing *Anabaena doliolum* at 43-58ºC showed a decline in the activity of SOD above 43ºC due to the presence of high-level H_2O_2 inactivated Cu/Zn as well as Fe-SOD. They also showed a decline in the CAT and APX activities at a temperature above 43ºC, indicating that each enzyme may have an optimum temperature for its activity, and OH• may modify the proteins, disturbing their catalytic activities and making them more susceptible to proteolytic attack.

Our study on gene expression is in accordance with the findings of Fan et al. (2016; 2018) who observed an increase in the expression of SOD, APX, and GR in *Ulva prolifera* grown with low and high-temperature stress, and hypothesized that plants subjected to temperature stress activated antioxidant defense mechanism by adjusting the level of gene expression of the antioxidant enzymes. Yang et al. (2019) studied the effect of temperature from 4-36ºC for 22 d on the gene expression of SOD, APX, and CAT in *Ulva prolifera* and observed that the expression level of SOD, APX and CAT were increased at high temperature of 36ºC but had no effect on H_2O_2 and MDA levels which also remained higher while low temperature had lower expression of SOD, CAT and APX without any effect on H_2O_2 and MDA level in comparison to control. They suggested that marine organism had evolved to adopt to wide variety of environmental factor on temporal and spatial scale which may explain this antagonistic effect on antioxidative metabolic pathway.

Our results on heat shock proteins also corroborate with the findings of Fan et al. (2018) on *Ulava prolifera* and Xing et al. (2022) on *Auxenochlorella protothecoides* showing upregulation in the expression of heat shock protein high temperature of 37ºC indicated that HSP is a mechanism needed for cyanobacteria to tolerate changes in growth temperatures.

Babele et al. (2017); Reddy et al. (2019) and Xing et al. (2022) under high temperature of 35℃ and 45℃ exhibited an increase in the proline content indicating an elevation of oxidative stress and help cells to maintain osmotic balance, provides a defense to enzymes as well as biological membranes. They suggested that increase in proline indicates detoxifying of free radicals by forming a stable complex, and the alleviate oxidative stress.

4.3.2 Nutrient and oxidative damage

The observed insignificant increase in the ROS production in our study under DP or DN, suggests no oxidative damage occurred in both the *Nostoc* species, which was also confirmed by determining an insignificant increase of lipid peroxidation (Fig. 3.3.7A) and protein oxidation (Fig. 3.3.7B) and thus maintaining higher biomass productivity of both the *Nostoc* species. Our study also observed an insignificant increase in the ROS production and oxidative damage under N starvation in both the *Nostoc* species, which suggest that as both the *Nostoc* species are diazotrophic in nature, thus could survive nitrogen-deficient condition without causing any oxidative or otherwise damage to membrane lipid and proteins, therefore, displayed better biomass production. However, the observed increase in ROS content in our study under P starvation and (-)NP suggests oxidative damage to both the *Nostoc* species, which was confirmed by determining the level of lipid peroxidation (Fig. 3.3.7A) and protein oxidation (Fig. 3.3.7B) which demonstrate that primarily phosphate deficiency may affect photosynthetic electron transport causing accumulation of ROS which may also damage chlorophyll molecules in LHC thus decreased photosynthetic activity and biomass production in both the *Nostoc* species. The greater production of ROS is also substantiated by the observed increase in the enzymatic antioxidant activity (Fig. 3.3.8.1) and expression of SOD and CAT (Fig. 3.3.10) genes and ascorbate and proline content (Fig. 3.3.8.2) in *Nostoc* species grown under (-)P medium in order to metabolised the ROS. The observed production of ROS, oxidative damage, activity of SOD, CAT and APX and their expression was mainly due to P deprivation, (-)P, as and deprivation of nitrogen, (-)N did not show any deleterious effect on any of the above parameter studied.

Several studies have been done on variable nutrients in algae. Yang & Wang, (2019) in *Mycrocystis aeruginosa* and Hamid & Sibi, (2018) in *Chlorococcopsis minuta* observed a significant decline in ROS, lipid peroxidation and SOD activity with increasing P and N concentration in the growth medium compared to control.

Fan et al. (2014) in *Chlorella pyrenoidosa*; Wu et al. (2012) in *Cylindrospermopsis raciborskii*; Hamid & Sibi, (2018) in *Chlorococcopsis minuta*; Peng et al. (2017) in *Caragana korshinskii* ; Yuasa et al. (2020) in *Chattonella antiqua*; Fernández-Juárez et al. (2020) in *Halothece sp.*; Chokshi et al. (2017) in *Acutodesmus dimorphus*; Zhang et al. (2013) in *Chlorella sorokiniana* and Yilancioglu et al. (2014) in *Dunaliella salina* reported an increase in ROS and MDA content under phosphorus, nitrogen and iron deficiency which they suggested largely due to the accumulation of excessive electrons in PS II, resulting in ROS production which further resulted into lipid peroxidation. Moussa et al. (2017) who studied the effect of excess and starvation of phosphorus and nitrogen on *Tetraselmis marina* and reported an increase in the ROS generation and MDA content under phosphorus starved condition followed by 3-fold P and 10-fold N which indicated that the sudden increase in the ROS and MDA content were mainly due to sudden change in the nutrient's concentration of the culture medium. These changes were accompanied by a significant decline in SOD activity under excess and depleted nutrient condition (N & P) which was due to inhibition of protein synthesis or impairment of enzymatic activity as the result of increase in the peroxides levels.

Activity of antioxidant enzymes corresponds to the ROS production causing oxidative damage under deficiency of nitrogen and phosphorus. Yang & Wang, (2019) and Peng et al. (2018) in *Mycrocystis aeruginosa*; Wu et al. (2012) in *Cylindrospermopsis raciborskii* reported an increase in the SOD and CAT activity under low phosphorus concentrations suggesting that the antioxidant system had responded to oxidative damage caused by phosphorus starvation. Chokshi et al. (2017) also reported an increase in SOD, APX and CAT activity under nitrogen-starved conditions in non-diazotrophic algae *Acutodesmus dimorphus* indicating an increase in the antioxidant activity which maintained the oxidative damage at a relatively lower level. They reported activity of CAT

was relatively higher than APX which they suggested was due to the pre-dominance of CAT as it contribute more strongly to eliminate H_2O_2 than APX.

Hamid & Sibi, (2018) in *Chlorococcopsis minuta*; Park et al. (2020) in *Scenedesmus quadricauda;* Meng et al. (2021) in *Chlorella grandis* and Chokshi et al. (2017) in *Acutodesmus dimorphus* reported an increase in proline content when grown in phosphorus and nitrogen-deficient culture media, indicated a defense mechanism for survival of algae under nutrient-deprived conditions. However, Pancha et al. (2014) in *Scenedesmus sp*.; Xiao et al. (2013) in *Nannochloropsis oceanic* a marine microalga and Bromke et al. (2013) in *Thalassiosira pseudonana* (diatom) observed a decrease in the proline content under nitrogen starvation.

4.4.1 Temperature and nitrogenase activity

The observed decrease in the activity of the nitrogenase enzyme in our study at a suboptimal temperature in both the *Nostoc* species (Fig 3.2.9) could be due to a decrease in the kinetic energy of the enzyme and substrate. As temperature decreases, the overall motion of groups of atoms within the enzyme and substrate thus declines the collision between atoms which further reduces the enzyme's reaction, for the substrate (N_2).

The observed decline in the nitrogenase activity at supra-optimal temperature (Fig 3.2.9) in both the *Nostoc* species may be due to the denaturation of the nitrogenase enzyme, as supra-optimal temperature increases the kinetic energy and causes the molecules to vibrate more rapidly and violently, which can disturb or break the intra- and intermolecular bonds between the amino acid side which is responsible for the folding of the polypeptide chain and the specific shape of the protein is broken leading to an alteration in its 3D structure resulting a decrease in the substrate-enzyme interaction.

Further, decrease in the nitrogenase activity could also be due to the decrease in the photosynthetic efficiency (Fig. 3.2.3) of both the *Nostoc* species, as nitrogen fixation is an energetically expensive process, and nitrogenase requires at least 16 molecules of ATP to fix one molecule of N_2 into two NH_3, thus decrease in the photosynthetic efficiency would decrease the extent of reductant supplied for nitrogenase enzyme at sub and supra-optimal temperature.

Further, a reduction observed in the nitrogenase activity of both the *Nostoc* species under both sub and supra-optimal temperature stress might also be due to the reduced number of heterocysts (Reddy et al. 2019). The temperature, both sub and supra, may also affect the membrane integrity of heterocysts, thus disturbing the anaerobic environment essential for nitrogenase activity. Our study observed a higher reduction in nitrogenase activity at supra-optimal temperature (Fig. 3.2.9) as well as change in saturation to unsaturation ratio of fatty acid (Table. 3.2.11), suggesting that activity of nitrogenase may also be influenced by fatty acid profile of cell (Bauersachs et al. 2014 and Reddy et al. 2019).

Our result on the expression of *nif H* gene (Fig. 3.2.10.2) was also similar to the activity of nitrogenase enzyme. The decrease in the expression of *nif H* gene at sub and supra-optimal temperature may be related to the transcription and translation processes being influenced by the temperature.

Our study on nitrogenase activity corroborates with the findings of other researchers. Thangaraj et al. (2017) studied the effect of temperature on the nitrogenase activity of two marine mesophilic *Nostoc* strains and psychrophilic *Nostoc punctiforme* and reported that mesophilic *Nostoc* strains showed maximum nitrogenase activity up to 37ºC and least at 4ºC which was suggested to be due to the sensitivity of nitrogenase enzyme to lower temperature which decreases the affinity of the enzyme for substrate. While psychrophilic strain, was able to fix nitrogen at 4ºC and its activity increased with increase in temperature till 37ºC due to the ability of psychrophilic strain to fix nitrogen regardless of the temperature due to tightly entangled trichomes with multi-layered sheath which creates a strict anaerobic environment protecting nitrogenase, thereby enhancing N_2 fixation. The same was also reported by Velazquez et al. (2011), in psychrophilic purple mat (PM) community of Maritime Antarctica comprising N_2 fixing cyanobacteria. Breitbarth et al. (2007) studied the effect of range of temperature from 20-34ºC on the nitrogen fixation by *Trichodesmium* and reported a significant increase in the nitrogen fixation between 24 and 30ºC which was due to higher growth rate, cellular elemental composition, and photosynthetic efficiency and thus higher carbon fixation rate while, temperature below 20ºC and above 34ºC caused significant decrease in the nitrogen

fixation related to insufficient carbon fixation and also due to increased membrane permeability resulting in membrane leakage. Stal, (2017) studied the effect of a wide range of temperature (12-60ºC) under constant oxygen concentration of 20% on the nitrogenase activity of a thermophilic cyanobacterium *Fischerella* sp., and reported a decline in the nitrogenase activity at temperature below and above 40ºC due to the inactivation of the enzyme.

While, Reddy et al. (2019) in *Anabaena* sp.; Compaore and Stal, (2010) in *Anabaena variabilis* and *Nostoc* sp., and Gallon et al. (1993) in *Gloeothece* sp., studied the effect of high temperature (35-42ºC) on the nitrogenase activity and reported a significant reduction in the nitrogenase activity at high temperature of up to 42ºC compared to the their respective optimum temperature of 25-39ºC and suggested that temperature stress have damaged the heterocysts, (a multi-layered sheath in the tightly entangled trichomes responsible for creating an anaerobic condition in order to protect the nitrogenase enzyme from extreme conditions) leading to diffusion of oxygen causing inactivation of the nitrogenase enzyme.

Levitan et al. (2010) studied the combined influence of CO_2 and temperature on the expression of *nif H* in *Trichodesmium* sp., and reported that temperature in the range of 25-30ºC did not affect the expression of *nif H* as the abundance of the nif H protein and nitrogen fixation rate was also not affected at 25 and 30ºC. Chen et al. (1998) also reported relative stability of *nif H* expression when *Trichodesmium* IMS101 grown at 24-31ºC. While, Alcaman et al. (2017) showed an increase in the expression of *nif H* in thermophilic cyanobacterium, *Mastigocladus* sp., at 50ºC compared to 45ºC.

4.4.2 Nutrient and nitrogenase activity

Our study observed that the culture media, when supplemented with DN concentration, distinctly inhibited the heterocysts formation (Fig. 3.3.1C&D) as the NO_3^- is provided in the growth media, which does not require the vegetative cells to differentiate into heterocysts to fix nitrogen as NO_3^- is biochemically easy to absorb and fix (Zheng et al. (2016, 2017). Likewise, cultures grown with DP were supplied with a normal level of nitrate as a source of nitrogen and also did not show differentiation of vegetative cells into

heterocysts (Fig. 3.3.1C&D) nor increase in the activity of nitrogenase (Fig. 3.3.9). However, nitrogen starvation (-N) resulted in an increase in the nitrogenase activity in both the *Nostoc* species (Fig. 3.3.9), which is corroborated with an increased in the heterocysts frequency as both the cyanobacteria being diazotrophic (Fig.3.3.9). A decline in the activity of nitrogenase enzyme under (-)P (Fig. 3.3.9), observed in our study may be due to role of phosphate in heterocysts differentiation, deprivation of which led to low/ no differentiation of heterocysts thus decrease in the nitrogenase activity. The role of phosphate in differentiation of heterocysts is also reported by Ogawa & Carr, (1969). Also, P deprivation may cause a decline in the photosynthetic efficiency by affecting ATP synthesis, thus affecting the nitrogenase activity. This is also substantiated with our study in a culture grown under (-)NP which also showed a decline in the nitrogenase enzyme activity, despite the deprivation of N, indicating that phosphorus starvation did not allow much differentiation of heterocysts probably due to limited phosphorylation as a source of energy. The increase in the nitrogenase activity in the N-deprived culture is also correlated with a higher level of expression of *nif H* gene in both the cyanobacteria (Fig. 3.3.10C).

The nitrogenase activity under different concentration of nitrogen and phosphorus are also studied by other researchers. Nisha et al. (2015) reported that several diazotrophic *Nostoc* sp., and *Anabaena doliolum* were able to fix nitrogen in the nitrogen free medium and reported that the exogenous supply of nitrogen in the form of ammonium negatively affected the nitrogenase activity due to its inhibitory effect as ammonia lowers the supply of reductant or energy. Reddy et al. (1989) and Ohmori & Hattori, (1974) in *Anabaema* species, a nitrogen fixing cyanobacteria, also reported the same.

Nisha et al. (2015) on *Nostoc* sp., and *Anabaena doliolum*; Steward & Alexander, (1971) on *Anabaena* sp., *Anabaenopsis circularis,* and *Chlorogloea fritschii* and Yandigeri et al. (2011) on *Anabaena* sp., and *Westiellopsis* sp., reported higher nitrogenase activity when grown with higher level of phosphorus but without any nitrogen source. They reported the increase was due to higher heterocysts production leading to higher N_2 fixation due to lesser CO_2 fixation to help improve the N:C ratio of the soil. However, in our study we have not observe any significant increase in the activity of nitrogenase that could be due to presence of N_2 source in the culture medium.

4.5.1 Temperature and fatty acid profile

A detailed observation of the major fatty acids indicated that saturated and unsaturated fatty acids had a different response to different growth temperatures in both the *Nostoc* species. Our observation of a higher decline in growth of *N. spongiaeforme* at sub and supra-optimal temperature compared to marine cyanobacteria *N. calcicola* may suggest a role for fatty acids in their adaptation. The observed increase in the unsaturated fatty acid (e.g., methyl palmitoleate, C16:1; oleic acid, C18:1 and methyl linolenate, C20:3) at suboptimal temperature and saturated fatty acids (stearic acid, C18:0) at supra-optimal temperature in *N. calcicola* (Table. 3.2.11B) suggest an influence on cell membrane stability, probably with regard to sol-gel status, for optimal cell functions as it helps in better function by such changes to the fatty acids and their ratio as compared to *N. spongiaeforme*. An increase of unsaturated fatty acids at suboptimal temperature and saturated fatty acids at supra-optimal temperature, as observed in our study is reported to be required for better adaptation to maintain the optimal fluidity of cell membranes to ensure the optimal functioning of membranes for relatively better growth. In our study, our results also showed lesser oxidative damage to *N. calciocla,* which may also be due to its better adaptation as a result of a changes in the ratio of unsaturated and saturated fatty acid at suboptimal and supra-optimal temperature.

Data observed with fatty acid is also substantiated with our study on the lipid phase transition of the membrane at sub and supra-optimal temperatures as physical properties of membrane biolayer are influenced by lipid composition that helps to maintain normal metabolic function under different temperature regime such as photosynthesis, ion permeability and respiratory function. The suboptimal temperature of 10ºC in our study increased the degree of unsaturation of lipids which affected the packing of lipids in bilayer as a double bond can produce a kink, which will create extra free space within the bilayer, and allows additional flexibility in the adjacent chains and thus have a lower transition temperature, resulting a shift in the phase transition from liquid crystalline to the gel phase. While, the presence of high content of saturated fatty acids at a supra-optimal temperature of 40ºC resulted in a shift from gel phase to liquid crystalline phase which may

aid the cyanobacteria to maintain the cell membrane stability and survival under changing growth temperatures (Gautier et al. 2013).

A few studies have reported the effect of temperature on the fatty acid composition of microalgae. Sheng et al. (2011) working with *Synechocystis* sp. PCC6803 at a range of temperature (18-44ºC) reported that a low temperature of 22ºC caused more of γ-C18:3 and α-C18:3, unsaturated fatty acids, and even at the lower temperature of 18ºC, only α-C18:3 was seen, indicating that the unsaturated acids have been produced as a response of low temperature which was not the case at high temperature of 44ºC as saturated and unsaturated fatty acid were suppressed. Jiang & Chen, (2000), investigated the effect of temperature (15-30ºC) on the fatty acid composition of *Crypthecodinium cohnii* ATCC 30556, a marine microalga and found that low temperature of 15ºC favored the formation of polyunsaturated fatty acids while, high temperature of 30ºC favored saturated fatty acid to maintain membrane fluidity and function. Seto et al. (1984) in *Chlorella minutissima*; Chen et al. (2008) in *Nitzschia laevis*; Converti et al. (2009) in *Chlorella vulgaris* and Xin et al. (2011) and Li et al. (2011) in *Scenedesmus*; Chaloub et al. (2003) in *Anabaena siamensis* and de la Rosa et al. (2020) in *Microcystis aeruginosa* under temperature stress revealed an increase in the polyunsaturated fatty acid at low temperature of 18-26ºC than 30-40ºC and suggested that increased levels of unsaturation of fatty acid in membrane lipids enhanced stability, fluidity and functions of cellular membranes, particularly thylakoid membranes to protect the photosynthetic machinery from photoinhibition at low temperatures.

Wei et al. (2015) studied the effect of high growth temperature on total lipid and fatty acid in two microalgae, *Tetraselmis subcordiformis* and *Nannochloropsis oculate* and reported that the total lipid varied with increasing growth temperature with level of saturated fatty acid increased. Sayegh & Montagnes, (2011) and Yang et al. (2013) reported an increased lipid content with increasing temperature in *Spirulina* spp., *Isochrysis galbana*, and *Ettlia oleoabundans* however, the effect of temperature on the lipid accumulation in algae appears to be inversely related to growth rate probably due to diversion of resources from growth and development to synthesis and accumulate lipids (Jiang, 2002). He also speculated that there are different adoptable strategies among

microalgae altering lipid content. Sushchik et al. (2003) working with *Spirulina platensis* showed that a relative content of saturated fatty acid was higher at high temperature of 40ºC than that of unsaturated fatty acid and suggesting that the changes in the FA composition due to temperature changes are necessary to maintain a definite state of cell membranes which have an adaptive significance. Mayzaud et al. (2013) demonstrated that high temperatures of 40ºC decreased the amount of ω3 PUFA in phytoplankton from Arctic region. Rousch et al. (2003) working with cyanobacteria *Chaetoceros muelleri* also showed decrease in unsaturated fatty acid.

Chaisutyakorn et al. (2018) on the other hand, working with diatoms *Tetraselmis suecica* FIKU032 and *Nannochloropsis sp.* FIKU036 at a wide range of temperature from 25-40ºC, reported that the saturated fatty acids decreased, while PUFA increased with increasing temperature from 25-40ºC but they failed to assign any reason for such change.

4.5.2 Nutrient and fatty acid

Our study observed an increase in both saturated and unsaturated fatty acids under double the concentration of phosphorus in both the *Nostoc* specie (Table. 3.3.11A&B) may suggest that the excess uptake of phosphorus might have provided the necessary intermediate phosphorylated products for the synthesis of these fatty acids (Chu et al. 2019).

Nitrogen is considered a single most critical nutrient to channel metabolic flux to lipid biosynthesis, even phosphorus is known to affect lipids metabolism as absence of phosphorus in the cultural medium causes the photosynthesis repression and thus photosynthesis derived precursors of starch cannot be synthetized and the energetic surplus induced by the limitation of cellular duplication was largely stored in form of lipids. The observed increase in saturated and unsaturated fatty acids in *N. spongiaeforme* and *N. calcicola* under phosphorus starvation and combine starvation of nitrogen and phosphorus may be due to significant decrease in growth, growth rate, biomass and photosynthesis observed in both the cyanobacteria (Table. 3.3.2A & Fig.3.3.3) since growth was limited due to the deprivation of P and N&P there was no requirement for the synthesis of new membrane compounds; the cells instead may divert and deposited the

carbon into triacyl glycerides (TAG) which may serve as a protective mechanism under nutrient stress (Elly & Alexander, 2011).

Our study with lipid phase transition did not observe any significant shift in the phase transition of lipid membrane, suggesting no effect on the sol-gel nature of the membrane as a result of changes in the status of N and P. However, it is suggested that the alteration in the fatty acid composition observed in both the *Nostoc* species under excess and starvation growth conditions of N or P may be required for membrane functions such as signaling molecules to regulate cell proliferation, growth, differentiation (Ohlrogge et al. 1995) and as energy storage molecules (TAG) (Elly & Alexander, 2011).

Others have also studied the effect of nutrient deprivation and excess on the fatty acid profile in algae. Chu et al. (2019), Fu et al. (2017), Rasid and Qni, (2015) and Shen et al. (2016) reported an increase in the lipid accumulation in *Chlorella* species under excess phosphorus suggesting that an abundant phosphorus uptake caused the downregulation of ADP-glucose pyrophosphorylase activity which inhibits the starch synthesis, and regulated carbon assimilation towards the lipid synthesis pathway.

Liang et al. (2013) on *Chlorella* sp., and Khozin-Goldberg & Cohen, (2006) on *Monodus subterraneus* under low phosphorus concentration showed an increase in the saturated fatty acid which indicated that the cell division rates decreased under low phosphorus concentration which leads to an accumulation of carbon, which was stored in the form of saturated fatty acid rich TAG while no change was seen in the unsaturated fatty acid, however El-Sheekh & Rady, (1995) in phosphorus starved *Chlorella kessleri* showed an enhanced level of unsaturated fatty acid.

Huang et al. (2013) in *Tetraselmis subcordiformis*, *Nannochloropsis oculate*, and *Pavlova virids*; Widjaja et al. (2009), Illman et al. (2000), Li et al. (2008) and Fan et al. (2014) in *Chlorella* species; Dean et al. (2010) in *Chlamydomonas reinhardtii* and *Scenedesmus subspicatus* showed higher total lipid contents under nitrogen deprived condition which was suggested to be the result of various reason mainly sugar consumption at a rate higher than the rate of cell generation which is reported to promote the conversion of sugar into lipid (Ratledge & Wynn, 2002). Another reason ascribed for lipid accumulation in microalgae under nitrogen starvation is proposed to be due to the

mobilization of lipid from chloroplast membranes as nitrogen from this membrane is relocated using Rubisco (Garcia-Ferries et al. 1996). Another hypothesis for increased lipid content under nitrogen starvation condition was suggested to be due to the inhibition of cell division leading to accumulation of lipid (Sheehan et al. 1998). Others have suggested that higher lipid accumulation under nitrogen deprivation condition may not necessarily be ascribed to higher levels of lipid synthesizing enzyme but may also be due to the cessation of other enzyme associated with cell growth and proliferation and operation of enzymes related to accumulation of lipid (Ganuza et al. 2008).

Almutairi, (2020) working on *Dunaliella salina* under phosphorus and nitrogen limiting conditions exhibited an increase in lipid content, including an increase in long chain fatty acids and unsaturated fatty acid, and indicated that under phosphorus and nitrogen limitation condition cells protected themselves against free radicals by accumulating dicarbon fragments to form unsaturated fatty acids, which act as free radical scavengers. Belotti et al. (2013) also reported an increase in lipid content in *Chlorella vulgaris* under nitrogen and phosphorus deficiency and ascribed the reason for accumulation of lipid content due to diversion of inorganic carbon from DNA and proteins synthesis toward lipids synthesis. There is a report by El-Kassas, (2013), who reported a reduction in the PUFA content under low nitrogen and phosphorus and suggested that low N or P may inhibit biochemical de novo synthesis of PUFA content.

5. Conclusion

Study investigated the morphological, physiological, biochemical and molecular responses of *Nostoc spongiaeforme*, a freshwater and *Nostoc calcicola*, a marine cyanobacteria to a range of growth temperatures from 10-40ºC, and variation in nutrients (excess and starvation of nitrogen and phosphorus). Among all the growth temperatures studied, suboptimal and supra-optimal temperature deleteriously affected both the *Nostoc* species but excess of phosphorus and nitrogen and individual and combined starvation of nitrogen and phosphorus resulted in variable results. It was observed that at the optimum temperature of 30ºC and under DP, DN, and (-)N, both the *Nostoc* species maintained their optimum growth rate, morphological structure and other physiological parameters while at sub and supra-optimal temperature, and (-)P and (-)NP both the *Nostoc* species suffered severe morphologically distortion, depigmentation, and leaving the cells with an distorted shape. The decrease in growth rate and biomass at sub and supra-optimal temperature and (-)P and (-)NP stress was associated with a reduction of photosynthetic pigments, decrease in Fv/Fm ratio, photosynthetic, and respiratory rate.

Both the *Nostoc* species grown at sub and supra-optimal temperature as well as under (-)P and (-)NP resulted an excess generation of ROS (H_2O_2 and OH•) causing oxidative damage in the form of lipid peroxidation and protein oxidation. Higher production of ROS in both the *Nostoc* species was responded by increased enzymatic antioxidants, which was further supported by higher expression of antioxidant enzymes SOD, and CAT to somewhat mitigate ROS and combating oxidative damage under temperature and nutrients stress, in both the *Nostoc* species. Non enzymatic antioxidants such as ascorbate and proline also showed similar results as seen with the enzymatic antioxidants in response to the temperature as well as nutrients stress to combat ROS. Response of TPC & TFC content to the stress may also play the similar function of antioxidant. In addition to expression of antioxidant enzymes, our result also showed higher expression of HSP more so in *N. calcicola* than in *N. spongiaeforme* due to the supra-optimal temperature at 40ºC which may explain better growth of *N. calcicola* than observed for *Nostoc spongiaeforme*.

We also conclude that temperature affected the nitrogenase activity of both the *Nostoc* spp. Nitrogenase activity decreased at suboptimal and supra-optimal temperatures in

both the *Nostoc* sp. Culture grown without nitrogen resulted in stimulating the nitrogenase activity but when grown with DP or DN, (-)P, and (-)NP caused no increase in the activity of the enzyme. Our study with the expression of *nif H* gene also corroborate the nitrogenase activity pattern.

Study revealed a qualitative and quantitative variation in the fatty acid composition under sub and supra-optimal temperature which was also supported by shifts in phase transition of lipid membrane in both the *Nostoc* species as the physical properties of membrane biolayer are influenced by lipid composition that helps to maintain normal metabolic function such as such as photosynthesis, ion permeability and respiratory function etc. under low and high temperature regime. Variation in nitrogen and phosphorus concentration also exhibited an alteration in the fatty acid content but without any shift observed in the phase transition of lipid membrane which suggest that changes in the status of N and P caused no effect on the sol-gel nature of the membrane. However, alteration observed in the fatty acid composition of the membrane due to nutrients variation may be required for membrane functions such as signaling molecules to regulate cell proliferation, growth, differentiation etc.

Our data conclude that among the two *Nostoc* species studied, marine cyanobacteria, *N. calcicola* showed better growth at high temperature of 40ºC probably due to greater expression of HSP. Suboptimal temperature, however, affected the growth of both the *Nostoc* species. Nutrient starvation mainly the (-)P affected the physiological and biochemical parameters of both the *Nostoc* species as deprivation of nitrogen, (-)N, did not show any deleterious effect on any of the studied parameter except for higher population of heterocyst, increased activity of nitrogenase and expression level of *nif H* gene.

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Attended seminars

- Presented poster in the National Conference of Plant Physiology 8-10, Dec at University of Agriculture Science, GKVK Bengaluru, 2016
- Presented poster in the National Conference of Plant Physiology 2017 on emerging role of plant physiology for food security and climate resilient agriculture 23-25 Nov 2017 at Indira Gandhi Krishi Vishwavidyalaya, Raipur, India
- Awarded best oral presentation in the National conference on 'Vistas in Biodiversity, Biology, Biotechnology and nanotechnology of Algae (VBBBNA2018)' held at Department of Botany, Madras Christian College (Autonomous), Chennai, 20-22 Sept 2018
- Oral presentation in the UGC-SAP National seminar on "NEW Vistas in Botany", held on 13th and 14th Feb 2020 at Goa university, Goa
- Participated in the workshop on applied bioinformatics: Techniques, tools and tasks" on Feb 18, 2020 at NFMC, Bharathidasan University, Triuchirappalli.

Publications

- A research article entitled "**Decrypting the effects of starvation and excess of nitrogen and phosphorus on *Nostoc calcicola*"** was published in the *journal of stress physiology and Biochemistry,* 2021, Vol. 17, pp, 5-19. **(UGC CARELIST).**
URL: http://www.jspb.ru/issues/2021/N2/JSPB_2021_2_05-19.pdf

- The research article entitled **"Physiological and biochemical response to starvation and excess of nitrogen and phosphorus in *Nostoc spongiaeforme*, a freshwater cyanobacterium"** was published in *Indian hydrobiology*, 2021, Vol.20, pp, 17-30. (**UGC CARELIST**).