Effect of UV-B and salinity stress on biomass and lipid production potential of microalgae: A study on stress induced molecular signaling

THESIS

SUBMITTED TO
BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
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Under the Supervision of
Prof. D.P. Singh

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(A Central University, NAAC Accredited ‘A’ Grade)
VIDYA VIHAR, RAEBARELI ROAD
LUCKNOW-226 025
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This Thesis is Dedicated to

My Parents

Whose inimitable love and affection stands before me as a constant source of energy & inspiration in every walk of my life.........

CERTIFICATE

This is to certify that the thesis entitled "**Effect of UV-B and salinity stress on biomass and lipid production potential of microalgae: A study on stress induced molecular signaling**" submitted by **Mr. Ranjan Singh** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The Thesis submitted to Babasaheb Bhimrao Ambedkar University satisfies all the requirements as stipulated in the *Doctor of Philosophy (Ph.D.) regulations - 1999 as amended in 2008/2010/2013/2016* and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

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Student Declaration

This is to certify that the material embodied in the present Ph.D. work entitled **"Effect of UV-B and salinity stress on biomass and lipid production potential of microalgae: A study on stress induced molecular signaling"** is original research work done by me. It has not been submitted in part or full for any other diploma or degree in any other University. In this thesis, matter written, data presented and plagiarism, if any, is the sole responsibility of the student Mr. Ranjan Singh. If any allegations/query/question arises regarding the thesis, I will be solely responsible and answerable.

I also declare that the thesis is essentially free from all kinds of plagiarism.

Ranjan Singh
Research Student

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Date: (**Ranjan Singh**)

Preface

Preface

Rising demand for energy and depletion of fossil fuel has become a major concern for people around the world. Microalgae can be used to generate a range of renewable fuels including biodiesel, bioethanol, bio-hydrogen, etc. Microalgae have recently gained interest due to its dual function of reclamation of soil, water and the production of algal biomass that can be used in bioenergy production, pharmaceuticals, fertilizers and animal feed. Microalgae are usually chosen for lipid production for its high photosynthetic efficiency, rapid absorption of nutrients, short life span, and low nutritional requirement so that they can be easily cultivated and grown rapidly in both industrial and laboratory conditions. In addition, algal reclamation is a more cost-effective and sustainable approach for effectively removing inorganic compounds containing salt, nitrogen, phosphorus and heavy metals. Microalgae are a single-cell photosynthetic organism capable of assimilating large quantities of carbon, nitrogen and phosphorus for their growth and production of oxygen. It has been reported that a wide range of microalgae such as *Chlorella vulgaris*, *Chlorococcum humicola*, *Chlamydomonas* and *Spirulina* has been used in the treatment of contaminated soil and water. The aims of the present study entitle "Effect of UV-B and salinity stress on biomass and lipid production potential of microalgae: A study on stress induced molecular signaling" is to give scientific knowledge about the ability of microalgae *Chlorella* vulgaris and *Chlorococcum humicola* for reclamation of soil and lipid production. The present investigation was attempted to study the tolerance level, photosynthetic efficiency and lipid production potential of microalgae under varying environmental stress conditions (NaCl and UV). The changes in growth rate, biochemical constituents, photosynthetic efficiency of microalgae were measured by using the UV-Visible spectrometry, FTIR, Chlorophyll fluorescence induction kinetics (OJIP) curve, SEM couple with EDS study. The present thesis is divided into eight chapters and each attempt has been made to independence and self-containment of each chapter.

Chapter 1 covers the 'Introduction' section presenting the outline of the work along with the objectives to be accomplished in this thesis.

Chapter 2 is related with the 'Review of Literature' of the review and citation of other work done by investigators in the field of microalgae, modification and improvement of algal strain, environmental stress condition (NaCl and UV-B) and lipid production.

Chapter 3 'Materials & Methods' is exclusively assigned to the general methodology used during the course of the present investigation.

Chapter 4 entitled as "NaCl incites reactive oxygen species in green Algae *Chlorococcum humicola* and *Chlorella vulgaris*: Implication in lipid synthesis, mineral nutrient status and antioxidant system" deals with the efforts to optimize the NaCl concentration and study the changes in biochemical constituents, nutrient status, antioxidant capacity and lipid production under NaCl stress.

Chapter 5 entitled as "Effect of UV-B on photosynthetic performance, nutrient status and lipid yield in Microalgae *Chlorella vulgaris* and *Chlorococcum humicola*" deals with the effect of UV-B on the growth, biochemical alteration, mineral nutrient content and lipid yield.

Chapter 6 entitled as "UV-B intensity induced photochemical and biochemical alteration in Microalgae *Chlorella vulgaris* and *Chloroccum humicola*" deals with the effect of UV-B intensity on the growth, biochemical constituents, and lipid production.

Chapter 7 entitled as "Combined effect of UV-B and Sodium Chloride stress on cellular response of Microalgae *Chlorococcum humicola* and *Chlorella vulgaris*: Evaluation of photosynthetic performance, photooxidative injury and Antioxidant status" deals with the efforts to optimize the combine effect of UV-B intensity and NaCl. Study investigates the synergistic and antagonistic impact of NaCl and UV-B on biochemical constituents, photosynthetic efficiency and lipid content in both the algae.

Chapter 8 covers the "Summary and Conclusion" section emphasizing the findings and future prospects during the study.

List of Abbreviations & Symbols

μg : Microgram
μL : Microliter
μM : Micromolar
AA : Amino acid
ABS/RC : Absorption of photons per reaction centre
ANOVA : Analysis of variance
APX : Ascorbate peroxidase
BSA : Bovine serum albumin
CAT : Catalase
DMA : Dimethylarsinic acid
DMRT : Duncan’s Multiple Range Test
DTNB : 5, 5’-Dithio-bis-trinitro benzoic acid
dw : Dry weight
EDTA : Ethylene diamine tetraacetic acid
FA : Fatty acid
Fv/Fm : Maximum quantum yield
Fv/Fo : Photosynthetic activity
Fw : Fresh weight
GPX : Guiaocol peroxidase
GR : Glutathione reductase
GSH : Reduced glutathione
GSSG : Oxidized glutathione
GST : Glutathione-S-transferase
H_2O_2 : Hydrogen peroxide
H_3PO_4 : Phosphoric acid
MDA : Malonyldialdehyde
Mo : Net closing rate of reaction centre
NADPH : Reduced nicotinamide adenine dinucleotide phosphate
NADH : Reduced nicotinamide adenine dinucleotide
NaCl : Sodium Chloride

NBT	:	Nitrobluetetrazolium
NO	:	Nitric oxide
NPSH	:	Non-protein thiol
$O_2^{\bullet -}$	:	Superoxide radicals
$OH^{\bullet}$	:	Hydroxyl radicals
Pi_{Abs}	:	Photosynthetic performance index
PMSF	:	Phenyl methyl sulphonyl fluoride
ppm	:	Parts per million
PVP	:	Polyvinyl pyrrolidone
RC	:	Reaction centre
ROS	:	Reactive oxygen species
SOD	:	Superoxide dismutase
TBA	:	Thiobarbituric acid
SA	:	Salicylic acid
TBARS	:	Thiobarbituric acid reactive substances
TCA	:	Trichloroacetic acid
TRo/RC	:	Trapping flux of reaction centre
UV-B	:	Ultraviolet-B
WHO	:	World Health Organisation
General	:	
µg	:	Microgram
d	:	Days
gm	:	Grams
h/hrs	:	Hours
kg	:	Kilogram
mg	:	Milligram
ml	:	Millilitre
min/mins	:	Minutes
mM	:	Milimolar
s	:	Second

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CHAPTER 1

General Introduction

1.0 General Introduction

The world's energy consumption is continuously rising. This rising demand of consumption is currently met by non-renewable energy resources such as the oil, petroleum and coal reserves, which are continuously dwindling due to overexploitation, urbanization and population growth (Elum and Momodu, 2017). The excessive use of this finite reserve is also a driving force for climate change, global warming, environmental pollution and ecosystem instability (Giorgianna and Mayfield, 2012). Fossil fuel such as coal, oil etc., have finite reserves and might be exhausted in the near future, leaving a remarkable question of energy security (Elum and Momodu, 2017). It has been estimated that >90% of energy demand is fulfilled by coal and 10% by other sources. Therefore, a transition for economically feasible, ecofrienndly, renewable and green fuel alternative is one of the utmost priorities. The renewable sources of energy include agricultural crops (Maize, Sunflower), non-agricultural crops (*Jatropha* sp.) and algae (Upadhyay et al., 2016). The major drawback in the utilization of agricultural and non-agricultural crop as feed stock of biofuel is low biomass yield, food insecurity, high cost of production, high land acquirement, harvesting time and low lipid yield (Smil, 2019). Thus, algae could be a viable renewable energy resource due to their fast growth rate, easier to cultivation, ability to grow in diverse habitat, non-arable land with brackish or salt water and soil (Rai et al., 2013; Hallmann, 2015). Algae, being the dominant aquatic organism, making its use in the production of biofuels, could fill the gap over traditional feed stocks (Jeffrey et al., 2014).

Microalgae are dominant, photosynthetic oxygen producing organisms on the earth (Brodie et al., 2017) and are considered as perfect organisms for sustainable biofuel production (Neofotis et al., 2016; Upadhyay et al., 2016; Singh et al., 2018). The biofuel production in algae is largely dependent on the biomass and production of energy rich compound i.e., lipid in the cells. Since, algae may possess >80% of total lipid, its application as a source of energy could be sustainable strategy of energy alternative (Korosi et al., 2017). Various studies have been reported that the lipid and biofuel production efficiency can be induced in algae under different environmental stresses (Upadhyay et al., 2016; Singh et al., 2018). In addition, application of genetic engineering has now become a common tool to develop algal strains with high growth rate, lipid content, pathogen resistance and higher biomass productivity (Shuba and Kifle, 2018). Lipids are present in algae in various forms. Microalgae store oil as non-polar storage lipids, such as triacylglycerides. The cellular membranes and photosynthetic membrane of algae also constitute to polar lipids, such as phospholipids, glycolipids and sterols (Vuppaladadiyam et al., 2018).

Oils from algae are produced though different processes such as biodiesel by trans-esterification, and petrol fuel via distillation and cracking (Isa and Ganda, 2018). It has been reported that, about 30 million hectares area are used for algal cultivation with biofuel yield of approximate 40,000 L/hec/year, which is sufficient to replace the 1,200 billion liters of petroleum used by the world’s largest consumer of petroleum; i.e., United States (Bhowmick et al., 2018). This area is comparable to that used for Soya plantation in the United States and roughly twice that used for production of corn ethanol (about 14 million hectares used to produce almost 64 billion liters of ethanol in 2011 (Hoekman et al., 2018). It has been also observed that the microalgae are vital resource for the production of various value added products used in food, feed, medical, cosmetic and chemical industries (Anupama and Ravindra, 2000; Harun et al., 2010).

Salinity is introduced in the environment through agriculture, natural as well as anthropogenic activities (Ismail et al., 2017). The main factors contributing to salinity include reduced precipitation, surface evaporation, weathering of native rocks, irrigation with saline water and faulty agricultural practices (Shrivastava and Kumar, 2015). The salt concentration is rising in the world, covering >6% of total land area (Ismail et al., 2017) and about 50% arable land is on the verge of salinification by 2050 (Wang et al., 2003; Jamil et al., 2011). Salinity is basically known as an excess amount of salts present in the soil. Salinization is recognized as the main threats to environment and human, affecting approx. 1 billion ha land worldwide, representing about 7% of earth’s continental extent (Metternicht and Zinck, 2003; Yensen, 2008). Shrivastava and Kumar (2015) reported that an approx 7 million hectares of land is covered by saline soil in India, but the most of saline soil occurs in indogangetic plane, that covers the states of Punjab, Haryana, U.P. Bihar and some parts of Rajasthan (Shrivastava and Kumar, 2015). Arid and semi-arid tracts of Gujarat, Madhya Pradesh, Maharashtra, Karnataka, Rajasthan and Andhra Pradesh are also affected by the saline lands (Sharma and Singh, 2015). Approximately 25% of total cultivated and 33% of irrigated agricultural lands are affected by high salinity worldwide, which are increasing at a rate of 10% per year (Parihar et al., 2015).

Salinization is considered to be a very serious environmental problem for the entire world and it is one of the most devastating environmental stresses, which adversely affects the cultivated land and productivity of crop (Jouyban, 2012). Due to the increasing population, the cultivable agriculture lands are under continuous demand for residential and commercial purposes (Goudie, 2018). Salinity, not only decreases the agricultural production, affects physicochemical properties of soil and influences the ecological balance (Balba, 2018). The impacts of salinity on crops include low productivity, low returns, soil erosions, nutrient loss, growth inhibition and death (Hu and Schmidhalter, 2005). The adverse effect of salinity is the result of complex interactions among morphological, physiological, and biochemical processes of plants including seed germination, plant growth, water & nutrient uptake, oxidative stress (Akbarimoghaddam et al., 2011; Carillo et al., 2011; Shrivastava and Kumar, 2015). Soil salinity significantly reduces phosphorus (P) uptake in plants because phosphate ions tend to get precipitated with Ca^{+2} (Bano and Fatima, 2009). Growth of algae in such salinized soil and water system may limit the successful production of renewable energy resource in a sustainable manner (Costa and de Morais, 2011).

Pandey et al. (2018) reported that salt is the common stressor for successful large scale production of biomass, lipid and biofuel from algae. High lipid production in algae under salt has been achieved by various authors (Singh et al., 2018; Yang et al., 2018). Ho et al. (2014) reported that under high salinity state, algae usually store energy-rich compounds, such as lipid and starch by changing the cellular allocation of carbon. Algae grown in saline habitat develop osmotic stress, alteration in nutrient uptake and generation of reactive oxygen species (ROS) (Verslues et al., 2006). ROS produced inside the living cell as a byproduct of cell metabolism are involved in signal cascade and elicit toxicity at higher concentration (Almeida et al., 2017). ROS comprises highly reactive and unstable oxygen molecules such as $^1O_2$, H_2O_2, $O_2^{\bullet -}$ and $OH^{\bullet}$, which cause damage to the redox homeostasis and cellular functioning of the plants (Das and Roy, 2014). In plant cell, high level of NaCl triggers exo-osmosis driven plasmolysis, altering the water potential of cells which restricts the entry of water and essential mineral nutrients (Singh et al., 2015). To protect the cell damage, plants adopt themselves by production of low molecular weight osmo-protectants and osmolytes (Filippou et al., 2014). ROS induced oxidative damage in algae are mitigated by low molecular weight scavengers (carotenoid, ascorbate, proline, cysteine and GSH) present in the algae (Singh et al., 2018). In algae stress induced ROS accumulation is counteracted by enzymatic antioxidants system such as super oxide dismutase (SOD), peroxidases, catalase (CAT), glutathione reductase (GR), Glutathione S-transferase (GST) (Chokshi et al., 2017).

Superoxide dismutase (SOD) is the most effective intracellular enzymatic antioxidant (Wang et al., 2018). Which acts as a first line of defence against ROS (Brook and Dobson, 2015) and SOD removes O_2^- by dismutation reaction and forms H_2O_2 and O_2 (Wang et al., 2018). H_2O_2 formed as a by-product of aerobic metabolism is further scavenged by catalases (CAT) and peroxidises (Shrama, 2016). CAT is an important enzymes in the removal of H_2O_2 generated in peroxisomes by oxidases involved in β–oxidation of fatty acids, purine catabolism and photorespiration, whereas ascorbate peroxiadse (APX) is thought to play the most vital role in scavenging of ROS and thus protecting algae cells against oxidative injury (Saini et al., 2018). APX is involved in scavenging of H_2O_2 in ascorbate-glutathione and water-water cycles and utilizes ascorbate as an electron donor (Singh et al., 2018). Gluthathione-S-transferase (GST) catalyzes the conjugation of electrophilic substrate to reduce gluthatione, and the resulting complexes are transported to a vacuole for further processing and degradation (Urbancsok et al., 2018). Induction of GST is found in algae in response to salt treatment, pathogen attack, wounding, drought, UV radiation, heavy metals, pesticides and other stress factors (Dar et al., 2017).

Solar UV radiation is one of the potent environmental factor (Sulzberger et al., 2019), which contributes to cleaning of the atmosphere, generation of photochemical smog, photo-damage, alteration in precipitation, coral bleaching, vegetation loss and agricultural escalation etc. (Danovaro et al., 2008; Comont et al., 2013; Kataria et al., 2014; Williamson et al., 2014), and also affect the functioning of the ecosystem services. Various studies have been reported that low dose of UV stimulates algal growth, while higher doses show inhibitory effect on growth of microalgae (Qi et al., 2014; Cheng et al., 2016). A number of studies carried out earlier on the impact of UV-B flux on aquatic communities like algae showed adverse effect on ultra-structural change, photosystem II chemistry, nutrient status and lipid productivity.

UV-B (290-400 nm) is known to be the most toxic radiation (Qi et al., 2014) in plant and animals. The long term exposure of UV-B impairs photosynthesis machinery, lipid peroxidation, DNA damage and formation of dimer (cyclobutane-pyrimidine) in plants and algae (Singh et al., 2017). For protection of cells from harm full effect of UV-B, algae are endowed with certain responses such as migration, development of protective cell wall, increased synthesis of carotenoids, regulating physiological, biochemical and antioxidant (ascorbic acid, cysteine and proline) defense (Kataria et al., 2014). Algae, being the most dominant photosynthetic microorganism in upper aquatic surface, receive high level of UV exposure and consequently result into photo bleaching coupled with reduced upwelling of nutrients, chlorosis and necrosis etc. (Singh et al., 2018). UV exposure might also reduce the photosynthetic efficiency in algae; damage the efficiency of biological pump, and fixation of CO_2 and ultimately reduce the carbohydrate and lipid content (Jahnke et al., 2019). The high photosynthetic potential of microalgae is not only important for lipid production, but it may also be beneficial for accumulation of nutrients, carbon sequestration, bioremediation and production of oxygen (Moreno et al., 2017). The first line of UV-B defence is to limit the penetration of UV-B within the microalgae cell (Bais et al., 2018). Under UV-B exposure, algae and cyanobacteria develop an adaptive mechanism against UV-B stress such as shading formation (Kruschel et al., 1998), migration to deeper water level (Karsten et al., 1998), production of anti-oxidative system (Wolfe-Simon et al., 2005) and synthesis of secondary metabolites such as mycosporin like protein (Karsten et al., 2014), scytonemin (Rastogi et al., 2014) and pigments (Zubia et al., 2014). Exposure to UV-B radiation results into enhanced accumulation of flavonoids, synaptic esters and anthocyanin pigments, which attenuate the penetration of UV-B radiation (Bais et al., 2018). Photosynthesis is dependent on light harvesting properties of the chlorophyll. Reduction in chlorophyll level under UV stress might be due to disruption of various photosynthetic process viz., electron transport system, photosystem II and pigment bleaching (Prasad et al., 2016). Various authors have earlier reported a decrease in the chlorophyll content of microalgae *Dunaliella salina, Nostoc muscorum, Plectonema boryanum, Aphanothece* sp., *Arthrospira* and *Spirulina platensis* exposed to UV-B radiation (Prasad and Zeeshan, 2005; Gao and Ma, 2008; Tian and Yu, 2009; Zeeshan and Prasad, 2009). Flavonoids act as catalyst in the light phase of the photosynthesis and regulate the iron channels involved in phosphorylation (Ehonen et al., 2019). Flavonoids in algae also function as stress protectants by scavenging ROS produced by photosynthetic machinery as reported by various authors (Singh, 2018; Sunil et al., 2019). It is now established fact that UV-B potentially impairs the performance of all the three main component processes of photosynthesis; the CO_2 fixation by the calvin cycle, the photophosphorylation reactions in the thylakoids membranes and stomatal conductance (Yadav et al., 2017). In photosynthesis, photosystem II (PSII) is found to be highly sensitive to UV-B. UV-B inactivates the water-oxidizing complex and modifies the binding sites on the PS II reaction centre with simultaneous blocking of pheophytine, the primary electron acceptor (Mathur and Jajoo, 2015). The most important target of UV-B in photosynthetic organisms is PS II, as it causes damage to Q_A, Q_B and catalytic Mn cluster of the water oxidizing complex (Sharma et al., 2017). Zhang et al. (2015) also reported that the activity of ATP-synthetase, PSII and ribulose 1, 5-bisphosphate carboxylase enzymes are severely reduced upon exposure of microalgae to high UV-B radiation.

Chlorococcum humicola is a spherical, unicellular and colonial (group of 2 to 4) freshwater green microalga belongs to family Chlorophyta (Hena et al., 2015; Singh et al., 2018). These are potential source of carotenoids, lipid and other bioactive compounds because of faster growth rate, adaptation to wider range of pH and temperature, CO_2 level and ease of outdoor cultivation (Hena et al., 2015). Under high stress conditions *C. humicola* culture changes its colour from green to orange as the cells accumulate high amount of carotenoids (Masojidek et al., 2000). *C. humicola* cells like *Haematococcus pluvialis* contain free astaxanthin, astaxanthin esters, free adonixanthin, adonixanthin esters, canthaxanthin, lutein, β-carotene and cis-isomers of keto-carotenoids (Yuan, 2002).

Chlorella vulgaris is a green, unicellular microalga producing high quantity of proteins, vitamins, minerals, carotenoids and polysaccharides (Liu and Hu, 2013). *C. vulgaris* is also reported to produce xanthophylls such as violaxanthin, lutein and zeaxanthin, with lutein as a major carotenoid (Cha-Um and Kirdmanee, 2008). Campenni et al. (2013) reported that *C. vulgaris* is used for its easy cultivation in both heterotrophic and autotrophic growth for biomass and carotenoid production. *C. vulgaris* cells usually double in eight hours at a temperature of 20-35^{o}C under autotrophic culture conditions (Cheregi et al., 2019). The carotenoid content in algae varies under different growth conditions (Safi et al., 2014). *Chlorella* has been widely used for healthy food and as nutrition supplements (Kitada et al., 2009). *C. vulgaris* contains very rigid cell wall and hence cells are processed in order to break the cell wall before using it as a food supplement in various applications (Tokusoglu and Unal, 2003). It has been observed that salinity induced the lipid production in algae with simultaneous decrease in biomass (Srivastava and Goud, 2017). However, no study is undertaken to explain the ultrastructural alteration, mineral nutrient status and metabolic alteration leading to synthesis of lipid, protein and carbohydrate under different degree of salt and UV-B stress. It is also not clear how the two stresses i.e. salinity and UV-B stresses interact and determine the direction of cell metabolism. The present study was carried out to observe the impact of salt and UV-B stress single or in combination on biochemical, physiological and structural changes (ii) SEM coupled with EDS analysis showing impact of mineral nutrient status (iii) FTIR analysis of macromolecules including lipid (iv) PAM analysis of photosynthetic performance of both *C. humicola* and *C. vulgaris*.

Objective of the work

The aim of the present work was to envisage the effect of UV-B and NaCl in microalgae *C. vulgaris* and *C. humicola.* The major objectives are as follows:

1. Collection, identification of natural growing microalgae.
2. Effect of dose dependent UV-B radiation on the growth and biomass production
3. Selection of UV-B and salinity tolerance microalgae.
4. Effect of UV-B in combination with salinity stress on biochemical change in the microalgae
5. Effect of UV-B and salinity induced molecules signaling and overall impact on lipid production

CHAPTER 2

Review of Literature

2.0 Review of Literature

Non-renewable fuels such as coal, oil and petroleum have been recognized as major contributor to increasing demand of global energy supply (Oh et al., 2018). These non-renewable sources are largely considered to be the prominent cause of pollution, diversity loss, global warming and climate change (Rahman et al., 2017). Currently, about 90% of total energy demand is fulfilled by fossil fuels and remaining ~10% from renewable resource such as biofuel, biodiesel, solar energy etc. (Milano et al., 2016). Based on the current status of the per capita energy consumption, there will be no more oil reserves after 2050 (Miller and Sorrell, 2013).

Application of fossil fuel enhances the pollution load in water, air and soil (Tripathi et al., 2016). The aquatic ecosystem is one of the mega reservoirs of all type of pollutants emitted from various resources into the atmosphere. Therefore, efforts should be made for the development of sustainable energy solution from renewable resources such as plants and microalgal biomass (Hari et al., 2015). Application of microalgal biomass as a sustainable resource of biofuel has captured considerable interest as it has the huge potential to be a next generation feed stock (Pandey et al., 2018).

Biofuels have greater potential to meet the global energy demand and are considered to be one of the most feasible alternative sources of energy as they are renewable, sustainable and environment-friendly (Chew et al., 2017). The strength of algal biomass is becoming a great success for many industrial applications due to its fast growth rate, ability to adopt diverse habitat, no competition with food crops and land and easy to culture and multiply in waste waters as well as bare lands (Chen et al., 2011). The high photosynthetic ability of microalgae is not only useful for lipid production, but also play a pivotal role in carbon sequestration, oxygen production and nutrient recycling etc. (Shen, 2014).

Microalgae are known as metaphor of “green gold mines” due to predominance of triglycerides (TAGs) in their lipid bodies (Shen et al., 2016). Biofuel production has several advantages such as reduction of country’s reliance on crude oil imports, employment and source of farmer’s income etc. (Gheewala et al., 2013). The biofuel derived from microalgae lipid is also non-toxic, biodegradable and does not contribute to net CO_2 or sulphur emissions (Atabani et al., 2012; Shuba and Kifle, 2018).

Lipids are energy reserves in microalgae cells and the raw materials for biofuel production (Chew et al., 2017). Although the biofuel production from algae are much better than the other raw materials, but it is not successfully employed as energy resource for commercial purpose due to low lipid productivity (Chen et al., 2015; Rai et al., 2015). Several studies have been reported that the biomass and lipids production in algae can be enhanced by different environmental stresses and through genetic engineering (Minhas et al., 2016; Zhu et al., 2016). Various stress factors such as light intensity, pH, temperature, nitrogen, UV and salinity etc. are known to influence the lipid production, biomass productivity and tolerance responses in microalgae (Lin and Wu, 2015; Rai et al., 2015; Schnurr and Allen, 2015; Benavente-Valdés et al., 2016). However, the mechanism behind stress induced stimulation in lipid production is still unexplored.

Under environmental stress, reactive oxygen species (ROS) are produced as a result of oxidative damage in algae. ROS refers to free radicals, molecules and ions that are derived from oxygen (Das and Roy Choudhary, 2014). Reactive oxygen species (ROS) are known to accumulate in microalgae under various environmental stress conditions like high temperature, salinity, heavy metal stress, high light intensity and various nutritional stress (Chokshi et al., 2015). The ROS comprises both free radical ($O_2^{\bullet -}$: superoxide radicals; $OH^{\bullet}$: hydroxyl radical; perhydroxy radical and $RO^{\bullet}$: alkoxy radicals) as well as non-radical molecular forms (H_2O_2: hydrogen peroxide and $_1O_2$: singlet oxygen) (Gill and Tuteja, 2010). Reactive oxygen species, such as superoxide ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) are formed by the partial reduction of oxygen molecule, which is an essential aspect of aerobic life (Sharma et al., 2012). Free radicals and reactive oxygen species (ROS) are believed as slow poison due to their adverse effects on biologically significant molecules (Dröge, 2002). It has been estimated that about 1% of oxygen consumed by plants is transformed into ROS in various subcellular parts such as chloroplasts, mitochondria and peroxisomes (Sharma et al., 2012). ROS are well recognized for dual role as deleterious and beneficial species both depending upon their concentration in the cells (Sharma et al., 2012). At higher concentration, ROS damages the biomolecules, whereas at low or moderate concentration it acts as secondary messenger in intracellular signalling cascades (Sharma et al., 2012). When

ROS reaches above threshold level, lipid peroxidation takes place in cellular membranes which in turn adversely affect normal cellular functioning (Redza-Dutordoir and Averill-Bates, 2016). ROS is injurious to algal cells affecting algal growth, development and physiochemical reactions (Sharma et al., 2016) due to oxidation of endogenous target molecules which are also considered as ROS reporter molecules. For example, increase in malondialdehyde (MDA), thymine dimers of DNA (Ayala et al., 2014), products of ROS coupled oxidation of polyunsaturated lipids and DNA are indicators of respectively, indicate the presence of ROS (Gaschler and Stockwell, 2017). Primary evidence for the generation of superoxide radicals ($O_2^{\bullet -}$) was observed in algae exposed to UV-B radiation (Xue et al., 2005). Increasing secondary evidences corroborate the involvement of ROS mediated injury caused by UV-B radiation (De Jager et al., 2017) to lipid in algae (He and Häder, 2002). Enhanced production of ROS in algae and plant cells exposed to high level of UV-B exhibit detrimental effects on enzyme activities and gene expression, which finally leads to cellular injury and programmed cell death (Xie et al., 2019).

Lipid peroxidation aggravates the effect of oxidative stress through production of various lipid-derived radicals which can react with and damaged proteins and DNA (Sharma et al., 2012). Increased in the lipid peroxidation has been reported in plants growing under different environmental stresses (Sharma and Dubey, 2005; Han et al., 2009). Malondialdehyde (MDA) is the end product of peroxidation of unsaturated fatty acids of cell membrane (Gaschler and Stockwell, 2017). Oxidative stress may also acts as inducer for high lipid production and accumulation in various microalgae like *Dunaliella salina* (Yilancioglu et al., 2014), *Chlorella pyrenoidosa* (Duan et al., 2012), *Chlorococcum humicola* (Singh et al., 2018).

2.1 Salinity

Salinity is a prime environmental obstacle of the present world, introduced in the environment by the natural resources, anthropogenic activities and faulty agricultural practices (Ismail et al., 2017). It affects the microorganism, plants, human health, as well as the environment (Chokshi et al., 2017). Salinity is a complex stressor, which influences various physiological and biochemical mechanisms associated with the growth and development of microalgae (Chokshi et al., 2017). It is one of the principal abiotic stressor that adversely affects plant productivity (Anjum et al., 2015). The mechanism by which salinity causes toxicity is not yet clears

(Kusvuran et al., 2016). However, it might be either due to (i) an effect of Cl^- toxicity on Photosystem II (ii) change in membrane integrity caused by a high Na^+ to Ca^{2+} ratio, plasmolysis influenced by osmotic potential and water uptake (Tavakkoli et al., 2010). The algae respond to oxidative stress by adjusting levels of antioxidants and associated components (Shiu and Lee, 2005). Microalgae differ in their adaptability to survive and flourish under saline environment by altering their physiology and osmotic adjustment (Kirrolia et al., 2011).

Salinity is one of the most unstable environmental factors which is continuously increasing due to encroachment and human interventions (Kirrolia et al., 2011; Cañedo-Argüelles et al., 2013). Scientists have reported that climate change causes droughts, less precipitation and rise in the level of soil salinity in some parts of the world (Van Aalst, 2006). Sea level rise may also upset the salt balance in estuaries which can impact aquatic plants and algae of these ecosystems (Hughes, 2004).

The beginning of 21st century is marked by global scarcity of water resources, environmental pollution and increased salinization of soil and water. It has been estimated that worldwide about 20% of total cultivated and 33% of irrigated agricultural lands are likely to be afflicted by high salinity (Shrivastava and Kumar, 2015). Furthermore, the salinized areas are increasing at a rate of 10% annually due to low precipitation, high surface evaporation, weathering of rocks, irrigation faulty agricultural practices. Jamil et al. (2011) have predicted that more than 50% of the arable land would be salinized by the year 2050. The problem of soil salinization has become bane for agriculture sector worldwide (Shrivastava and Kumar, 2015). The world agricultural land world over destroyed by salt accumulation in each year is estimated to be 10 million ha (Pimentel et al., 2004), which might get accelerated due to climate change. An excessive use of ground water, increasing use of low-quality water for irrigation, use of chemical fertilizers, faulty farming practices and poor drainage (Machado and Serralheiro, 2017) are considered as secondary salinizing elements (Shrivastava and Kumar, 2015). Salinity not only decreases the agricultural production of the most crops but also affects physicochemical properties of soil and ecological balance of the land (Machado and Serralheiro, 2017).

A very high concentration of salt (NaCl) in the soil indicates three major types of stresses, i.e., ionic, osmotic and oxidative stress (Xiong and Zhu, 2002). Ionic stress results from imbalance in ion homeostasis. Crops grown on saline are negatively influenced on account of high osmotic stress, nutritional disorders and poor soil conditions (Shrivastava and Kumar, 2015). Inundation of land by saline water for long periods leads to percolation of salts into the soils layers resulting in to decline in nitrogen fixation and mineralization, impairing soil fertility within few years (Islam and Yasmin, 2017). Soil salinity also decreases the uptake of water due to salinity induced ion toxicity, osmotic stress and nutrients (N, Ca, K, P, Fe, Zn) deficiency (Shrivastava and Kumar, 2015; El-Ramady et al., 2018). High salt concentrations in the cells results in to membrane damage, suppression of enzyme activity, disturbances in vital functions such as cell division, carbon assimilation, metabolism of proteins, nucleic acids, decreases in respiration intensity and accumulations of toxic products in the cells (Sudhir and Murthy, 2004; Gupta and Huang, 2014).

River, lakes and ponds are vital resources of surface drinking water on the earth. However, the increase in the salinity of freshwater reservoirs has threatened the aquatic ecosystem severely (Kaushal et al., 2005; Bhateria and Jain, 2016; Canedo-Argüelles et al., 2018). Discharge of industrial effluents and sewage are important contributors to water salinization in the world. The impact of salinization on aquatic organism includes inhibition of photosynthesis, nutrients limitation, reduced uptake of water, plasmolysis and death (Jouyban, 2012). The salinity in water and soil are mainly caused by high concentration of sodium chloride (NaCl), but potassium chloride (KCl), magnesium chloride ($MgCl_2$) and calcium chloride ($CaCl_2$) also significantly contribute to water salinity (Lorenzo et al., 2015). Since the molecular toxicity depends on the chemical nature of the compound (Egorova and Ananikov, 2017), presence of KCl and $MgCl_2$ in water provides structural integrity to microalgae and subsequently help in biomass accumulation, whereas NaCl and $CaCl_2$ induce synthesis of various metabolite in order to maintain cellular homeostasis (Church et al., 2017). The Na^+ of NaCl competes with K^+ ions for its uptake due to analogous similarity of ions, which results in K^+ deficiency within the cytosol (Almeida et al., 2017). In the absence of Mg^{+2} (as $MgCl_2$), cell growth and cell division are arrested which ultimately leads to decline in the accumulation of cellular lipid and reduction in total biomass yield (Gorain et al., 2013). It has also been reported that the higher concentration of Mg^{2+} ions in the cellular environment blocks the entry of K^+ ion in the chloroplast, leading to impairment in the photosynthetic apparatus (Srivastava et al., 2017). Calcium (as $CaCl_2$) is crucial ion required for perceiving the light energy through chlorophyll molecules, however, in the absence of nitrogen in culture media or any other stressors, the excess reducing power is channelized towards the lipid synthesis by compromising the cell growth (Chen et al., 2017). Magnesium directly affects the synthesis of the chlorophyll which results into decrease in the growth rate as well as lipid productivity in microalgae (Gorain et al., 2013). On the other hand, salinity induces the synthesis of neutral lipids in the form of secondary metabolites to withstand adverse saline environment (Ben Moussa-Dahmen et al., 2016). Growing salinity adopted algae in saline environment might ensure the success of algal in term of production of biofuel in sustainable manner (Costa and de Morais, 2011). Zhang et al. (2017) suggested that salinity is the cheapest environmental stressor, which can be employed for grater production of biomass, lipid and biofuel to meet the industrial need. High lipid production under salt is achieved by changing the pathway of carbon flow towards synthesis and storage of compounds like starch and lipid (Ho et al., 2014). Increases lipid content of algae under salt stress can be achieved through modification of photosynthetic machinery, synthesis of osmo-protectants like glycerol, sugar and protein (Paliwal et al., 2017). Yilancioglu et al. (2014) reported that shift in the sugar/ carbohydrate metabolism towards synthesis of storage molecules under stress condition.

2.2 Salinity induced photosynthetic performance

Photosynthesis is an energy transformation process that converts light energy into chemical energy (Amaro et al., 2012; Petsas and Vagi, 2017). Plant and algal cells are generally adapted to a certain range of salt concentration indicate that most probably all life originated in a highly saline environment i.e., oceans (Kirrolia et al., 2011). Salt can directly influence that electron transport and/or photophosphorylation which result in to decline in the quantum efficiency of photosynthesis (Cardol et al., 2011). The concentration of the photosynthetic pigment in microalgae also depends upon the duration of salt exposure and the concentration of the salt in the media. The chlorophyll a/b ratio and the total content of chlorophyll (a+b) decreased with increase in NaCl concentration (Khosravinejad et al., 2008). This is indirect evidence on dependence of photochemical activity in microalgae cells on salt. Enhanced salinity in photosynthetic parameters, osmotic and leaf water potential, transpiration rate, leaf temperature and relative leaf water content (Demetriou et al., 2007). Salinity also affects photosynthetic components such as enzymes, chlorophylls and carotenoids (Amirjani, 2011). The reduction in photosynthesis with increasing salinity has been attributed to: (a) stomatal closure, which leads to a reduction in intracellular

CO_2 partial pressure; (b) non-stomatal factors (Saravanavel et al., 2011) as the reduction in protein concentration (Ayala-Astorga and Alcaraz-Meléndez, 2010); (c) decline in photosynthetic pigments (Shah et al., 2017); and (d) changes in ionic concentrations (Demetriou et al., 2007). NaCl stress damaged the oxygen evolving complex (OEC) and the PSII (photosystem II) reaction centre and subsequently suppressed photosynthetic electron transport at the donor and receptor sides of the PSII reaction centre and influencing the absorption, transfer and application of light energy (Gururani et al., 2015; Ji et al., 2018). Salinity stress decreases the PSII activity (Lu and Vonshak, 2002) and inhibits the quantum yield of PSII electron transport (Xia et al., 2004). Misra et al. (2001) and has reported that NaCl affects PSII efficiency by slowing down primary charge separation in PSII and pigment-protein complexes of thylakoid membranes. The inhibitory target site of high salt concentration in PSII seems to vary with species (Demetriou et al., 2007). Allakhverdiev et al. (2000) analysed Chl fluorescence in salt-stressed cyano-bacterium *Synechococcus* sp., and suggested that the photochemical reaction centre complex, including QA, pheophytine, and the P680, was undamaged in NaCl-treated cells. NaCl interfered with the PSII mediated transport of electrons from water to DCIP, but not from DPC to DCIP (Demetriou et al., 2007). NaCl triggers the accumulation of ROS in algae which reduces photosynthetic efficiency due to peroxidation of thylakoid lipids and degradation of the PSII complex (Sharma et al., 2012). Increased Na^+ level causes deterioration in chloroplast structure and an associated lose in chlorophyll and photosynthetic activity (Khosravinejad et al., 2008; Sharma et al., 2012).

Algae are grouped as halophilic or halotolerant based on their adaptability potential and concentration of salt (Das Sarma and Das Sarma, 2001). In either case, algae produce some metabolites to protect themselves from salt injury and osmotic stress (Farghl et al., 2015). Microalgae can acclimate to high salt concentration by slowing the rate of cell division slowed with the gradual increase in the salt concentration (Sharma et al., 2012). To protect cells from damage caused by ROS, plant and algae have several innate defense systems such as antioxidative enzymes (Chokshi et al., 2015). Many adaptive strategies applied by algae in response to salinity induced stress includes (i) production of antioxidative enzymes like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) (ii) accumulation of low molecular weight antioxidants like ascorbate, glutathione, polyphenols; (iii) production of compatible solutes like proline, glycine betaine to facilitate osmotic adjustment (Hamid and Sibi, 2018).

A delicate balance between ROS generation and ROS scavenging system is disturbed by the different types of stress factors like salinity, drought, extreme temperatures, heavy metals, high solar irradiance etc (Fig. 2.1) (Das and Roy Choudhury, 2014). Antioxidants are helpful in quenching of free radical reactions and also play crucial role in mitigation of cellular damage (Lobo et al., 2010). Algal antioxidants are of very different nature such as carotenoids and vitamin E (α-tocopherol) is fat-soluble fraction, whereas vitamins, phycobili-proteins and polyphenols are the powerful water-soluble antioxidants (Sharma et al., 2017). The antioxidants can also be categorized according to their size viz., small molecule antioxidants and large molecule antioxidants. The small molecule antioxidants neutralize the ROS in a process called radical scavenging and the main antioxidants in this category are vitamin C, vitamin E, carotenoids and glutathione (GSH) (Kurutas, 2015). The large-molecule antioxidants are enzymes such as SOD, CAT, and GR (Nimse and Pal, 2015).

The integral defence mechanism of the cell includes an array of enzymatic scavengers such as SOD, CAT, APX, GR and non-enzymatic antioxidant viz., carotenoids, proline, polyphenols, cysteine, flavonoids, etc. (Chokshi, et al., 2017). The enzymes of oxidative stress play an important role to protect algae and maintain the redox state of the cell (Kneeshaw et al., 2017). SOD provides the first line of defence by catalysing the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2 (Ighodaro and Akinloye, 2017). Superoxide dismutase (SOD), eliminate $O_2^{\bullet-}$ via catalyzing its dismutation, one $O_2^{\bullet-}$ being reduced to H_2O_2 and another oxidized to O_2 (Fig. 2.1). It removes $O_2^{\bullet-}$and hence declines the risk of $^{\cdot}OH$ formation by the metal-catalyzed Haber Weiss type reaction. This reaction has about ten thousand fold faster rate of reaction than natural dismutation. SODs are categorized by their metal co-factors into three categories; the manganese (Mn-SOD), copper/zinc (Cu/Zn-SOD), and the iron (Fe-SOD), which is localized in diverse cellular sections (Kumar et al., 2016). The sub-cellular distribution of these isozymes is also distinctive. The Mn-SOD occurs in the mitochondria and peroxisomes cells; some Cu/Zn-SOD isozymes are originated in the cytosolic fractions and also in chloroplasts of algae and plants (Sandalio et al., 1987). The presence of several SODs may result from the fact that the cells of algae are divided into two compartments by internal membranes. Since, $O_2^{\bullet-}$ radicles are negatively charged and cannot cross plasma membrane readily as they are efficiently trapped within the compartment where they are produced. So, this may have resulted into development of multiple SODs in compartmentalized cells.

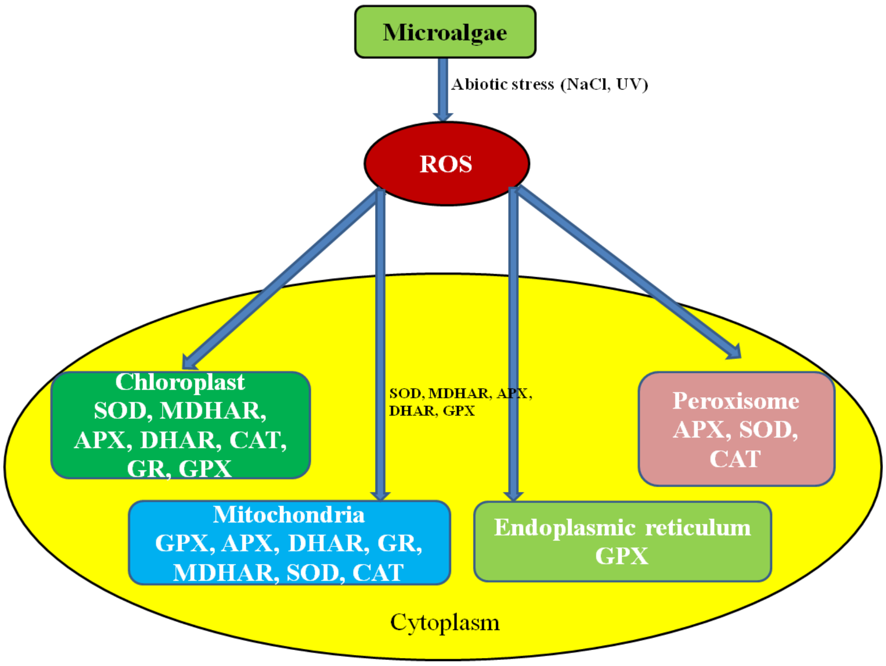


Fig. 2.1: Defence enzyme involved against ROS production under abiotic stress in algae

The antioxidant enzymes, CAT and APX convert H_2O_2 to H_2O and O_2 (Gill and Tuteja, 2010). Glutathione redutase (GR) is a flavo-protein and works as oxidoreductase, to initiate the GSH oxidation in both algae and cyanobacteria (Fig. 2.1). It is a potential enzyme of the ascorbate-glutathione cycle and plays a vital role in defense system against ROS by sustaining the reduced status of GSH. It is localized mainly in chloroplasts, but small quantity of this enzyme has also been found in cytosol and mitochondria. GR catalyses the reduction of glutathione (GSSG), a molecule involved in various metabolic antioxidative and regulatory processes in algae where GR catalyzes the NADPH dependent reduction of disulphide bond of oxidized glutathione (GSSG) and is thus significant for maintaining the GSH pool (Kumar et al., 2014). Essentially, GSSG consists of two GSH linked via disulphide bridge which can be transformed back to GSH through GR. GR and GSH play a vital role in defining the tolerance of algae under various abiotic stresses. Proline acts as a free radical scavenger and provides tolerance towards salinity stress (Zouari et al., 2016). Proline is known to accumulate under various adverse environmental conditions like nitrogen starvation (Pancha et al., 2014), salt, heavy metals and temperature etc. (Chokshi et al., 2015). Proline also helps in maintaining the cytosolic pH and stabilization of subcellular structures. They work as a compatible solute and ROS scavenging agent (Upadhyay et al., 2014). Proline content in microalgae under salinity indicates its adaptive response towards salinity (Dawood et al., 2014; Pancha et al., 2015). According to Hong et al. (2000) increased resistance to oxidative stress is due to accumulation of proline and other metabolite. It is likely that proline accumulation may be one of the major mechanisms of salinity tolerance by the microalgae. Tuna et al. (2008) suggested that proline accumulation in salt stressed cells is achieve by decreasing proline oxidation to glutamic acid, proline utilization and enhanced proteins turnover.

Ascorbic acid is well known antioxidants in eukaryote as well as in prokaryotes. It has been reported that ascorbic acids mostly available in reduced form in chloroplast and leaves under normal psychological conditions (Bowler et al., 1992). In plants and algae, mitochondria play a central role in the metabolism of ascorbate. Algal mitochondria are not only synthesizing ascorbate from L-galactone-g-lactone dehydrogenase, but also take part in the regeneration of ascorbate from its oxidized forms (Bowler et al., 1992). The regeneration of ascorbate is very significant because completely oxidized dehydroascorbate has a small half-life and would be lost unless it is reduced back.

Ascorbate is measured as the most powerful ROS scavenger because of its capacity to donate electrons in a number of enzymatic and non-enzymatic reactions. It provides defence to membranes through directly scavenging of $O_2^{\bullet-}$ and $^{\cdot}OH$ and by regenerating α-tocopherol from tocoperoxyl radical. During the stress condition, ascorbate oxidized to MDHA (monodehydroascorbate). MDHA have significant role in ROS scavenging due to resonance stabilization, MDHA scavenges the radical by reacting with oxygen radicals such as superoxide and help SOD enzymes in removal of superoxide (Smirnoff, 2018). MDHA formed in the reaction further converted into DHA with the help of enzyme completing the ascorbate cycle operating inside the cell.

2.3 Microalgae and lipid production

Lipids are produced in all living organisms and play structural and functional roles such as formation of cell membranes, carbon and energy storage (Escribá, 2017) in algae and plants. However, only a few other microorganisms including yeasts, molds and algae can accumulate microbial lipids about 20% of their dry cell weight (DCW) (Donot et al., 2014). Microalgae are emerging alternative source for the lipid production and various other bioactive compounds (Laoteng et al., 2011). High lipid accumulation and biomass productivity are the two main desired phenotypes in algae preferred for biodiesel production (Yilancioglu et al., 2014). Increase of reactive oxygen species under varied environmental conditions is helpful in increasing the accumulation of lipid content in the microalgae **(**Li et al., 2010**)**. The production of lipid molecules can be enhanced in economical way by using various substrates as a source of nutrients for the microalgae (Sun et al., 2018). Microalgae are able to accumulate cellular lipids in the range of 25-70% of DCW under different environmental stresses such as high light stress (UV-B), salt stress and deprivation of nutrients (Donot et al., 2014; Fan et al., 2014). Microalgae mainly accumulate neutral lipids (~90% of their lipid storage) (Donot et al., 2014). Lipid accumulation in the microalgae occurs in two stages until all elements required for culture growth are available in medium, the cells of microalgae divide rapidly and synthesize mainly membrane lipids and chloroplast lipids (Solovchenko, 2012). When any growth factor becomes limiting but photosynthetic carbon dioxide fixation continues, that process called lipogenic phase onsets characterized by slowdown or cessation of cell division, frequently by the reduction of the photosynthetic apparatus and by accumulation of neutral lipids (Solovchenko, 2012). In addition to their traditional and most-studied function in carbon and energy storage these lipids and especially PUFAs may act as antioxidants or otherwise protective defence molecules under stress response (Hu et al., 2008). Satpati et al. (2016) reported that a growth medium with high salinity increased microalgae cellular content by improving the formation of TAG. Microalgal strains under salinity stress produce different physiological mechanism such as uptake, efflux and differential retention of sodium ion inside and outside of the cell and cellular membrane fluidity which leads to change in the fatty acid composition (Srivastava et al., 2013). In the presence of salt, microalgae system enhances the intracellular lipid that acts as a storage energy material till the favourable conditions are attained (Mohan and Devi, 2014). The increase of cellular lipids can also be accounted as a defence mechanism towards the cell injury caused by osmotic stress (Sharma et al., 2012). The generation of low levels of ROS by environmental stresses initiates adaptive responses, including lipid biosynthesis and accumulation (Singh et al., 2018). In contrast, high levels of ROS may cause cellular damage, leading to the consumption of stored lipids as energy source in order to maintain cellular homeostasis (Singh et al., 2018). Microalgae are capable of de novo PUFA (poly unsaturated fatty acid) synthesis and hence represent a potentially vast and untapped resource of these fatty acids and could be used in the treatment of various diseases and as a source of health supplements (Robertson et al., 2013). PUFA play a significant role in inflammatory disorders, obesity, Type 2 diabetes mellitus, rheumatoid arthritis, neuro inflammatory processes and cardiovascular health (Robertson et al., 2013). Thus, growing algae in devastating salt affected arable lands are crucial and one of the best practices for economic success of the world via biomass utilization in production of biofuel and other value added products in a sustainable manner.

2.4 Ultraviolet radiation (UVR)

UV radiation was first time discovered by J.W. Ritter, a German physicist, in 1801. The natural source of UVR is the sun, while anthropogenic sources include welding instruments and UV lamps etc. (Balk, 2011). Some important anthropogenic sources are enlisted below:

2.4.1 Sunbeds: Sunbeds are important source of UV rays. Sunbeds, sunlamps and tanning booths give out the harmful radiation which increases the risk of skin cancer.

2.4.2 Medical exposure: In medical field particularly therapeutic diagnostics, UV lights is used as a good source of UVR.

2.4.3 Industrial/commercial exposures: Welding is one of the most important sources of UV radiation and the levels of UVR are very high. Several industries have been reported to use UV lamp, fluorescence lamps for their daily need and they serve as good source of UVR.

2.4.4 Lighting: Halogen lamps, made of tungsten, are used in the workplace and in the home for a variety of lighting and display purposes. Black lights, which emit mainly UVA, are commonly used for special effects and also for the authentication of bank notes and documents.

2.5 Classification of UVR

On the basis of intensity and wavelength UV radiation is of three types (Fig. 2.2).

2.5.1 UV-A (315-400 nm)

It ranges between 315 to 400 nm. The earth receives highest percentage of UV-A (95%) radiation. Since they are less absorbed by the stratospheric ozone layer, they can easily reach to the earth surface.

2.5.2 UV-B (280-315 nm)

It ranges between 280-315 nm of solar UV radiation and it is strongly absorbed by the stratospheric ozone layer. After reaching to the earth's surface, it may cause sunburns, snow blindness, immune reductions and a variety of skin problems, including premature aging of skin and cancer (Balk, 2011). In plants, it induces many physiological, morphological and molecular changes, such as leaf structure alteration and DNA damage (Nawkar et al., 2013).

2.5.3 UV-C (100-280 nm)

It ranges from 100-280 nm of solar UVR. It is mostly absorbed by the stratospheric ozone layer and thus, the levels of UV-C radiation reaching the earth's surface are very small.

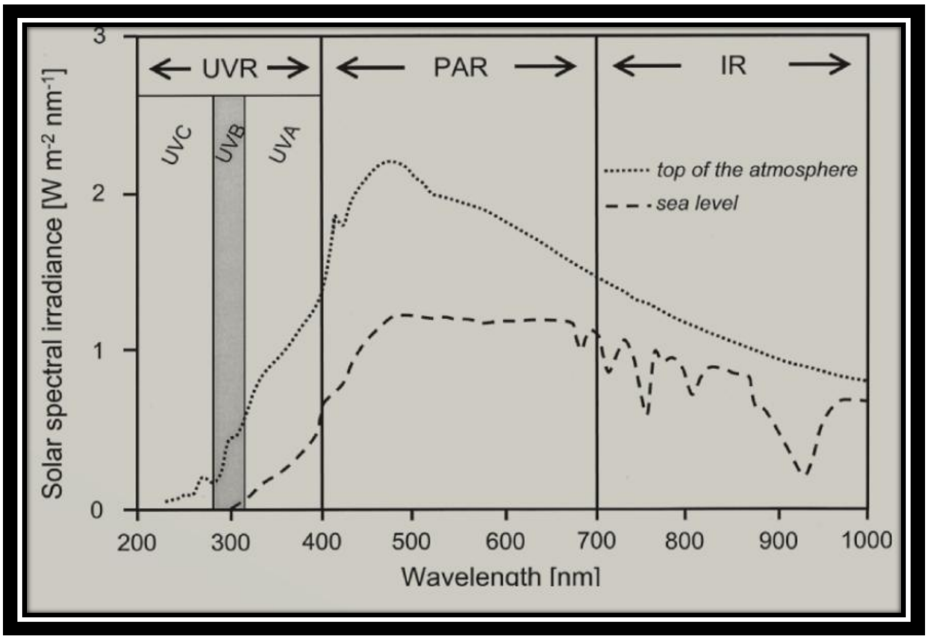


Fig. 2.2: Ranges of different solar radiation at different intensity level

2.6 Factors affecting UVR

- Temperature and sunlight
- Latitude and altitude
- Cloud cover
- Altitude
- Stratospheric ozone

2.7 UV-B and temperature

Temperature is a key factor regulating growth, development and physiology of algae (Juneja et al., 2013). Number of studies, reports a compensatory effect on enhanced temperature and UV-B-mediated inhibition on plant growth and physiology. Mark and Tevini (1996) showed that an increase in temperature (28-32°C) resulted in higher values of absolute growth parameters regardless of UV-B treatment in sunflower (*Helianthus annuus*) and maize (*Zea mays*). On the other hand, pre-exposition to low and ambient doses of UV-B promoted heat tolerance in conifer seedlings (L’Hirondelle and Binder, 2005) and cucumber (Teklemariam and Blake, 2003; Grinnan et al., 2013). A temperature-dependent change in primary and secondary plant metabolites also develop the negative effects of enhanced temperature (20-25°C) on *Pierisnapi* larvae and development on *Sinapis alba* as reported by Bauerfeind and Fischer (2013). In addition to phenolic acids, tannins have been described to be harmfully affected by the interaction between UV-B and temperature. For example, in *Populus tremula* seedlings, Randriamanana et al. (2015) studied a genotype-specific decrease of soluble reduced tannins when both UV-B and temperature were increased.

2.8 UV-B and photosynthetically active radiation

Several observations have revealed the role of photosynthetically active radiation (PAR) (400-700 nm) in the modulation of plant sensitivity and photomorphogenic responses to UV-B radiation and vice versa. Direct mechanism viz., increased photorepair, photoreactivation and levels of photoprotective compounds, as well as indirect mechanisms have been postulated to clarify the UV protective effects of high PAR light conditions (Bolink et al., 2001; Krizek, 2004; Hoffmann et al., 2015). The recent researches evidence the PAR and UV-B might have a synergistic effect on plant photoprotection. A synergistic effect in the production of different metabolites and chemical substances when both PAR and UV-B irradiance is applied to plants as reported by various authors (Götz et al., 2010; Guidi et al., 2011; Barnes et al., 2013; Müller et al., 2013; Vidović et al., 2015). This suggests a common acclimation response of plants to both light signals (Wargent et al., 2015). Vidović et al. (2015) has reported that under high intensity, UV-B stimulated photosynthesis rates in *Plectranthus coleoides* by increasing stomatal conductance, CO_2 assimilation rate and internal CO_2 concentration whereas under natural sunlight conditions, photo-inhibition (i.e., light-induced inactivation of photosystem II) in *Cucurbita pepo* has been also suggested to be caused by the UV-A, but not the UV-B component of solar radiation (Hakala-Yatkin et al., 2010). Besides, this photoinhibitory effect is suggested to be attenuated by UV-B-inducible screens, i.e., accumulation of phenolic compounds in the plant epidermis. When both PAR and UV-B irradiances are applied, a greater increase in the concentration of flavonoids is detected in old plant leaves. For instance, high PAR induced the accumulation of flavonoids in young leaves of *Hordeum vulgare* and combined treatment with UV-B increased the production of these compounds in older leaves as well (Klem et al., 2012). Similarly, higher flavonoid production in young, and older leaves of *Betula pendula* plants grown under ambient PAR and UV-B has been also reported (Morales et al., 2013).

2.9 UV-B and UV-A

Ultraviolet-A (315-400 nm) constitutes the major component of the solar UV spectrum. Krizek et al. (1997, 1998) has reported decreased stem elongation and leaf enlargement under ambient UV-A in *Cucumis sativus* and lettuce. Interestingly, UV-A can interact with UV-B to modulate plant responses. For instance, UV-A can mitigate the deleterious effects of UV-B on the photosynthetic apparatus under low PAR conditions (Adamse et al., 1994), as demonstrated in barley (Štroch et al., 2015, cluster bean (*Cyamopsis tetragonoloba*) (Joshi et al., 2007, 2013) and the woody shrub *Pimelea ligustrina* (Turnbull et al., 2013).

These results suggested a major role of UV-B on the induction of flavonoids. However, UV-A has been described to modulate the UV-B associated responses in plants. For instance, in turnip hypocotyls, while both UV-A and B induced anthocyanin biosynthesis, the pattern of anthocyanin accumulation along the hypocotyl greatly differed depending on the wavelengths of UV applied (Zhou et al., 2007; Wang et al., 2012). Also, Morales et al. (2010) described different changes in the abundance of specific flavonoids when UV-A or UV-B were depleted. Under exclusion of UV-B, young silver birch leaves accumulated less of six epidermal flavonoids (i.e., myricetin-3-galactoside, quercetin-3-galactoside, quercetin-3-rhamnoside, and kaempferol-3-rhamnoside), while UV-A exclusion decreased the accumulation of only quercetin-3-galactoside and quercetin-3-arabinopyranoside. Likewise, Wilson et al. (2001) reported that UV-A reduced the production of UV-B-inducible flavonoids in rape by shifting the abundance of particular quercetin compounds. A common regulatory component of plant responses to both types of UV was therefore proposed. In particular, Morales et al. (2013) suggested that the UV-photoreceptor UVR8 may be involved in the UV-A regulation of individual metabolites in *Arabidopsis*. This was supported by the necessary activation of UVR8 for UV-A induction of kynurenic and chlorogenic acids, tryptophan, phenylalanine, kaempferol and kaempferol-3-rhamnoside in *Arabidopsis* (Morales et al., 2013).

2.10 UV-B and blue light

Blue light (400-500 nm) regulates diverse plant processes such as phototropism, photomorphogenesis, stomatal opening, and leaf photosynthetic functioning (Whitelam and Halliday, 2008). During plant growth it constitutes an essential part of the development in higher plants (Hogewoning et al., 2010). It has been reported that blue light supplemented with red light conditions enhance the dry matter production in radish, lettuce, and spinach (*Spinacia oleracea*) (Yorio et al., 2001; Johkan et al., 2010), as well as leaf photosynthesis in pepper (Brown et al., 1995) and rice (*Oryza sativa*) (Matsuda et al., 2004).

Blue light supplementation in plants before and after UV-B exposure, can prevent the damaging effects of high UV-B radiation. For instance, blue light (i.e., 62% of PAR) increases the acclimation of pepper and cucumber plants to UV radiation under high light intensity conditions (Adamse et al., 1994; Hoffmann et al., 2015). This has been explained by a lower degradation of photosynthetic related pigments (chlorophyll a, b and carotenoids) by UV (Hoffmann et al., 2015), as well as the increase in epidermal flavonols when plants were grown under enriched blue light radiation (Adamse et al., 1994; Ebisawa et al., 2008; Son and Oh, 2013; Hoffmann et al., 2015; Ouzounis et al., 2015; Siipola et al., 2015). These observations have led to some authors to propose blue light as the major constituent of the sunlight responsible for the up-regulation of the epidermal content of flavonoids (Ouzounis et al., 2014; Siipola et al., 2015).

2.11 Effect of UV-B radiation on algae

Industrial revolution and population growth are the major contributor of environmental deterioration (Zhang et al., 2007). Anthropogenic activities directly or indirectly add to the harmful substance into the atmosphere destroying the stratospheric and ozone layer. All aquatic organisms, including microalgae, are vulnerable to UV-B, but to different extents (Sinha and Häder, 2002) (Fig. 2.3). Studies have been showed that green microalgae treated with UV-R exhibited tolerance to a high intensity of UVR, which might be due to the presence of highly effective escape mechanisms (Rastogi et al., 2014). Photosynthetic pigments of algae are more prone to UVR and they the photosynthetic pigment act as photosensitizer, and produces ROS under excess of UV-B/visible light radiation. The destruction of chlorophyll content as well as decreasing photosynthesis process, results in the overall drop in biomass productivity of the algae (Xue et al., 2005). In *Dunaliella bardawil*, UV-B radiation causes major damage to cells with respect to photobleaching by damaging the CO_2 fixation process i.e., RUBISCO (Xue et al., 2005). Lesser et al. (2002) reported that *Scenedesmus* sp. growing in Antarctica regions have developed potential to ensure their survival against UVR by replacing the damaged D1 protein or

RuBisCO, and repair of UV induced DNA damage. In another study Gorton and Vogelmann (2003) and Leya et al. (2004) reported the production of red coloured hypnoblasts as protectants from UV-B irradiation in microalga *Chlamydomonas nivalis*. In the unicellular algae *Haematococcus pluvialis*, production of excess asthaxanthin protects the chloroplasts against UVR stress (Shah et al., 2016).

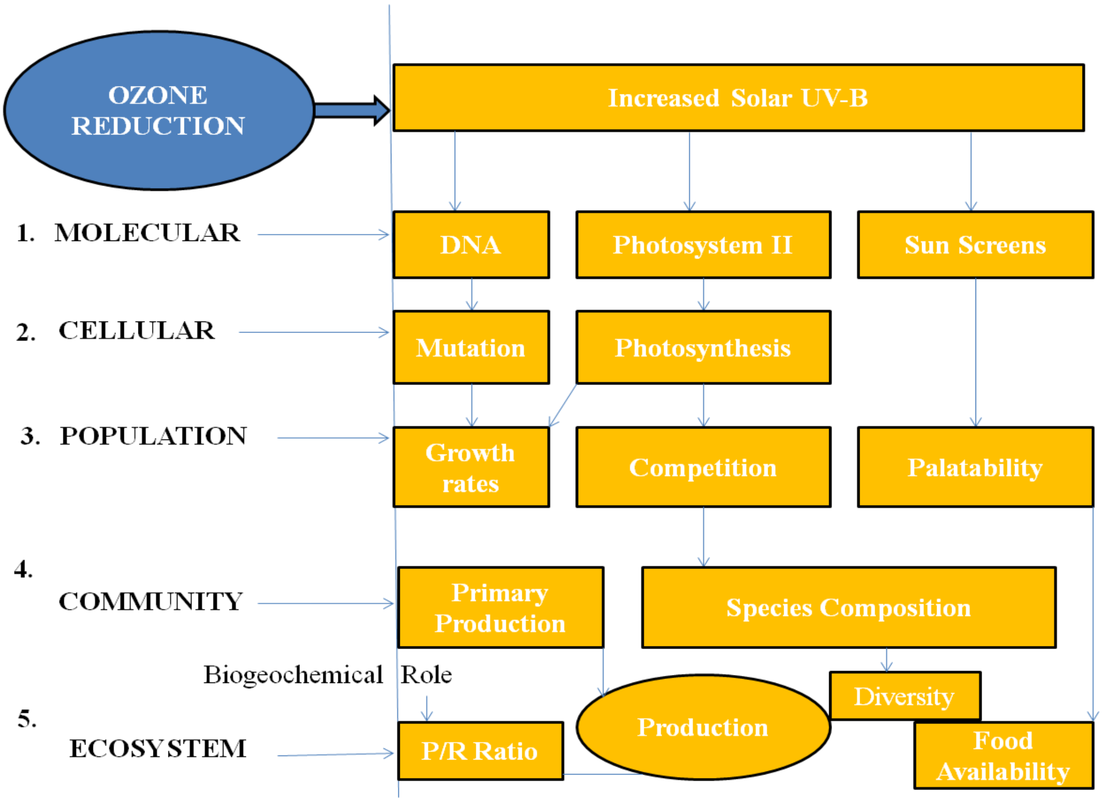


Fig. 2.3: Effect of UVR on growth, physiology and molecular structure of algae

There is a marked impact of UV-B on algal physiology as due to UV-B effect on various morphological, physiological, biochemical and molecular alteration under UV exposure (Fig. 2.3). Holzinger et al. (2006) reported that algae *Prasiola crispa* exposed to 24 h UV-B (2.0 W m^{-2}) showed high degree of dilations in the thylakoids of chloroplast, apparent reduction in the number of plastoglobuli and quantum yield (Fv/Fm).

It has been reported that low doses of UV-B induces synthesis of proteins important for maintenance and protection of photosynthetic apparatus via UVR gene signaling (Fig. 2.4) (Kataria et al., 2014). For example, the UVR8/HY5 (HYH) based UV-B signalling pathway leads to the expression of SIG5, which encodes sigma factor of plastidic RNA polymerase, involved in D_2 protein biosynthesis and ELIP1 (Early Light Inducible Protein 1), related with D1 protein of photosystem II

(Mellenthin et al., 2014). In addition, ELIP1 is induced in chloroplasts during maturation, protecting photosynthetic apparatus from photooxidative stress (Rossini et al., 2006). Musil and Wand (1994) reported stimulation of net CO_2 assimilation rates and growth in winter ephemeral *Dimorphotheca pluvialis* L. under low, ambient doses of UV-B radiation.

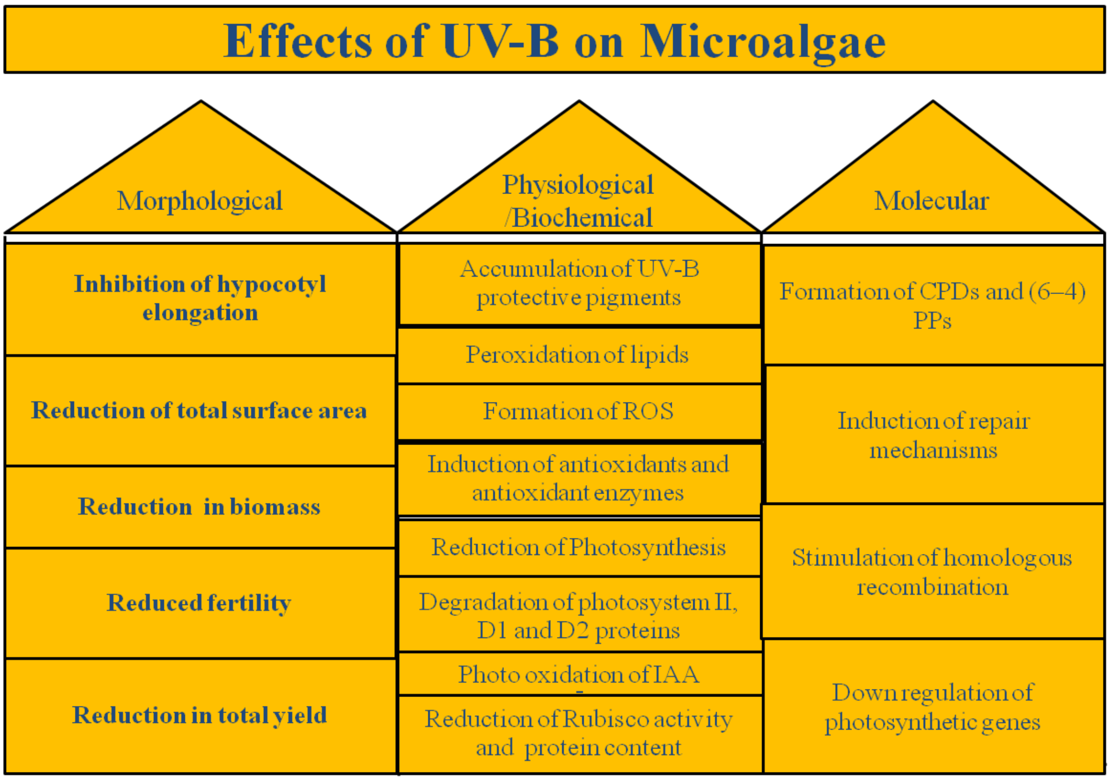


Fig. 2.4: Schematic diagram showing impact of UV-B on microalgae

2.12 Defense responses in algae

Formation of reactive oxygen species (ROS) is one of the major characteristics of plant and algae under UV-B stress (Das and Roychoudhury, 2014). A regulated balance between ROS production and destruction is essential if function and metabolic efficiency are to be maintained in both optimal and stress conditions (Poljsak et al., 2013). There is a wide range of sources of ROS generation in algae (Fig. 2.5). The electron transport chains of the mitochondria and chloroplast are two major sources of ROS production (Sharma et al., 2012). In the chloroplast, environmental stress such as UV-B and salt may limit CO_2 fixation and reduce the $NADP^+$ regeneration by the Calvin cycle (Tausz, 2004). Chloroplast is highly sensitive to UV-B radiation and extreme radiation of UV may lead to over saturation of the light reactions of photosynthesis, which eventually may cause photo-inhibitory injury to the photosynthetic apparatus (Kataria et al., 2014).

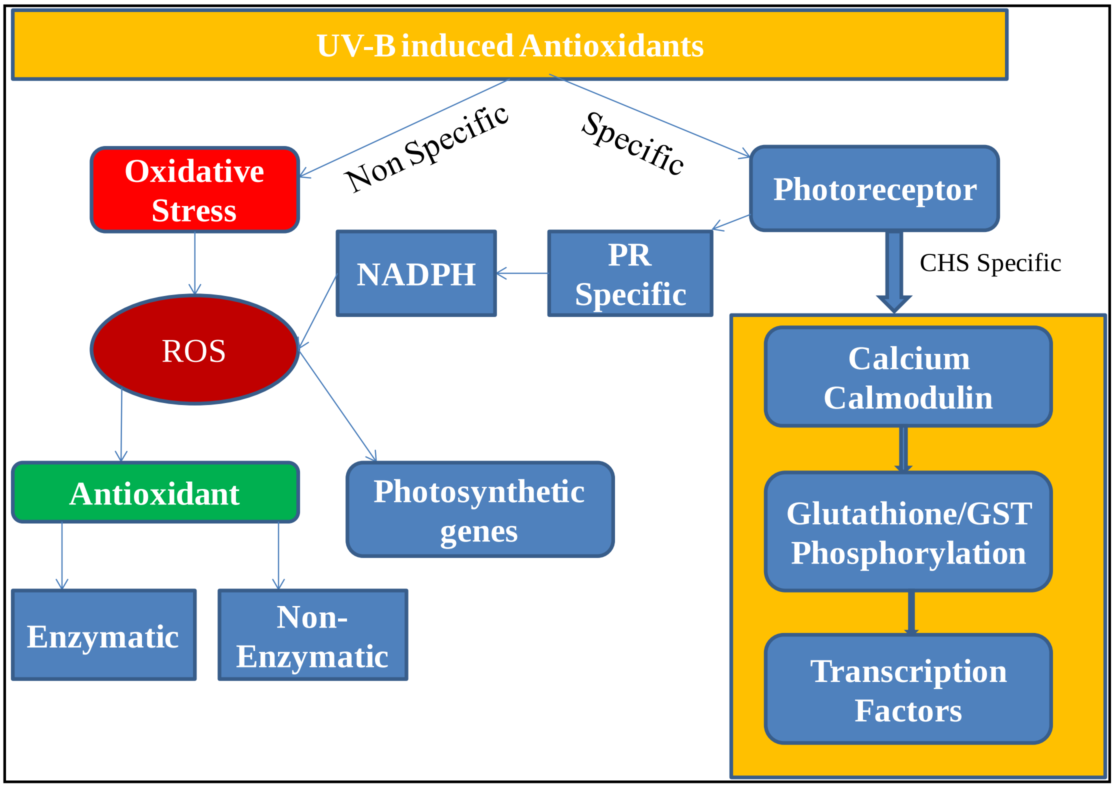


Fig. 2.5: Generalized view of UV induced toxicity and responses mechanism in the algal cells

Algae exposed to UV-B radiation accumulate H_2O_2 in cells, due to stimulated production of oxidative products and it is countered by stronger adaptive responses present inside the cells (Shiu and Lee, 2005). This suggests that the UV-B radiation induced oxidative bursts of H_2O_2 are responsible for the injury and degradation of photosystem II (PSII). It is extensively accepted that UV-B radiation damages the donor side of photosystem II by deactivating the Manganese (Mn) cluster of water oxidation (Schmidt et al., 2016). Peroxide radical is the main reactive oxygen induced by UV-B radiation in the thylakoids of chloroplast (Das and Roy Choudhury, 2014).

Effective antioxidant defense systems, including enzymes and antioxidants have been developed by algae to counteract the toxicity of ROS (Mallick and Mohn, 2000) (Fig. 2.6). Bowler et al. (1992) have concluded that UV-B radiation produces $O_2^{\bullet-}$, $^{\bullet}OH$ and H_2O_2 radicals.

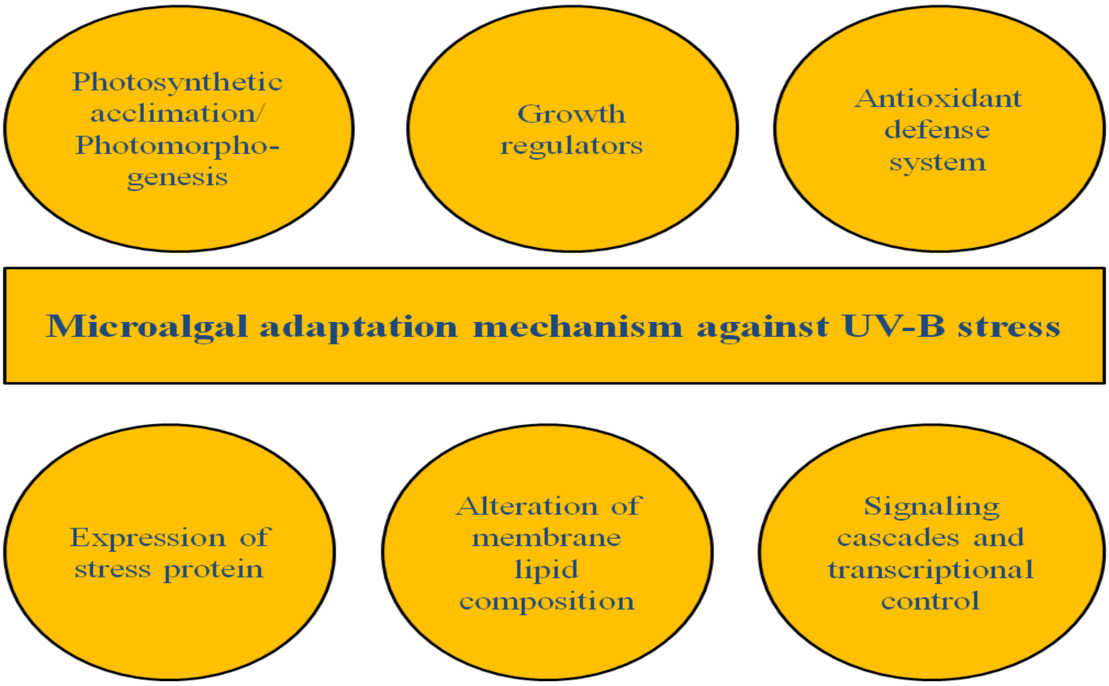


Fig. 2.6: Different adaptation mechanism in microalga under UV-B stress

CHAPTER 3

Materials and Methods

3.0 Materials and Methods

3.1 Study material

Microalgae 1. *Chlorella vulgaris* 2. *Chlorococcum humicola*

3.2. Collection of microalgae

Pure culture of green microalga *Chlorella vulgaris* (*C. vulgaris*) was obtained from algal biology lab, CSIR-NBRI Lucknow, India (26.7697°N, 80.9262°E) and *Chlorococcum humicola* (*C. humicola*) was procured from the School of biosciences, MG University, Kerala, India (co-ordination). All the algal species were grown in BG11 nutrient media in the laboratory under controlled culture condition at 24±2°C; 120 μmol photon $m^{-2}s^{-1}$ photosynthetically active radiation (PAR) with a photoperiod of 16:8 h (Singh et al., 2018b). The composition of BG-11 nutrient medium is as follows:

Table 3.1: Composition of BG-11 media and preparation stock solution

Macronutrients	**Concentration**	**Stock solution (ml)**
$NaNO_3$	1.5 g L^{-1}	**10**
KH_2PO_4	0.04 g L^{-1}	**1.0**
$MgSO_4.7H_2O$	0.075 g L^{-1}	**1.0**
$CaCl_2.2H_2O$	0.036 g L^{-1}	**1.0**
Citric acid	0.006 g L^{-1}	**1.0**
Ferric ammonium citrate	0.006 g L^{-1}	**0.5**
EDTA (disodium salt)	0.001 g L^{-1}	**0.5**
Na_2CO_3		**1.0**
Trace metals		
H_3BO_3	2.86 mg L^{-1}	**1 ml**
$MnCl_2{\cdot}4H_2O$	1.81 mg L^{-1}	
$ZnSO_4{\cdot}7H_2O$	0.222 mg L^{-1}	
$NaMoO_4{\cdot}2H_2O$	0.39 mg L^{-1}	
$CuSO_4{\cdot}5H_2O$	0.079 mg L^{-1}	
$Co(NO_3)_2{\cdot}6H_2O$	49.4 mg L^{-1}	
Stock solution (1 L)	**(16 ml macronutrient + 1 ml micro nutrient + 983 ml distilled water)**	

3.2.1 Purification and isolation process

In order to explore any algal species it may be necessary to grow this alga as a pure culture i.e., culture of a single species (uni-algal culture). Generally, three methods are adopted to purify micro algal strain and/or to produce single cells described below:

1. Agar plate method
2. Successive dilutions
3. Picking up of single cells from the original culture by using the capillary method.

3.2.2 Agar plating technique

Materials required

- Agar agar
- Plastic petri dish bag
- Bunsen burner
- Conical flask (1 L)
- Pipette
- Inoculation loop
- Para film

3.2.3 Preparation of isolation plates

For isolation, simple nutrient agar plates were used to grow the algae. The plates were filled with 20 ml nutrient media containing 1.5% (w/v) agar.

Procedure

- 15 g of nutrient media with 1.5% solid agar was taken in a conical flask containing 1 L of distilled water.
- The flask was autoclave at 125^{o}C and 15 lbs pressure for 30 min at 1 atm.
- After the cooling, the media was poured over sterilized plates and were allowed to cool and solidify. The process was carried out in a closed laminar flow to avoid contamination.

- The plates were store in plastic bags and refrigerated until needed. The agar plates were further used for the growth of the culture by using streak plate method. The procedures of streak plates are as follows:

3.2.4. Streak plates

Procedure

- 0.1 ml algal culture was placed on the plate.
- Sterilize the inoculation platinum loop by sprit lamp until it turned red.
- Sterilized inoculation loop was used to spread the algal sample by streaking onto the agar.
- Now lid of plate was put back on the isolation plate and masking tape (Parafilm) along the edge of the plate. Parafilm will prevent the agar from drying out, since it may take several days for the individual algal cells to grow and produce visible colonies.
- The inoculated petri dishes were placed in a culture room at desired environmental conditions.
- Depending on the density of the inoculum, the algal cells will grow enough to form colonies on the surface after 5-21 days
- Once algal colonies are observed, take a sample by means of a sterile platinum hook and check under the microscope.

Since different colonies on this first streak plate often tend to grow together, it is difficult to pick up monoclonal colonies of single species. Therefore, reputed attempts were made to pick pure monoclonal colony. The mono-specific pure colony was picked up with sterilized capillary and was transferred to a test tube filled with 5-10 ml of culture medium and shakes it regularly during incubation on an illuminated glass rack. After the sufficient growth, the culture was transferred to 1000 ml conical flask containing 200 ml BG-11 medium.

3.2.5 Dilution method

Materials

20 screws capped test tubes filled with 9 ml of algal culture medium.

- Laminar cabinet
- Burner
- Sterilized 1ml tip box

Procedure

Take 10 glass test tubes filled with 9 ml of nutrient medium and numbered them from 1 to 10. The test tube containing the strain to be diluted should be kept in the biological safety cabinet. Take 1 ml from the initial contaminated strain and inoculate in the test tube no. 1 and stir gently. Using a new sterile 1 ml pipette repeat the previous step by taking 1 ml inoculum from tube no. 1 and inoculate it into tube No. 2. Repeat the same procedure with the remaining tubes, each time pipetting 1 ml from the previous tube (gently stirred) into the next one; flame necks and caps them).

When cell growth reappears, check samples of the tubes under the microscope and get rid of the tubes that are still contaminated, typically the initial ones, and keep only the purified cultures, usually in the more diluted tubes. Repeat the process using the last dilutions if necessary, and in any case at least every three months to always have a safe amount of purified cultures ready at hand.

3.3 Microscopic examination

The microscopic study of pure algal cells was done by using multifunctional digital microscope (Model No. US3, U1407007) and picture were taken through preinstalled monitor.

Classification of isolated microalgae

Division	Chlorophyta	**Division**	Chlorophyta
Class	Chlorophyceae	**Class**	Trebouxiophyceae
Order	Chlorococcales	**Order**	Chlorellales
Family	Chlorococcaceae	**Family**	Chlorellaceae
Genus	*Chlorococcum*	**Genus**	*Chlorella*
Species	*humicola*	**Species**	*vulgaris*

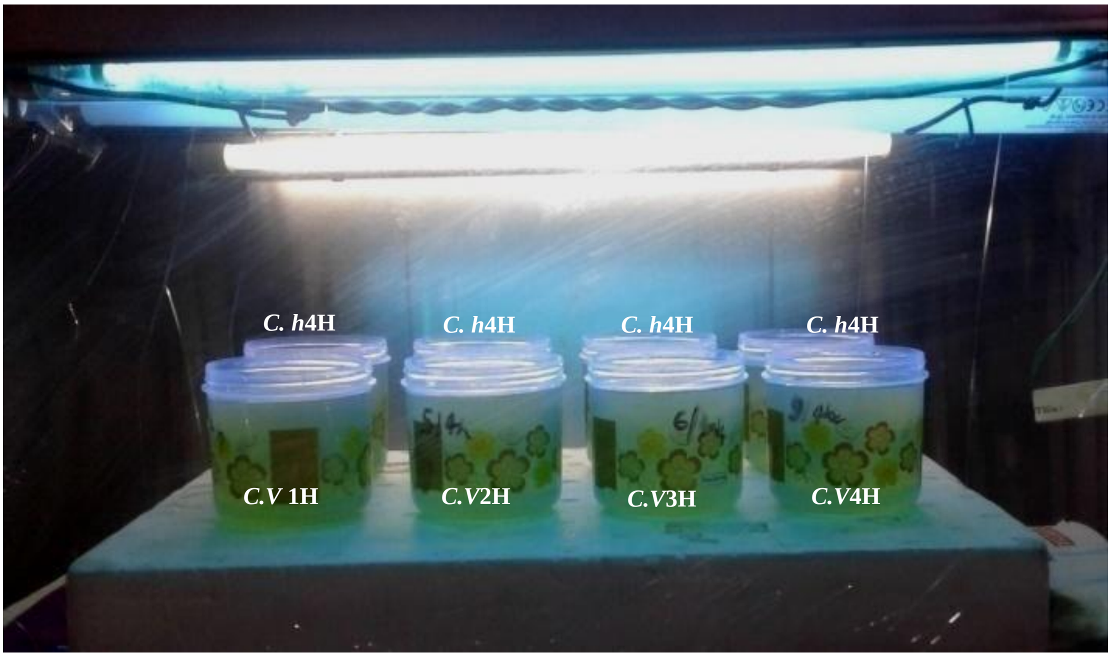
3.4. Experimental setup and designing

3.4.1 Preparation of salt solution

Salt (NaCl) solution of different concentrations (25mM, 50 mM, 100mM, 500mM and 1000mM) were prepared from10M stock solution of NaCl (NaCl 98% pure, Sigma Aldrich, USA) via dilution.

3.4.2 Treatment of algae under UV-B exposure

The UV-B exposure to microalgae *C. vulgaris* and *C. humicola* was provided by the specifically designed UV-B tubes (Philips TL; 40W) in a closed chamber with the average intensity of (15 Wm^{-2}).



CV: *Chlorella vulgaris*, CH: *Chlorococcum humicola*

Fig. 3.1: UV-B exposure of microalgae in closed laminar chamber equipped with UV-B light

3.4.3 Treatment of algae under NaCl + PAR

Pure algal seed inoculums (approx 5-10 ml) of *C. humicola* and *C. vulgaris* were used to inoculate separately in 1 L Erlenmeyer flasks containing 200 ml BG-11 medium. The inoculated cultures were transferred to controlled growth chamber filled with fluorescent tubes at 25±1°C. Different concentration of NaCl salt (25, 50, 100, 500 and 1000 mM) was added separately to Erlenmeyer flasks. Prior the inoculation, a control set devoid of salt was also maintained by using BG-11 media only.

3.4.4 Treatment of algae under UV-B exposure + PAR

Pure algal cultures of *C. humicola* and *C. vulgaris* were inoculated in 1000 ml Erlenmeyer flasks containing 200 ml BG-11 media for growth and acclimatization in the culture room. The inoculated cultures were left for 12 h for growth and acclimatization under the controlled conditions. After 72 h of growth, artificial exposure of UV-B radiations was applied to microalgal culture for different duration of ½ h, 1 h, 2 h, 3 h and 4 h with interval of 24 h interval for a period of 15 d. The exposure of UV-B to microalgae was provided by transferring the cell suspension in a sterile wide mouth PVC container. The closed laminar chamber was equipped with three UV-B tubes (Philips TL; 40W) with a wavelength ranges from 280 to 320 nm. After the UV-B exposure, the treated algal cultures were transferred to PAR light in the culture room. A control set of microalgae (without treatment UV-B) was also set prepared and placed under PAR light only. All the experiments were performed in triplicate. The culture were harvested at the end of exponential phase of the growth (15 d) and the collected samples of microalgae were refrigerated and stored at 4^{o}C for physiological and biochemical analysis. All the experiments were set in random block designing (RBD).

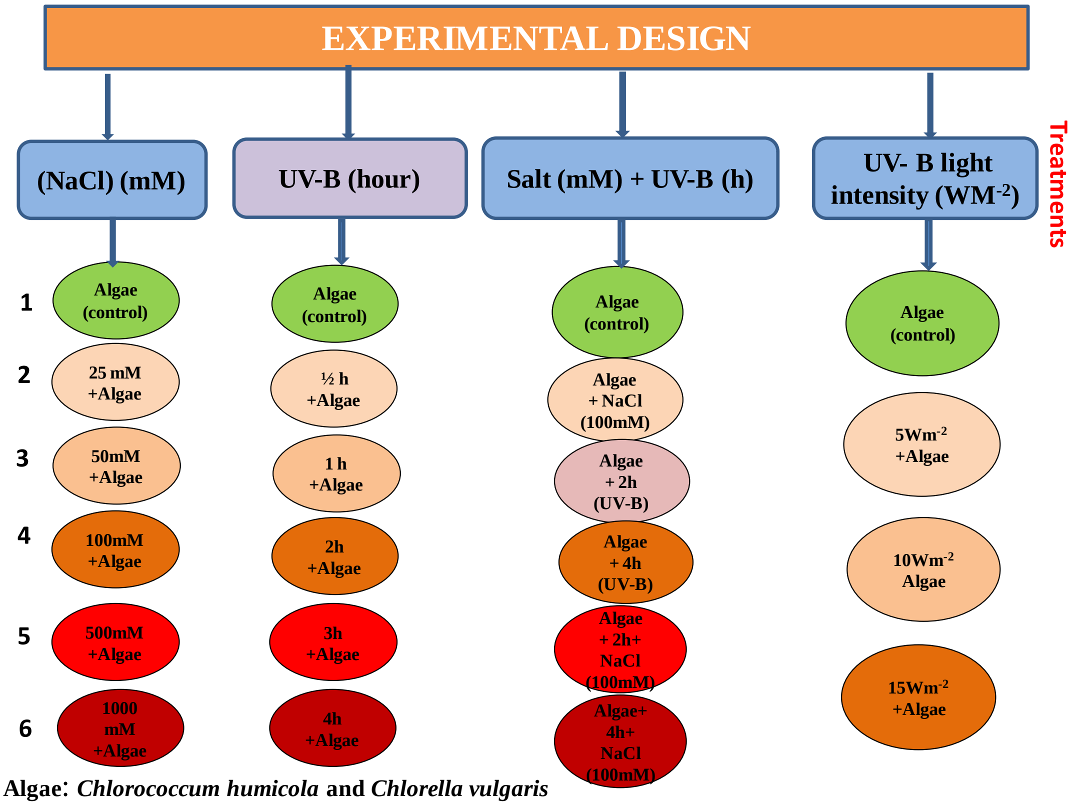
3.4.5 Treatment of algae under NaCl (100mM) + UV-B +PAR

Pure algal cultures (approx. 5-10% w/v, OD-0.075) of *C. humicola* and *C. vulgaris* were inoculated in 1000 ml Erlenmeyer flasks containing 250 ml BG-11 media. The inoculated cultures were stored for growth and acclimatization for 24 h under simulated growth condition (28±2°C under PAR of 150 μmol photon $m^{-2}s^{-1}$ and photoperiod of 16:8 h (light: dark). After 72 h of growth, fixed amount of NaCl (100 mM) (Sigma-Aldrich, ACS reagent, >99.0% purity) and UV-B (15 Wm^{-2}) exposure of 2 h and 4 h per day was provided to both microalgae separately. The UV-B radiation was artificially provided with the help of three Philips TL UV-B tubes (40W) with a spectral range of 280 to 315 nm. After the exposure of UV-B, i.e. 2 h and 4 h, algal cultures were shifted in a growth chamber. Two type of control sets were used (1) untreated control, (2) UV-B devoid. The algal culture was maintained using BG-11 media.

3.4.6 UV-B treatment of microalgae

Pure algal cultures (approx. 5-10% w/v, OD-0.070) of *C. humicola* and *C. vulgaris* were inoculated in 1000 ml Erlenmeyer flasks containing 250 ml BG-11 media for the growth and acclimatization in culture room. After 72 h of the growth, artificial exposure of UV-B radiation of intensities 5, 10, 15 Wm^{-2} were provided with 24 h interval for a period of 15 days (saturation phase) in a closed UV-B chamber. The closed chamber was equipped with three UV-B tubes (Philips TL;

40W) with a wavelength ranges from 280 to 320 nm covered with glass. Different intensity of UV-B was ensured by changing the distance between culture and UV-B source. The UV-B intensity was measure with the help of the UV-B Solar meter (Germany). The exposure of UV-B to both microalgal strains was provided by transferring the culture on a sterile PVC container with wide mouth. After the exposure to UV-B, the treated algal cells were transferred in control PAR light in culture room. The cells without UV-B exposure were also exposed to PAR light and they were treated as control set.



Algae: ***Chlorococcum humicola*** **and** ***Chlorella vulgaris***

Fig. 3.2: Experimental designing showing different treatment condition

3.5 Sterilization

All the glassware and nutrient medium were sterilized in an autoclave at 15lb inch^{-2} and 121^{o}C for 15 min, while the inoculation loop and trays were surface sterilized with the help of absolute alcohol.

3.6 Harvesting and collection of biomass

All the experiments setup was performed in triplicates. The harvesting was done at the end of exponential phase of the growth (15 d after NaCl and UV-B exposure). After centrifugation, the microalgal cell samples were refrigerated for further physiological and biochemical analysis. The whole experiment was set according to random block designing (RBD).

3.7 Growth measurements and biochemical analysis

3.7.1 Analysis of growth in algae

Growth of microalgae was measured in terms of optical density (OD) (650nm) at every day with the help of UV-visible spectrophotometer (UV-160, Shimadzu, Japan). The growth optimization and toxicity test was performed by measuring the lethal concentration (LC 50) of NaCl and UV-B.

3.7.2 Measurement of LC-50

The LC-50 was the dose of NaCl or UV-B inhibiting microalgal growth by 50%.

3.7.3 Biomass productivity

The biomass productivity of algae *C. vulgaris* and *C. humicola* during the exponential growth phase was calculated by the following formula.

$$BP = (B_2 - B_1) / (t_2 - t_1)$$

Where, BP is the biomass productivity expressed in mg $L^{-1}d^{-1}$ and B_1 and B_2 are biomass (mg L^{-1}) harvested from the two sampling points t_2 (at the end of exponential growth phase) and t_1 (at the start of exponential growth phase), respectively.

3.7.4 Specific growth rate

Specific growth rate was calculated using the equation given by (Wong and Cheng, 1991).

$$\text{Specific growth rate (k)} = \ln X_1 - \ln X_2 / T_1 - T_0$$

Where, X_0 = Initial absorbance; at time T_0 and X_2 = absorption at time T_1

3.7.5 Percentage inhibition

Percentage inhibition (PI) of the algae was determined by the formula given below:

$$PI = C_0 - Ct / C_0 \times 100$$

Where, C_0 and Ct was cell density of microalgae at time t_0 and t_1, during the growth.

3.8 Estimation of physiological parameters (photosynthetic performance) of the algae

The physiological studies of microalgae were conducted by measuring transient chlorophyll fluorescence induction in microalgae cell. The photosynthetic parameters were measured by pulse modulated Photon Systems Instruments (Aqua Pen-C portable fluorometer, Czech Republic)chlorophyll-a fluorescence induction (OJIP) curve was used for calculation of photosynthetic performance index (Pi_{ABS}), maximum quantum yield (Fv/Fm), photochemical quenching (PQ), Non-photochemical quenching (NPQ), Net closing rate of reaction centre (Mo), Trapping flux (TRo/RC), effective antenna size expressed as absorbance per reaction centre (ABS/RC) and % inhibition of RC (reaction centre) in microalgae *C. humicola* and *C. vulgaris* under different experimental conditions were examined using Photon Systems Instruments (Aqua Pen-C portable fluorometer, Czech Republic). In addition, percent inhibition of RC was also calculated using formula mention below:

$$\text{Percent inhibition of RC} = \text{ABS/RC}_{\text{control}}/\text{ABS/RC}_{\text{treated}}$$

The Aqua Pen-C was set to maximum pulse intensity of 3000 μmol photon m^{-2-1} of red light for 10s, while rapid light/dark induction adopted curves for Chl a irradiance levels ranging from 0 to 480 μmol photon m^{-2} s^{-1}. The microalgae cells were adapted to dark (10 to 15min) at room temperature prior to analysis.

3.9 Biochemical characterization

3.9.1 Estimation of pigments

Chlorophyll content in algal cells was analyzed by the method of Arnon (1949). 100 mg of algal sample were crushed in 3 ml 80% acetone (v/v) with the help of pestle and mortar under dark cold conditions and centrifuged at 8000×g for 10 min. The supernatants were used for the estimation of chlorophyll at the wavelength of 663 and 645 nm. The carotenoid content was determined at the wavelength 480 nm and 510 nm using the formula given by Duxbury and Yentsuch (1956). The estimation of photosynthetic pigment was calculated by the formula given below:

$$\text{Chlorophyll a (mg g}^{-1}\text{fw)} = 12.7(A_{663})-2.63(A_{645})\times v/1000\times w$$

$$\text{Chlorophyll b (mg g}^{-1}\text{fw)} = 22.9(A_{645})-4.68(A_{663})\times v/1000\times w$$

$$\text{Total chlorophyll} = 20.2(A_{645})+8.02(A_{663})\times v/1000\times w$$

$$\text{Cartenoid (mg g}^{-1}\text{fw)} = 7.6(A_{480})+1.94(A_{510})\times v/1000\times w$$

Where, v= final extract volume (ml); w = wt. in gram

3.9.2 Protein estimation

The concentration of protein in algal cells was estimated by method of Lowry et al. (1951), modified by Herbert et al. (1971). The Bovine Serum Albumin (BSA) as was used for preparation of standard curve.

Principle:

The peptide nitrogen reacts with the cupric oxide or copper (II) ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteauy phosphomolybdic phosphotungstic acid to hetero polymolybdenum blue [Mo(V) or Mo(VI)], by the copper-catalyzed oxidation of aromatic acids. The Lowry method is sensitive to pH changes and so the pH of test solution should be maintained at 10-10.5.

Reagents

A. 2% Na_2CO_3 in 0.1 N NaOH

B. 1% Sodium potassium tart rate in H_2O

C. 0.5% $CuSO_4.5\ H_2O$ in distilled water

D. Reagent I: 48 ml of A + 1 ml of B +1 ml C were mixed for preparation of reagent D.

E. Reagent II- 1 part Folin-Phenol [2 N] + 1 part water

BSA Standard - 1 mg ml^{-1}

Procedure

0.2 ml of BSA standard in 5 test tubes and make up to 1ml using distilled water.

The test tube with 1 ml distilled water serves as blank.

Add 4.5 ml of Reagent I and incubate for 10 minutes.

After incubation add 0.5 ml of reagent II and incubate for 30 minutes.

Measure the absorbance at 660 nm and plot the standard graph.

Estimate the amount of protein present in the given sample from the standard graph.

Standard preparation

Concentrations (mg L^{-1})	**Absorbance**
10	0.1020
20	0.1193
30	0.1307
40	0.1451
50	0.1823
100	0.3013
150	0.3856

$y = 0.002x + 0.073$

$R^2 = 0.991$

3.9.3Estimation of lipid

Lipid content in algal cells was measured by the method of Chen et al. (2009).

Reagents

Vanillin	0.06 gm
Alcohol (Absolute form)	1 ml
Orthophosphoric acid	40 ml
Distilled water	9 ml

Procedure

For the lipid estimation, 0.5 ml of microalgae sample was mixed with 2 ml conc. H_2SO_4. The samples were further boiled for 10 min and allowed to cool for 5 min. After cooling, 5 ml of reagent was added and incubated at 37^{o}C for 20 min. Absorbance of sample after incubation was taken at 530 nm using spectrophotometer.

3.9.4 Estimation of carbohydrate

Reagents

Ethanol (80%)

1.0% phenol solution

Concentrated H_2SO_4

Procedure

25 mg fresh algal cells were homogenized in 3 ml of 80% ethanol and centrifuged at 3000×g for 10 min. Add 1.0 ml of 1.0% phenol solution containing 5.0 ml of concentrated H_2SO_4 in 1.0 ml supernatant and mixed thoroughly. The mixture was kept at room temperature for 30 min and the absorption was recorded at the wavelength of 480nm. Total lipid of algal pellets was estimated by using the vanillin method as described by Ben-Amotz et al. (1985).

3.10. Toxicity analysis

3.10.1 Estimation of thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation in terms of thiobarbituric acid reactive substance was determined in microalgae. MDA is a product of polyunsaturated fatty acid peroxidation formed after reaction with thiobarbituric acid (TBA). MDA concentration was estimated by the method of Heath and Packer (1968).

Reagents

Phosphate buffer (pH 7.0)	50 mM
2-Thiobarbituric acid (TBA)	0.5 % (w/v) in 0.2 N HCl
Trichloroacetic acid (TCA)	20% (w/v) in TBA solution
Trichloroacetic acid (TCA)	5% (w/v) in dw

Procedure

3 ml microalgae were homogenized with 2ml 5% (w/v) TCA. The resulting homogenate was centrifuged at 10,000×g for 10 min. To an aliquot of 0.5 ml of supernatant, 2 ml of 20% TCA containing 0.5% TBA was added. The mixture was heated at 90^{o}C for 20 min, quickly cooled in ice-bath followed by centrifugation. Absorbance of the supernatant was recorded at 352 nm and 600 nm. The value for non-specific absorption of each sample at 600 nm was subtracted from absorption recorded at 532 nm. MDA concentration was calculated using the extinction coefficient 155 mM^{-1} cm^{-1}.

3.10.2 Estimation of electrolyte leakage

Fresh microalga samples (200 mg) were placed in test tubes containing 10ml of double distilled water. The tubes were incubated in water bath at 32^{o}C for 2 h and initial electrical conductivity of the sample (EC_1) was measured with the help of conductivity meter (Hanna Instruments). After that samples were autoclaved at 121^{o}C for 20 minutes to release all electrolytes and cooled to 25^{o}C and final electrical conductivity (EC_2) was measured using following formula:

$$Eh = EC_1/EC_2 \times 100$$

3.10.3 Estimation of hydrogen peroxide

The concentration of hydrogen peroxide (H_2O_2) in microalgae was estimated by following the method of Velikova et al. (2000).

Reagents

Trichloroacetic acid (TCA)	0.1% (w/v)
Potassium phosphate buffer (pH 7.0)	10mM
Potassium iodide	1 M

Procedure

3 ml Microalgae cells were homogenized in 3ml of 0.1% TCA (w/v). The homogenate was centrifuged at 10000×g for 15 min. Add 10 mM potassium phosphate buffer (pH 7.0) and 1 mM potassium iodide solution in supernatant. The absorbance of the solution was estimated at 390 nm against the blank.

3.11 Estimation of antioxidants

3.11.1 Estimation of ascorbic acid

The ascorbic acid content in algae *C. vulgaris* and *C. humicola* were estimated spectrophotometrically at 525 nm following Kampfenkel et al. (1995). 200 mg algal biomass were homogenized in 3 ml of TCA (0.2%) and centrifuged at 3000×g for 15 min and supernatant were used for ascorbic acid estimation.

Reagents

Extracting solution	
Trichloroacetic acid	3 gm
EDTA	0.075 gm in 100 ml distilled water
(Preparation of DCPIP (2,6-Dichlorophenolindophenol)	100 mg of DCPIP in 1000 ml of distilled water. The solution was further diluted as 10/25 (solution: water) (v/v) before use.

Procedure

100 mg of the algal cells were homogenized with 2 ml of extracting solution and centrifuged. Add 1 ml of supernatant with 5 ml of DCPIP. After addition of DCPIP, the samples were shaken and record absorption at 520 nm.

3.11.2 Estimation of cysteine

Cysteine was estimated by the method given by Gaitonde (1967).

Reagents

Reagent A	
$HClO_4$ (95% pure)	5 %
Reagent B	
Ninhydrin	250 mg
Glacial acetic acid (95%)	6 ml
Hydrochloric acid (HCl)	4 ml
Glacial acetic acid (Reagent C)	
Hydrochloric acid (Reagent D)	

Procedure

500 mg of the algae cells were crushed in 2ml of 5 % chilled $HClO_4$ with the help of mortar and pestle. After crushing, the samples were centrifuged at 10,000×g for 10 min. 1 ml of supernatant was taken and mixed with 1 ml of glacial acetic acid and 1 ml of ninhydrin and heated at 100^{o}C for 30 minutes and allowed to cool at room temperature. Absorbance was taken at 560 nm.

3.11.3 Estimation of glutathione

Glutathione content in algal cells was analyzed by the method of Anderson (1985).

Reagents

5% sulfosalicylic acid
0.1 M $PO4^{-3}$ buffer (pH-7.0+0.5mM EDTA)
3.0 mM DTNB (2,2 dithiobis nitorbenzoic acid)
Reduced GSH

Procedure

5% sulphosalicylic acid was used to homogenize the algae culture. The homogenized samples were centrifuged at 10000×g for 10 min. The supernatants were used for the estimation of reduced glutathione at 412 nm wavelength.

3.11.4 Estimation of proline

Proline content in microalgae was estimated according to the method of Bates et al. (1973).

Reagents

Toluene	Pure
Sulfosalicycic acid	3% (w/v)
Glacial acetic acid (GAA)	3 % (v/v)
Phosphoric acid	6 M
Acid ninhydrin	1.25 g ninhydrin warmed in 30 ml glacial acetic acid (100%) and 20 ml 6 M phosphoric acid

Procedure

Fresh microalgae cells (3 ml) were crushed in 3% aqueous sulfosalicyclic acid, centrifuged at 10,000×g. Add 3% glacial acetic acid and acid ninhydrin. Samples were heated for 1 h in water bath at 95^{o}C, cooled and extracted with 4ml toluene by vortexing for 15 s. After vortexing two layers are formed remove the upper toluene layer and the absorbance was read at 520 nm using toluene as blank. Theproline content in each sample was calculated from the standard curve.

3.12 Assay of antioxidant enzymes

3.12.1 Preparation of enzyme extract

Algal cells (200 mg) were homogenized in 2 ml of 100 mM potassium phosphate buffer, pH 7.5 containing 1 mM of EDTA and a pinch of polyvinyl polypyrrolidone (PVP). The homogenate was centrifuged at 12,000×g for 15 min at 4^{o}C. All the steps in the preparation of enzyme extract were carried out at 0-4^{o}C. This extract was used to assay the activities of different antioxidant enzymes.

3.12.2 Superoxide dismutase (SOD) (EC.1.15.1.1.)

SOD was assayed according to the method of Nishikimi et al. (1972).

Principle

The assay of SOD is based on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazon. The colour formed at the end of the reaction can be extracted into butanol and measured at 560nm.

Reagents

1. Sodium pyrophosphate buffer (0.025M, pH 8.3)
2. Phenazine methosulphate (PMS) (180µM)
3. Nitroblue tetrazolium (NBT) (300µM)
4. NADH (780µM)
5. Glacial acetic acid
6. H_2O

Procedure

The assay mixture contained 1.2 ml of sodium pyrophosphate buffer, 0.1ml of PMS, 0.3 ml of NBT, 0.2 ml of the enzyme preparation and water in a total volume of 2.8ml. The reaction was initiated by the addition of 0.2 ml of NADH. The mixture was incubated at 30^{o}C for 90 sec and the reaction was stopped by the addition of 1.0 ml of glacial acetic acid. Absorbance of reaction mixture was measured by 560 nm in a spectrophotometer.

One unit of enzyme activity is defined as the amount of enzyme that gave 50% inhibition of NBT reduction in one minute.

3.12.3 Glutathione reductase (EC. 1.6.4.2)

Glutathione reductase activity in algal cells was assayed by the method of Smith et al. (1988).

Principle

Reduced glutathione on reaction with DTNB (5, 5'-dithiobis nitro benzoic acid) produces a yellow colored product that absorbs at 412 nm.

Reagents

1. 2mM NADPH
2. Phosphate buffer containing 1mM EDTA (0.2M, pH 8.0)
3. DTNB (3mM in 0.01M phosphate buffer)
4. Standard GSH (20 nM moles ml^{-1})

Procedure

The supernatant (algal extracts; 0.1ml) was made up to 1.0ml with 0.2M sodium phosphate buffer (pH 8.0). Standard GSH corresponding to concentrations ranging between 20 nM were also prepared. 2 ml of freshly prepared DTNB solution was added and the intensity of the yellow colour developed was measured at 412nm for 3 min at interval of 15 sec. The values are expressed as n moles GSH/g fw sample.

3.12.4 Ascorbate peroxidase (APX) (EC.1.11.1.7)

Ascorbate peroxidase (APX) was assayed according to the method of Nakano and Asada (1981).

Reagents

1. Potassium phosphate buffer (50 mM, pH 7.0).
2. Stock solution of ascorbic acid (10 mM)
3. Stock solution of H_2O_2 (1 mm)

Assay

Take 50µm of enzyme prepared assay mixture, containing 2.905 ml of PPB and 15µl of ascorbic acid (10 mM)+ 30µl H_2O_2 (10 mm) and mixed the reaction mixture properly. Take the absorption at 290 nm for 3 min at the interval of 15 second.

3.12.5 Catalase (EC 1.11.1.6)

For the measurement of catalase activity, the extraction was carried out in the buffer containing 50 mM Tris HCl (pH 7.0), 0.1 mM EDTA, Sodium phosphate buffer (pH=7), 20 mM H_2O_2, 1 mM Phenylmethylsulfonyl fluoride (PMSF) and 0.3 g PVP. Activity was measured by the method of Aebi (1974). The decrease in the absorbance was noted at 240 nm.The molar extinction coefficient of H_2O_2 at 240 was taken as 0.004 cm^2 µmol. Enzyme activity was expressed as µ mole of H_2O_2.

Reagents

(A) 50 mM Sodium phosphate buffer, pH-7.0

(B) H_2O_2 100mM- 103 µl in 10 ml dw

(C) 51.5 µl in 5 ml dw

(D) 257.5 µl in 25 ml dw

Reaction mixture

The reaction mixture contained
Buffer - 2.750 ml
H_2O_2 - 0.200 ml
Plant extract - 0.050 ml

Procedure

The experiment starts with the preparation of reagents which include 50 mM potassium phosphate buffer and H_2O_2. Potassium phosphate buffer and H_2O_2 were taken in the eppendrop of tubes. In order to avoid any loss of enzymatic activity, algal sample was added at last at the time of absorbance. As soon as the algal extract was added, absorbance was calculated at 240 nm at 15 sec interval for 3 min.

3.13 Scanning Electron Microscopy coupled energy dispersive X-ray spectrometer study

3.13.1 Sample preparation

The algal strains were fixed in 2.5% of glutaraldehyde for 4 h at 4^{o}C, washed with phosphate buffer and subsequently dehydrated by ethanol (10%, 30%, 50%, 70%, 95% and 100%) for 5 min at each concentration (Chandra et al., 2017). The final dehydration was carried in 100% ethanol for 30 min. The dehydrated samples were mounted on aluminum stub with the help of carbon tape. SEM coupled with energy dispersive X-ray spectrometer (EDS) detector was used to for the X-ray spectra. X-ray plotting was done using EDS in conjunction with Scanning Electron Microscopy (SEM) (JEOL, Japan; model JSM-6490LV).

3.14. Fourier transforms infrared spectroscopy (FTIR) examination

To study FTIR spectrum in algae, the dried algae samples were grinded with potassium bromide (KBr, 1:10 ratio) and pressed to form pellet using hydraulic press (150 lbs). The pillet were furhter positioned and fixed into socket of a separete in FTIR machine for the determination of lipid and proteins frequencies. The spectrum of absorption were analyzed using FTIR (Thermo-Scientific Nicole 6700, USA). The IR spectrum (400-4000 cm^{-1}) were attained with 5-7 cm^{-1} resolution and 32 scan number for each spectrum (Chandra et al., 2014).

3.15 Quality control and quality assurance

The standard reference materials (E-Merck, Germany) were used for the calibration and quality assurance for each analytical batch. Analytical data quality of chemical was ensured with repeated analysis of quality control samples (n=3) and the results were found within (±2.82) the certified values.

3.16 Statistical analysis

All the experiments were conducted in triplicate. To confirm the variability of data and validity of results, all data were subjected to one way analysis of variance (ANOVA) followed by Duncan’s Multiple Range Test (DMRT, $P<0.05$) to see the significant level (Gomez and Gomez, 1984). For group wise comparison of means one way ANOVA posthoc multiple comparison tests were performed ($P<0.05$) for the statistical significant level between controls and treated algae.

CHAPTER 4

NaCl incites reactive oxygen species in green algae *Chlorococcum humicola* and *Chlorella vulgaris*: Implication in lipid synthesis, mineral nutrient status and antioxidant system

4.0 NaCl incites reactive oxygen species in green algae *Chlorococcum humicola* and *Chlorella vulgaris*: Implication in lipid synthesis, mineral nutrient status and antioxidant system

4.0 Introduction

The onset of industrial revolution, urbanization and lifestyle, dependency of the world on energy has tremendously increased which needs to be supplemented by renewable energy source such as bio-oil, solar energy and wind energy. This non-renewable resource with limited stocks is fast dwindling due to overexploitation. Therefore, a transition from non-renewable energy source to renewable energy is pre-requisites to meet the growing energy demand. Microalgae are dominant, photosynthetic and oxygen producing organisms on this planet (Brodie et al., 2017). which can thrives in variety of habitat due to high degree of adaptability to withstand under extreme stress conditions (Rai et al., 2013) and capability to grow with minimum nutritional requirement (Korosi et al., 2017).

Salinity is an important environmental stress, mainly contributed by the natural sources, anthropogenic activities and modern agricultural practices (Ismail et al., 2017). Growing algae in salinized water and soil could make them highly valuable resource with minimum cost of production in a sustainable manner (Costa and de Morais, 2011). Zhang et al. (2017) reported that salt is the most common stressor and strong limiting factor for stressor successful large scale production of biomass, lipid and biofuel (Ho et al., 2014). Algae grown in saline habitat develops osmotic stress, alteration in nutrient uptake and generation of reactive oxygen species (ROS) due to ionic disturbance, physiological alteration and oxidative injury (Verslues, 2017). ROS are produced inside the living cell as a byproduct of the cell metabolism, imparting a signal cascade which elicits toxicity at higher concentration (Almeida et al., 2017). In plant cell, high level of NaCl triggers exo-osmosis driven plasmolysis, altering water potential of the cell which restricts the uptake of water and essential mineral nutrients (Singh et al., 2015). To cope with NaCl toxicity, algae synthesize low molecular weight compatible solutes which served as osmoprotectants (Filippou et al., 2014). ROS induced oxidative damage to algae is counter attacked by the intracellular scavengers (enzymatic and non-enzymatic antioxidants) of oxy-radicals (Singh et al., 2018). Under the salinity stress, the photosynthetically generated energy is reduced and thus, the production of storage compounds such as neutral lipid is enhanced (Waltz, 2009; Upadhyay et al., 2016).

C. vulgaris and *C. humicola* are photosynthetic microalgae exhibiting fast growth rate, cosmopolitan distribution, and ability to grow under different harsh environment (Rai et al., 2013). It has been observed that salinity can induce the lipid production in algae (Srivastava and Goud, 2017). However, no study has been undertaken to explain the ultrastructural alteration, mineral nutrient status and chemistry of functional group involved in the lipid, protein and carbohydrate synthesis under different concentrations of salt. The present investigation using *C. vulgaris* and *C. humicola* was carried out to study the impact of salt on biochemical, physiological, and structural changes (ii) SEM coupled with EDS analysis showing the effect of salinity on the mineral nutrient status and deterioration of different functional group of ecological importance through FTIR analysis.

4.1 Materials and methods

Microalgae *C. vulgaris* and *C. humicola* were cultured in Erlenmeyer flasks (1000 ml) containing 200 ml nutrient media. The mass culture of algal strains was prepared in BG-11 medium at 24±2^{o}C under 150 µmol photon $m^{-2}s^{-1}$ PAR (Photosynthetically active radiation) with a photoperiod of 16:8 h (light: dark) in the culture room under controlled condition. The BG-11 media contains $NaNO_3$ (1.5 gL^{-1}), K_2HPO_4 (0.04 gL^{-1}), $MgSO_4.7H_2O$ (0.075 gL^{-1}), $CaCl_2.2H_2O$ (0.036 gL^{-1}), citric acid (0.01 gL^{-1}), ferric ammonium citrate (0.006 gL^{-1}), Na_2.EDTA (0.001 gL^{-1}), Na_2CO_3 (0.02 mgL^{-1}) and 1 mL of trace metal solution per liter. The trace metal includes H_3BO_3 (61.0 mg L^{-1}), $MnSO_4.H_2O$ (169.0 mgL^{-1}), $ZnSO_4.7H_2O$ (287 mgL^{-1}), $CuSO_4.5H_2O$ (2.5 mgL^{-1}), and $(NH_4)_6.MoO_4.4H_2O$ (12.5 mgL^{-1}). The pH of the medium was adjusted to 6.8-7.2. The culture was shaken manually (4 times/d) for proper aeration.

4.1.1Experimental design

The experiments were performed in the laboratory of Department of Environment Science, BBAU, Lucknow, India (26.7697° N, 80.9262° E). Pure algal inoculums (approx 5-10%) of *C. humicola* and *C. vulgaris* were used in 1000 ml

Erlenmeyer flasks containing 200 ml BG-11 culture media. The inoculated flasks were kept in growth chamber for acclimatization for 24h. After 72h of growth, different concentrations of NaCl (25, 50, 100, 500 and 1000 mM) were added separately in culture flasks. A control set devoid of NaCl was also maintained by using BG-11 media. All the experiments were performed in triplicates under controlled laboratory conditions. The algal cells were harvested during stationary phase of growth.

4.1.2 Growth and toxicity analysis

The growth of algae was monitored by measuring the optical density (OD) of culture at 24 h interval with the help of UV-visible double beam spectrophotometer wavelength of 700 nm. The optimization of growth and toxicity test were performed using varying concentration of NaCl (100-500 mM) and UV-B light sources (Philips tube).

4.2 Data analysis

4.2.1 Biomass productivity

Biomass productivity during the exponential growth phase was calculated by the formula as follows:

$$BP = (B_2 - B_1) / (t_2 - t_1)$$

Where, BP is the biomass productivity expressed in mg $L^{-1}d^{-1}$ and B_1 and B_2 are biomass concentrations in mg L^{-1} t_2 is the 15th day representing end of exponential growth phase and t_1 is 3rd day denoting the start of exponential growth phase).

4.2.2 Specific growth rate

Specific growth rate was calculated using equation (Wong and Cheng, 1991) as follow:

$$\text{Specific growth rate } (\mu) = \ln X_1 - \ln X_2 / t_2 - t_i$$

Where, X_0 = Initial absorbance at time t_1; X_2 = absorption at time t_2

4.2.3 Percentage inhibition

Percentage inhibition (PI) of the algal growth was determined by the formula given below:

$$PI = C_0 - Ct / C_0 \times 100$$

Where, C_0 and Ct are the cell density of untreated control cells and UV-B or salt treated cells (at stationary phase), respectively.

4.3 Sample preparation and analysis of SEM coupled with EDS

The algal strains were fixed in 2.5% of glutaraldehyde for 4 h at 4 °C, washed with phosphate buffer and subsequently dehydrated by ethanol (10%, 30%, 50%, 70%, 95% and 100%) for 5 min at each concentration. The final dehydration was carried in 100% ethanol for 30 min. The dehydrated samples were mounted on aluminum stub with the help of carbon tape. SEM coupled with energy dispersive X-ray spectrometer (EDS) detector was used to for the X-ray spectra. X-ray plotting was done using EDS in conjunction with scanning electron microscopy (SEM) (JEOL, Japan; model JSM-6490LV).

4.4 Fourier transform infrared spectroscopy (FTIR) analysis

To study FTIR spectrum in algae, the dried algae samples were grinded with potassium bromide (KBr, 1:10 ratio) and pressed to form pellet using hydraulic press (150 lbs). The pellets were furhter positioned and fixed into socket of a dislocated in FTIR machine for FTIR spectra of cells and proteins. The spectrum of absorption were analyzed using FTIR (Thermo- Scientific Nicole 6700, USA). The IR spectrum (400-4000 cm^{-1}) were attained with 5-7 cm^{-1} resolution and 32 scan number for each spectrum (Chandra et al., 2014). The background absorption by KBr was substracted by omics software.

4.5 Analysis of biochemical parameters

4.5.1 Estimation of chlorophyll and carotenoids

Chlorophyll content in algal cells was estimated by the method of Arnon (1949). 100 mg of algal sample were crushed in 3 ml 80% acetone with the help of pestle and mortar under dark cold conditions and centrifuged at 8000×g for 10 min. The supernatant was used for estimation of chlorophyll at the wavelength of 663 and 645 nm. Carotenoid content was determined at the wavelength 480 nm and 510 nm using the formula given by Duxbury and Yentsuch (1956).

4.5.2 Estimation of protein

Protein content was measured by the method of Lowry et al. (1951) using bovine serum albumin as the standard. 100 mg of algal sample was crushed in 5 ml 10% chilled trichloroacetic acid (TCA) and centrifuged at 10,000×g for 10 min. After decanting, the supernatant the pellet was washed and re-suspended in 5 ml of 1N

NaOH and heated for 15 min, cooled and again centrifuged at 10,000×g for 10 min. The absorption of supernatant was recorded at 750 nm.

4.5.3 Estimation of carbohydrate and total lipid

Carbohydrate content in algal cells was estimated by the methods of Dubois et al. (1956). 250 mg algal sample was homogenized in 3 ml of 80% ethanol followed by centrifugation at 3000 rpm. 1 ml of supernatant was further mixed with 1% phenol solution (1 ml) + conc. H_2SO_4 (5 ml). The absorption was recorded at the wavelength of 480 nm.Total lipid content in algal cell was estimated by the vanillin method (Ben-Amotz et al., 1985).

4.5.4 Estimation of lipid peroxidation

Lipid peroxidation in the form of TBARS (thiobarbituric acid reactive substance) content in algae was estimated by the method of Heath and Packer (1968). 100 mg of algae sample were crushed in 3ml 0.1% TCA and mixture was centrifuged at 10,000×g for 10 min. 1 ml aliquot of the supernatant was mixed with 4 ml of TBA reagent and mixture was heated at 95ºC for 30 min followed by cooling in an ice bath The reaction mixture was centrifuged at 10,000×g for 10 min. The absorbance of supernatant was taken at 532 nm and 600 nm respectively. The absorbance at 600 nm was substracted from absorbance at 532 nm in order to correct the value for non-specific turbidity.

4.5.5 Estimation of electrolyte leakage and H_2O_2

Electrolyte leakage in algal cell was estimated by the methods of Dionisio-Sese and Tobita (1998). H_2O_2 was estimated following the method of Velikova et al. (2000). Algal biomass (100 mg) were extracted in 2 ml chilled TCA (0.1% w/v) under cold conditions and it was centrifuged at 12000×g for 10 min. The reaction mixture included 0.5 ml phosphate buffer (10 mM, pH 7.0) and 0.5 ml supernatant. The reaction was started with 1ml KI solution in reaction mixture. The absorbance of chromophore was recorded at 390 nm after 5 min of incubation.

4.5.6 Estimation of ascorbic acid proline and cysteine

Ascorbic acid ($\mu Mg^{-1}fw$) content was estimated by following the methods of Kampfenkel et al. (1995). Proline content in algal cell was determined by using the methods of Bates et al. (1973). Cysteine content was determined by following the protocol of Gaitonde (1967).

4.6 Preparation of enzyme extract

100 mg algal cells were homogenized in 2 ml of 100 mM PP buffer (pH 7.5), containing 1 mM of EDTA and polyvinyl polypyrrolidone (PVP) followed by centrifugation at 12,000×g at 4^{o}C. The preparation of enzyme extracts was carried out in cold laboratory at 4^{o}C. The crude enzymes extracts was used for assay of different enzyme activities.

4.7 Enzymatic assay

Superoxide dismutase (SOD) activity was assayed by the method of Nishikimi and Rao (1972), using the crude enzyme extracts. Catalase (CAT) activity was assayed by Aebi (1984) and the result was calculated using extinction coefficient (ε) = 39.4 mmol $L^{-1}cm^{-1}$. Glutathione reductase (GR) activity was assayed by the method of Smith et al. (1988).

4.8 Statistical analysis

All the determinations were carried out in triplicates. To confirm the validity and variability of the data one way analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT, $P \leq 0.05$) were performed for all parameters to see the significant difference between the variables.

4.9 Results and discussion

4.9.1 Effect of NaCl on growth and biomass

The growth characteristics of algae *C. humicola* and *C. vulgaris* under different concentration of NaCl (25-1000 mM) have been depicted in Fig. 4.1 A and 4.1B. The growth curve exhibited all three phases of growth. The exponential phase in both the algal strains started from 3rd day and continued up to 15-16th day in control condition. However, increasing the concentration of NaCl in both the algal strains resulted into shortening of total span of exponential phase i.e., 11-13 day. At higher concentrations of NaCl (500 and 1000 mM), the exponential phase in both *C. humicola* and *C. vulgaris* was reduced from 15 to 09 days, indicating NaCl induced toxicity and reduction in the life cycle of cells. Results showed a NaCl concentration (Control to 1000 mM) dependent inhibition of cell growth in both the strains.

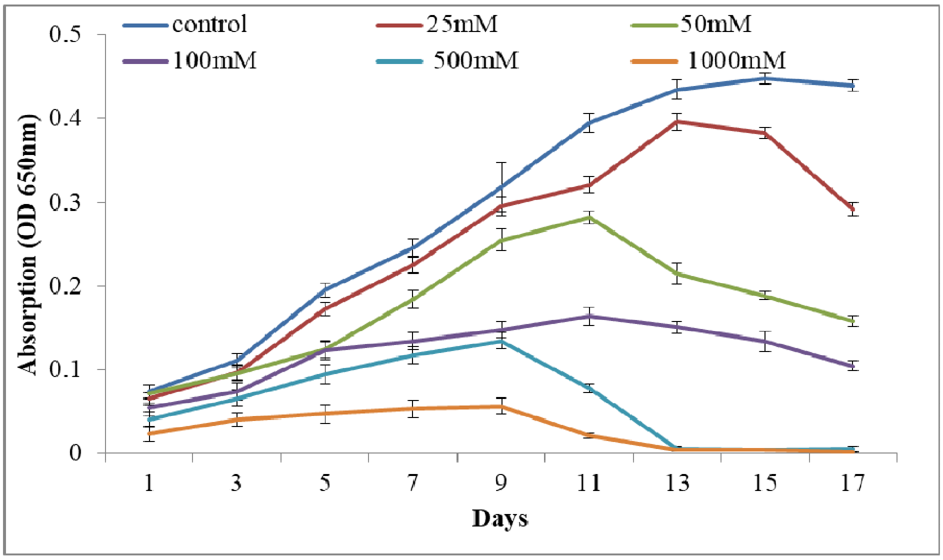


Fig. 4.1A: Growth responses of alga *C. vulgaris* treated with different concentrations of NaCl. All the values are means ± S.D.

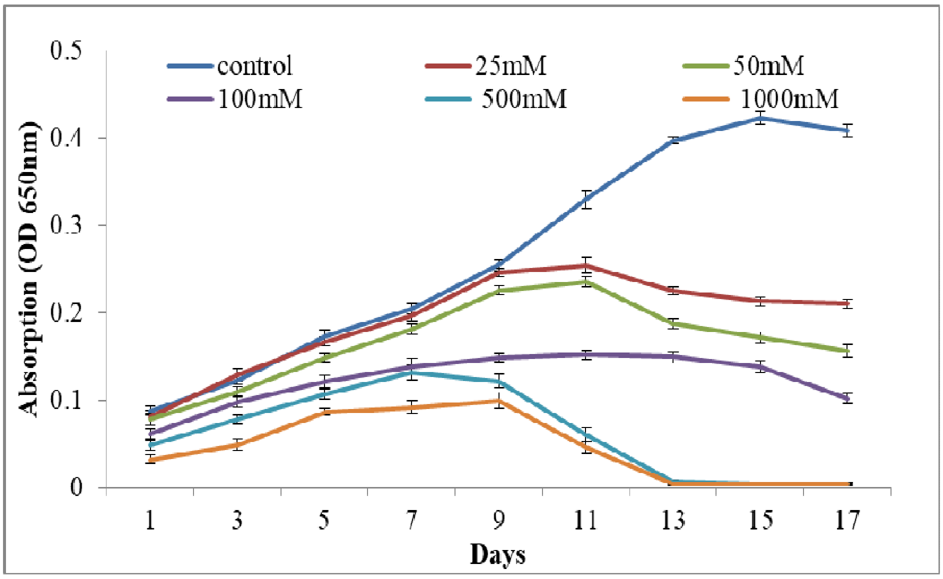


Fig. 4.1B: Growth responses of alga *C. humicola* treated with different concentrations of NaCl. All the values are means ± S.D.

The growth inhibition in both algal strains was found to be more or less >80% at 500 mm^{-1} concentration of NaCl (Table 4.1). The result revealed that LC-50 in both the strains was at 100 mM concentration of NaCl. The specific growth rate decreased with the further increase in the concentration of NaCl which clearly reflects the NaCl toxicity to the algal cells (Table 4.1).

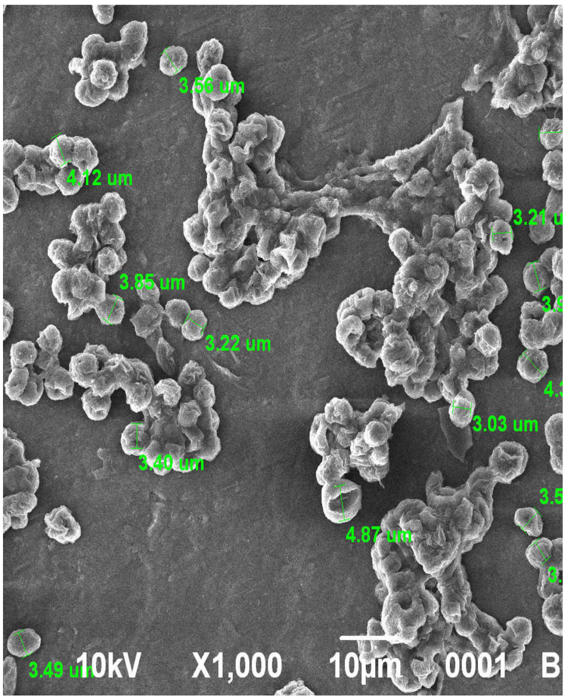
Table 4.1: Specific growth rate (µh^{-1}), biomass productivity (mg L^{-1} d^{-1}), percent inhibition (%) and biochemical analysis (protein, carbohydrate and total lipid) of *C. vulgaris* and *C. humicola* treated with different concentration of NaCl (25-1000 mM). All the values are means ± SD. ANOVA post hoc DMRT was done to check the variation between the variables. Identical letter denotes no significant difference ($p \leq 0.05$)

Treatments (mM)	**Parameters**					
	Specific growth rate	**Biomass productivity (BP)**	**Percent inhibitory (PI)**	**Protein (mg g^{-1} fw)**	**Carbohydrate (mg L^{-1})**	**Total lipid (mg L^{-1})**
A. ***C. vulgaris***						
Control	0.159	78.0±2.99	0.00	0.040^{d} ±0.0006	23.78^{e} ± 0.094	0.144^{c} ±0.008
25	0.137	68.9±4.56	17.69	0.029^{c} ±0.0005	24.48^{f} ±0.115	0.288^{f} ±0.006
50	0.073	49.2±7.00	27.69	0.014^{b} ±0.0005	19.67^{d} ±0.080	0.273^{e} ±0.002
100	0.059	53.4±5.50	57.95	0.013^{b} ±0.0004	12.05^{c} ±0.054	0.168^{d} ±0.005
500	-0.189	12.60±5.50	80.00	0.012^{b} ±0.0002	10.89^{b} ±0.100	0.116^{b} ±0.004
1000	-0.189	8.1±1.10	94.62	0.004^{a} ±0.003	5.96^{a} ±0.034	0.094^{a} ±0.003
B. ***C. humicola***						
Control	0.140	57.73±2.61	0.00	0.031^{d} ±0.0057	15.79^{d} ±0.113	0.160^{a} ±0.005
25	0.087	51.57±4.55	22.42	0.020^{c} ±0.00027	18.25^{f} ±0.047	0.329^{d} ±0.005
50	0.064	37.47±31.0	28.48	0.020^{c} ±0.00021	17.00^{e} ±0.080	0.267^{c} ±0.007
100	0.047	35.63±1.51	53.94	0.019^{c} ±0.00054	12.73^{c} ±0.080	0.217^{b} ±0.014
500	-0.206	23.67±2.51	81.52	0.012^{b} ±0.00081	8.66^{b} ±0.041	0.210^{b} ±0.016
1000	-0.189	6.07±0.06	86.06	0.010^{a} ±0.0003	5.28^{a} ±0.060	0.177^{a} ±0.008

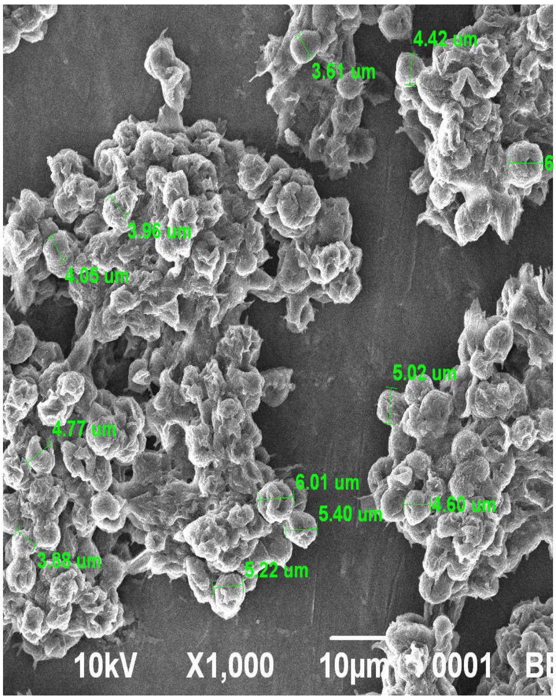
Earlier workers have suggested that NaCl toxicity to the algal cells was attributable to membrane shrinkage, ionic imbalance and ex-osmosis of the turgid cells (Singh et al., 2015; Verslues, 2017). Rady et al. (2018) have reported that growth retardation in salt treated algal cells occur due to altered membrane transport and nutrient deprivation of the cells. The biomass productivity in both the algae also exhibited the decreasing trend with increasing concentration of NaCl which may be ascribed to NaCl induced changes in the synthesis of biomolecules (Upadhyay et al., 2016).

4.9.2 Effect on morphology and mineral nutrient status under SEM coupled with EDS

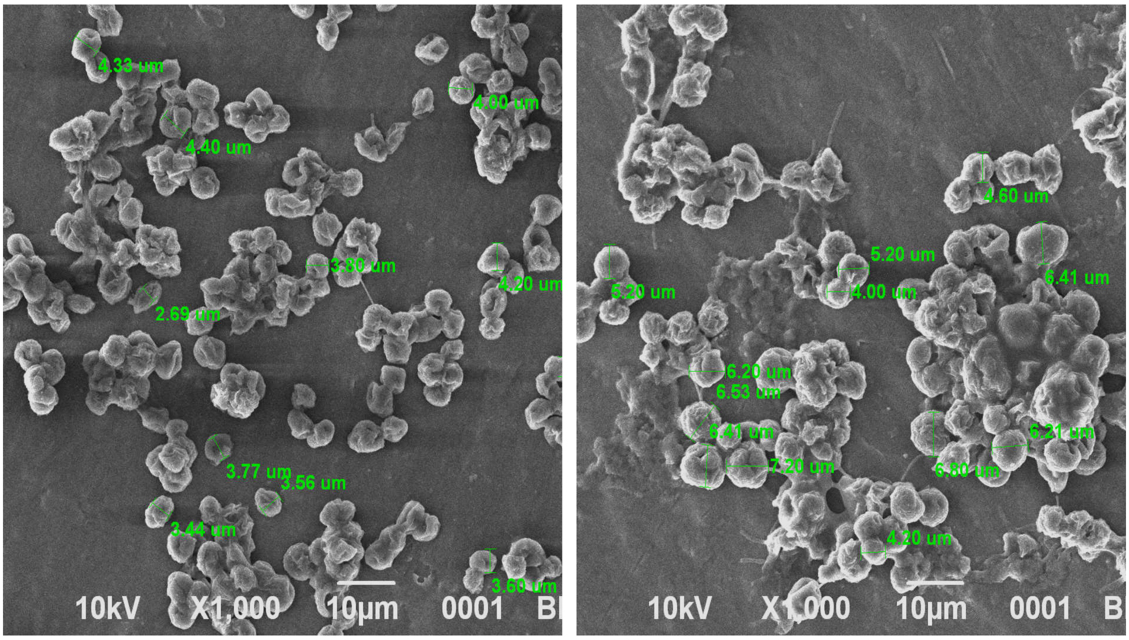
Scanning electron microscopy (SEM) coupled with energy dispersion X- ray spectroscopy (EDS) were used to study the morphological alterations and mineral status of different elements in algae *C. vulgaris* and *C. humicola* (Fig. 4.2A and 4.2 B). Scanning Electron microscopy of *C. humicola* and *C. vulgaris* revealed that under salt stress (100 and 500 mM, respectively) the cell size was increased as compared to untreated control. An average 33.52% increase in the cell size was observed in *C. humicola* cells whereas the average cell size in *C. vulgaris* was increased by 27.79% as compared to their respective control cells (without NaCl).



***C. vulgaris* control**



***C. vulgaris* treated with NaCl (100mM)**



***C. humicola* control**

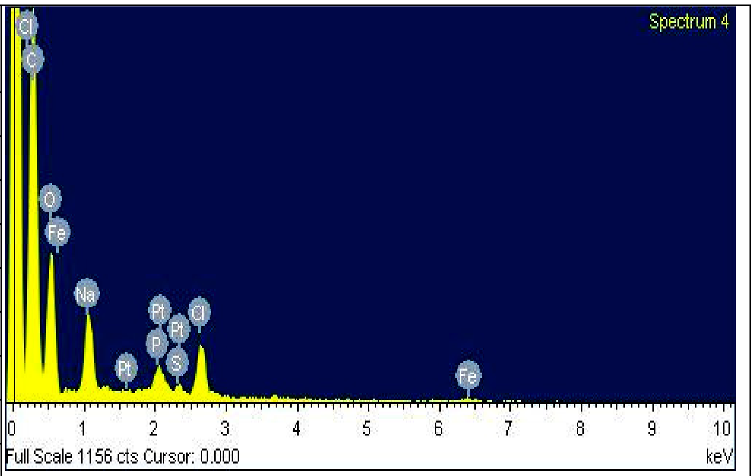
***C. humicola* treated with NaCl (100mM)**

Fig. 4.2A: Scanning electron micrograph study in control and NaCl treated algae *C. vulgaris* and *C. humicola*

Further, the energy dispersive x-ray spectroscopy data showed that mineral content was reduced in the NaCl treated algal cells than control. EDS data expressed in term of percent atomic number (% atomic) and percent atomic weight (% weight) showed the energy dependent localization of different elements in their respective shells (K, L, M and N) (Fig. 4A and 4B). The EDS data in *C. vulgaris* showed significant reduction in mineral contents including C, S and Fe which were in the order of 14.09%, 53.12% and 46.77%, respectively in term of % weight, while with respect to % atomic weight it was 12.43%, 51.85% and 46.67%, respectively. In the case of Na, the concentration in both the strain was increased from 0.5 to 3.56% (% weight) and 0.31 to 2.13% (% atomic weight). EDS spectrum of algae *C. humicola* revealed a marked alteration in mineral contents. Results showed that under salt stress, mineral Mg, Si, S, Ca, Mn, Fe and Zn vanished in algal cells while carbon, diminished by ~50%. Algae treated with salt showed high content of Na and Cl in both algal species as evident from EDS spectra which represented higher accumulation of salt. The potential of cell ligands bind with elements largely depends on the presence of different functional group (negatively charged), which chelates the ions on the surface or transport them inside the cell (Singh et al., 2018). Decrease in mineral content might be due to the fact that NaCl alters the stomatal conductance, membrane composition, permeability and cell deformation which ultimately limit the uptake and sorption of important mineral in plants and algae (Zouari et al., 2016). Demidchik et al. (2014) reported that high salinity decreases the water potential of the solution, alters intracellular pH and the entry of water and other important nutrients. In addition, high salinity inhibits the process of photosynthesis and cell division which may be possibly responsible for increase in the cell size as observed in the present finding (Fahy et al., 2017).

Element	Weight %	Atomic %
C K	69.9	78.47
O K	23.47	19.78
Na K	0.54	0.31
Mg K	0.64	0.35
S K	0.64	0.27
Fe K	0.62	0.15
Zr L	4.68	0.69
Bi M	-0.48	-0.03
Totals	100	

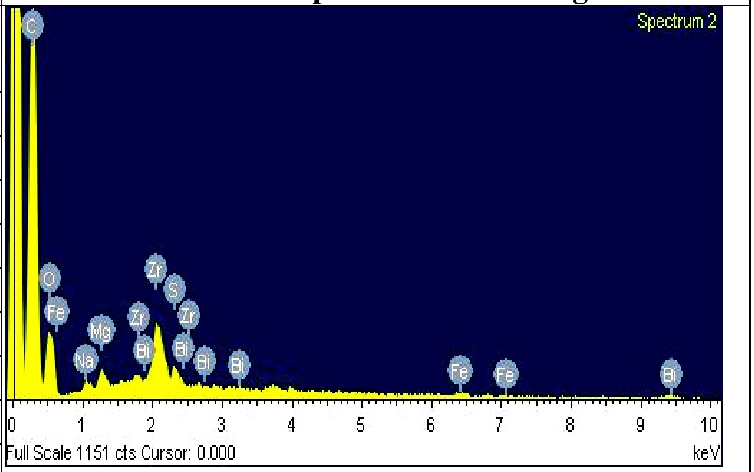
***C. vulgaris* control**



EDS spectra of control algae

Element	Weight %	Atomic %
C K	60.05	68.71
O K	32.55	27.96
Na K	3.56	2.13
P K	0.48	0.21
S K	0.3	0.13
Cl K	1.83	0.71
Fe K	0.33	0.08
Pt M	0.91	0.06
Totals	100	

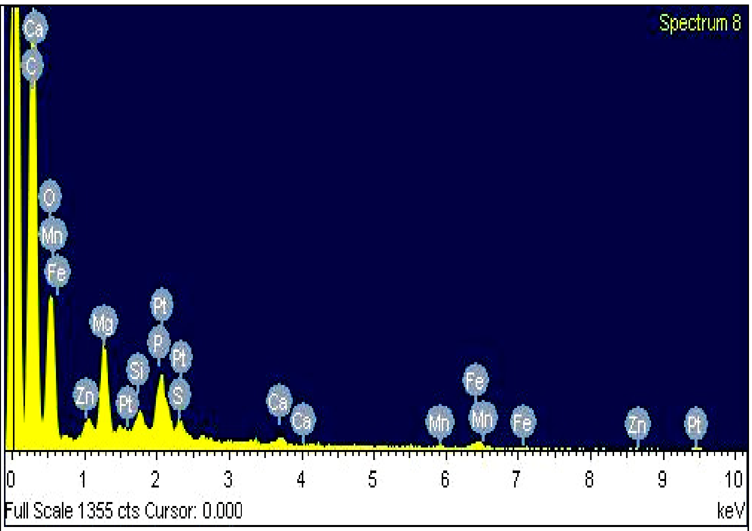
***C. vulgaris* treated**



EDS spectra of treated algae

Element	Weight %	Atomic %
C K	59.03	68.33
O K	33.11	28.77
Mg K	2.67	1.53
Si K	0.57	0.28
P K	0.96	0.43
S K	0.45	0.19
Ca K	0.28	0.1
Mn K	0.26	0.07
Fe K	0.59	0.15
Zn K	0.05	0.01
Pt M	2.04	0.15
Totals	100	

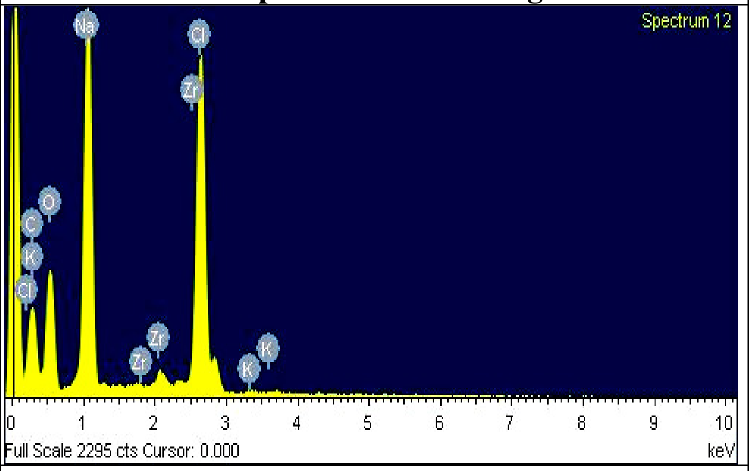
C. humicola **Control**



EDS spectra of Control algae

Element	Weight %	Atomic %
C K	30.28	43.06
O K	31.46	33.59
Na K	20.49	15.22
Cl K	16.13	7.77
K K	0.17	0.08
Zr L	1.47	0.27
Totals	100	

C. humicola **treated**



EDS spectra of treated algae

Fig. 4.2B: EDS spectra study in control and NaCl treated algae *C. vulgaris* and *C. humicola*. The EDS data showed in term of percent atomic and percent weight of different elements present in the algae

4.9.3 FTIR spectral elucidation

FTIR spectra of microalgae *C. humicola* and *C. vulgaris* grown under different salinity regimes have been depicted in Fig. 4.3A and 4.3B. The analysis of FTIR spectra exhibited different charactersitics absorption bands associated with proteins, lipids, carbohydrates and phosphorylated molecules. The change in IR absorption peak (wave number) of protein (primary amide, sec-amide and amide- I, II, III) and Lipid (carbonyl, methylated and phosphodiester) were observed in both algae grown undersalt stress (100 mM).

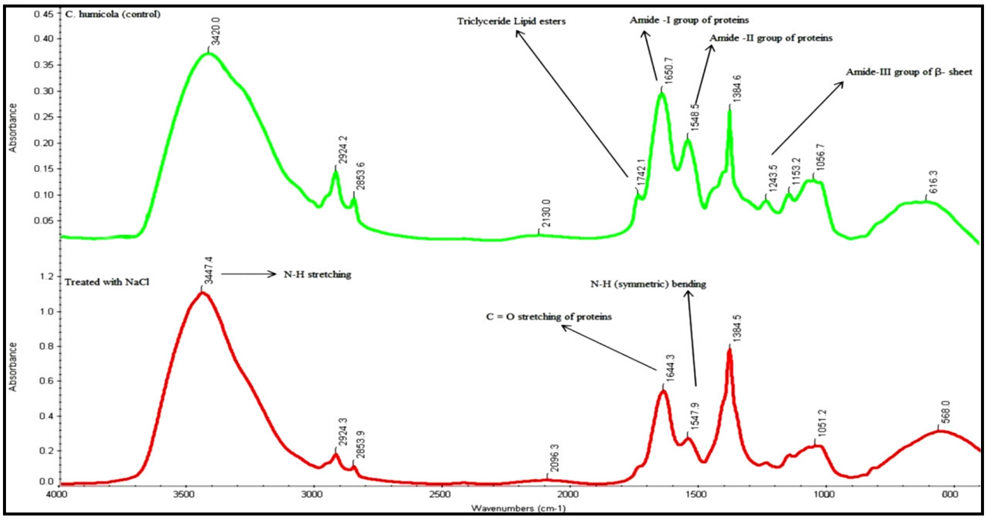


Fig. 4.3A: FTIR spectra of *C. humicola* treated with different concentration of NaCl

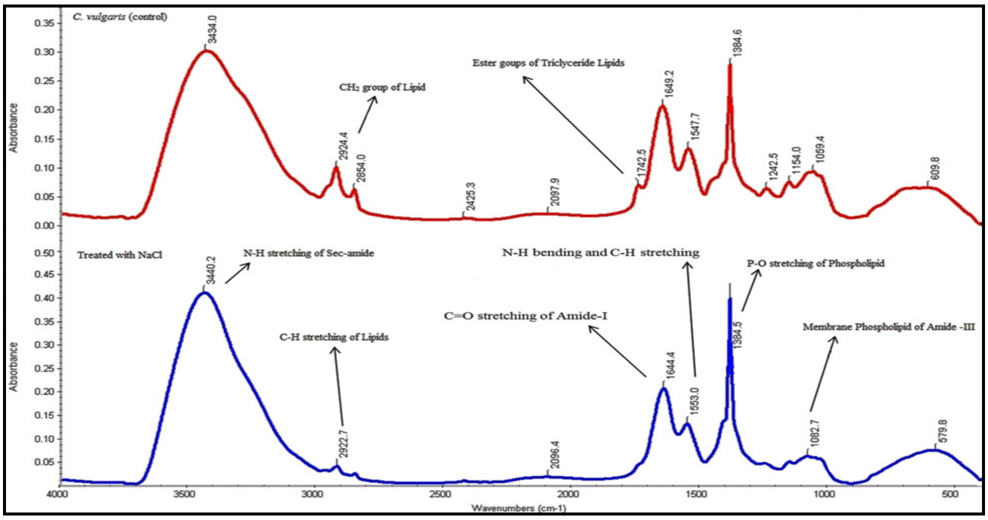


Fig. 4.3B: FTIR spectra of *C. vulgaris* treated with different concentration of NaCl

The bond assignment related to different functional groups in both algae *C. humicola* and *C. vulgaris* are depicted in Table 4.2 and 4.3. The IR absorption bands for protein [amide I (C=O) and amide II (N-H) with spectral peak at 1650-1640 and 1560 cm^{-1}, respectively], carbohydrate (1174-1134 cm^{-1}) and lipid (1750 cm^{-1}) were altered in the both algal strains treated with 100 mM of NaCl when compared with the control. The peak height of amide I, amide II, and lipid in NaCl treated algae *C. vulgaris* decreased from 4.4-3.0 cm (amide I), 3.0-2.2 cm (amide II) and 1.4-1.1 cm (lipid) as compared to control. Similarly, in case of *C. humicola*, a reduction in peak height from 5.0 to 3.24 cm (1650 cm^{-1}), 3.5 to 1.9 cm (1550 cm^{-1}) and 1.65 to 1.0 cm (1740 cm^{-1}), respectively. Decrease in characteristic IR peaks height for protein, carbohydrate and lipid demonstrates NaCl induced toxicity in algae. The changes in protein, lipid and carbohydrate production in NaCl affect algal strain can be examined by the testing the ratio viz., protein/ lipid and lipid/ carbohydrate. The protein to lipid ratio in alga *C. vulgaris* treated with NaCl showed decrease in the P/L ratio from 3.14-2.72 and 2.14-2.0 in both *C. vulgaris* and *C. humicola* respectively. The results suggested that decrease in biomass content (Chandra et al., 2014). Fang et al. (2017) also reported that the decrease in protein content in algal cell due to salt stress. In the case of *C. humicola*, similar trend was observed. However, protein (amide I) to lipid content ratio showed marginal increase from 3.03 to 3.2. The carbohydrate spectral peak was found to have diminished in NaCl treated *C. humicola* and *C. vulgaris* which clearly demonstrates the reduction in photosynthesis coupled with increase in the storage compound (Upadhyay et al., 2016). The weak vibration band near 2550 cm^{-1} indicated the S-H group stretching (Haris et al., 1999). There was obvious change in the ratio between untreated and treated algae *C. humicola* and *C. vulgaris*. The spectral band near the 3011 to 2920 cm^{-1} showed strong absorptions due to the presence of N-H stretching of amines A and linked OH stretching (Chapman et al., 2001).

Table 4.2: Assignment of functional groups associated with major vibration bands in IR spectrum of *C. humicola*

Range of wave number in cm^{-1}	**Wave number (cm^{-1}) of *C. humicola* (Control)**	**Wave number cm^{-1} after treatment of NaCl**	**Band assignment and Functional groups**
3029-3639	3420.0	3447.4	N-H stretching (asymmetric) of Amide -A (Dovbeshko et al., 1997)
2809-3012	2924.2	2924.3	Asymmetrical C-H stretching of $-CH_2$ in methylated lipids (Socrates, 2001)
	2853.6	2853.9	C-H symmetric stretching of CH_2 (Naumann, 2011)
2200-2000	2130.0	2096.3	A combination of hindered rotation and O-H bending (water) (Fabian et al., 1995)
1763-1712	1742.1	0	C = O stretching of ester group (Forfang et al., 2017)
1583-1709	1650.7	1644.3	C=O stretching of proteins in the form of α-helix, Amide-I (Paluszkiewicz et al., 2001)
1560-1530	1548.5	1547.9	N-H bending, β sheet of Amide-II (Schulz and Baranska, 2007)

1390-1380	1384.6	1384.5	Symmetric deformation of C-O of CH_2 and CH_3 of proteins/ lipids Amide-III (Joshua et al., 2013)
1250-1220	1243.5	0	P=O stretching (asymmetric) of PO_2^- (Vongsvivut et al., 2013)
1134-1174	1153.2	0	C-O-C stretching of polysaccharides carbohydrate (Chandra et al., 2014)
1070-1050	1056.7	1051.2	Symmetric stretching of C-O-C and C-O-P, saccharides and PO_2^- (Huleihel et al., 2002)

Table 4.3: Assignment of functional groups associated with major vibration bands in IR spectrum of *C. vulgaris*

Range of wave number in cm^{-1}	**Wave number (cm^{-1}) of *C. vulgaris* (control)**	**Wave number (cm^{-1}) after treatment of NaCl**	**Band assignment and Functional groups**
3500-3400	3434.0	3440.2	N-H and O-H stretching of Sec-amide and hydroxyl group (Schulz et al., 2007)
2930-2920	2924.4	2922.7	Asymmetrical C-H stretching of -CH_2in methylatedlipids (Murdock and Wetzel, 2009)
2875-2850	2854.0	0	Symmetrical stretching of C–H from methyl (–CH_3) groups of lipids (Forfang et al., 2017)
2700-2200	2425.3	0	P–H stretching Phosphorus acid and ester (Stuart, 2004)
2200-2000	2097.9	2096.4	Si–H stretching (Stuart, 2004)
1763-1712	1742.5	0	C=O stretching of esters from lipid triglycerides and fatty acids (Vongsvivut et al., 2013)
1583-1709	1649.2	1644.4	C=O stretching of α- Helix proteins of Amide-I (Jebsen et al., 2012)
1481-1585	1547.7	1553.0	N-H (symmetric) bending, C-N (symmetric) stretching of Amide-II (Duygu, 2012.)
1380-1370	1384.6	1384.5	Symmetric deformation in methylated (CH_2) lipids and proteins (Vongsvivut et al., 2013)
1244-1230	1242.5	0	P=O (asymmetrical stretching) of the phosphodiester backbone of nucleic acid (Heraud et al., 2005)
1134-1174	1154.0	0	C-O-C stretching of polysaccharides carbohydrate (Chandra et al., 2014)
1072-1099	1049.4	1082.7	C-O-C stretching in phosphodiester group of Amide -III(Chandra and Singh, 2014)

4.10 Effect on biochemical parameters

4.10.1 Effect on photosynthetic pigment and protein content

The chlorophyll content in microalgae *C. humicola* and *C. vulgaris* treated with different concentrations of NaCl (100-1000 mM), decreased as compare to control (Fig. 4.4). At 25 mM of NaCl, chlorophyll level in *C. vulgaris* was increased by 5%, however, in *C. humicola* by 23%. However, no significant changes in the chlorophyll content was observed at 50 mM of NaCl ($P \leq 0.05$). A range of NaCl concentrations exhibited concentration dependent progressive decreased in chlorophyll content in both the algal strain due to ROS induced chlorosis and formation of triplet state chlorophyll molecule which strongly damage the photosystem I and II, followed by decrease in photosynthetic performance and chlorophyll synthesis (Upadhyay et al., 2016). Under NaCl treatment, algal cells experience a cascade of physical and physiological alterations viz., decreasing nutrients uptake, CO_2 flux, increased NADPH and limited entry of absorbed light to the photosystem decrease in the chlorophyll biosynthesis and low biomass accumulation (Srivastava and Goud, 2017). Chokhsi et al. (2017) have also reported reduction in the pigment concentration in algae *Acutodesmus dimorphus* under NaCl stress. On the other hand, increase in the level of chlorophyll at low NaCl (25 mM), concentration indicates additives response of cell to salt stress.

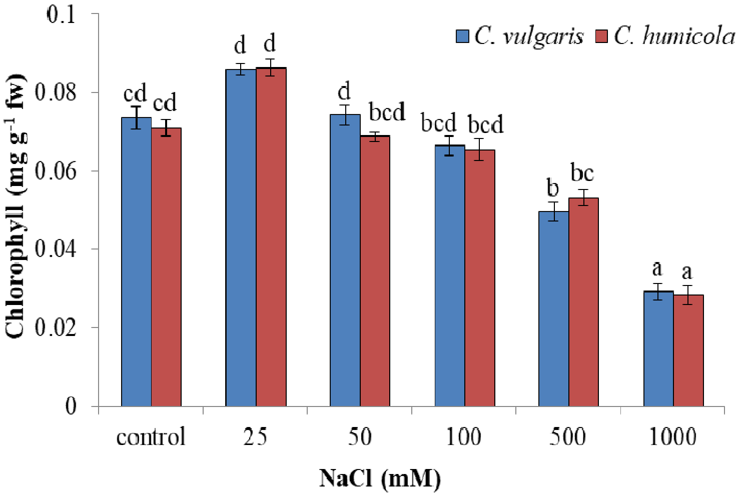


Fig. 4.4: Effect of different concentration of NaCl on chlorophyll content in algae *C. vulgaris* and *C. humicola*. All the values are means ±S.D. ANOVA significant at level of ($p \leq 0.05$) according to one-way ANOVA test. Identical superscripts denote no significant difference between means in column according to DMRT ($p \leq 0.05$)

The results showed increase in the accessory pigment carotenoid content in algae *C. vulgaris* and *C. humicola* treated with different concentration of NaCl (Fig. 4.5). Maximum increase in carotenoid content was observed at 25 mM concentration of NaCl in both the algae when compared with control. Carotenoids are accessory pigment fundamentally important in light harvesting and cell protection against oxidative damage (Ramanna et al., 2017). The NaCl induced increase in the carotenoid content of cells is the results of adaptive tolerance response against oxidative radicals to cope with the cell damage and that may be ascribed to carotenoid induced deactivation of singlet oxygen and quenching of excited triplet state of chlorophyll resulting into reduction in singlet $^1O_2$ species (Rady et al., 2018).

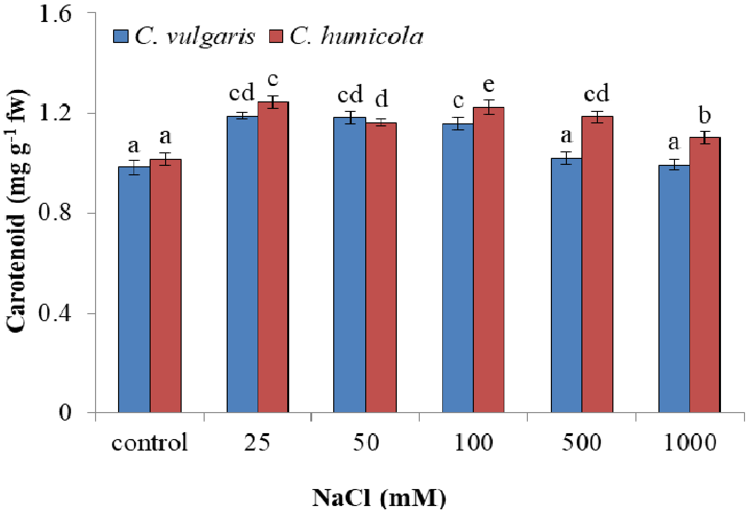


Fig. 4.5: Effect of different concentration of NaCl on carotenoid content in algae *C. vulgaris* and *C. humicola*. All the values are means ± S.D. ANOVA significant at level of ($p \leq 0.05$) according to one- way ANOVA test. Identical superscripts denote no significant difference between means in column according to DMRT ($p \leq 0.05$)

Protein content in algae *C. humicola* and *C. vulgaris* also decreased in the presence to NaCl stress gradient. The NaCl induced maximum toxicity was observed in *C. vulgaris* cell as compared to *C. humicola* cell (Table 4.1). In this case, algal strains treated with 50 and 100 mM NaCl, did not show significant change ($p \leq 0.05$). The results revealed that the *C. vulgaris* is more vulnerable to NaCl stress as compared to *C. humicola*. Decrease in the protein content in both the algae under different physical stress has been reported by Rai et al. (2013).

4.10.2 Effect on total lipid and carbohydrate content

The lipid content in algae *C. vulgaris* and *C. humicola* were increased by 2 fold at 25 mM concentration of NaCl (Table 4.1). *C. vulgaris* cells treated with 500 and 1000 mM of NaCl showed reduction in lipid content. However, lipid content in the case of *C. humicola* was increased by 10.6% at 1000 mM of NaCl. Further, the results showed decrease in the lipid content with gradual increase in salt concentration present in algae. The carbohydrate content in *C. vulgaris* and *C. humicola* treated with 25 mM NaCl increased by 2.95% and 15.56%, respectively (Table 4.1). However, *C. humicola* cells showed increased accumulation of carbohydrate content at 50 mM NaCl. A significant reduction in the carbohydrate content was observed in both algae treated with 100, 500 and 1000 mM concentration of NaCl when compared with the control. Carbohydrate and lipid play an important role in carbon partitioning, photosynthesis, osmotic homeostasis and lipid metabolism (Rosa et al., 2009). In the present study, the NaCl induced decrease in the carbohydrate content may be ascribed to low photosynthetic rate, inhibition of cell division and osmotic imbalance (Sami et al., 2016). An increase in the carbohydrate and lipid content under salinity stress may be due to modification and down regulation of photosynthetic machinery (PSII) and synthesis of different osmolytes such as glycerol, sugar, osmoprotectant and protein synthesis (Paliwal et al., 2017). George et al. (2014) have also reported an increase in lipid and carbohydrate content of microalgae under nutrient starvation, salt stress, pH, temperature and photoperiods. A comparative increase in lipid content in NaCl treated *C. humicola* might be due to shift of sugar/carbohydrate metabolism in favour of lipid storage form to counter attack on the oxidative stress and tolerance in microalgae than *C. vulgaris* (Yilancioglu et al., 2014).

4.10.3 Effect on TBARS content, H_2O_2 and electrolyte leakage

Lipid peroxidation was measured in terms of TBARS in algae *C. vulgaris* and *C. humicola* under salt stress. Results showed that the TBARS content increased with increase in NaCl concentration (100-1000 mM) in both the algal strains as compared to control (Table 4.4). However, maximum level of TBARS content was observed in the algae *C. humicola* at 1000 mM concentration of NaCl. Algae treated with 25 mM NaCl stress did not show significant change as compared to control ($P \leq 0.05$). TBARS is a byproduct of lipid peroxidation and acts as a bioindicator of cellular toxicity during various stresses like temperature, chemicals, heavy metals and salt etc

(Yilancioglu et al., 2014). The H_2O_2 content in algae *C. vulgaris* and *C. humicola* treated with different concentration of NaCl increased with increase in concentration of NaCl (Table 4.4). Algae *C. vulgaris* exhibited >80% increase in H_2O_2 content at 500 mM concentration of NaCl, while, in case of *C. humicola* it was 37.43% increase as compared to control (without NaCl). High level of H_2O_2 in *C. vulgaris* reflects higher level of NaCl toxicity. Maximum H_2O_2 content was observed at 1000 mM of NaCl which was 319.85 and 280.19 µmol g^{-1} fw in both the of *C. vulgaris* and *C. humicola*, strains respectively. H_2O_2, a non-radical reactive oxygen species involved in signaling in the oxidative stress and causes toxicity at higher concentration (Sofo et al., 2015). The enhanced H_2O_2 concentration was recorded in response to increasing concentration of NaCl (Singh et al., 2018). The H_2O_2 metabolizing enzyme catalase is involved in the transformation of superoxide into H_2O.

The electrolyte leakage (EL) is the measure of membrane damage leading to leakage of intracellular ionic content. Both algae *C. vulgaris* and *C. humicola* treated with different concentration of NaCl showed different degree of electrolyte leakage (Table 4.4) which increased with increasing salinity. The level of EL was minimum at 1000 mM concentration of NaCl in both the algae. Maximum EL was observed in both the algae *C. vulgaris* (47.02%) and *C. humicola* (38.07%) cells treated with 500 mM concentration of NaCl. Electrolyte leakage is directly linked with membrane injury due to high NaCl concentration and osmotic imbalance. High level of NaCl in algal cell induces the efflux of K^+ leakage which ultimately changes the membrane polarization and significantly diminishes the osmotic balance due to release of K^+ present in cytosol (Demidchik et al., 2014).

Table 4.4: TBARS (mmol g^{-1} fw) electrolyte leakage (µs cm^{-1}) and H_2O_2 (µmol g^{-1} fw) content of *C. vulgaris* and *C. humicola* treated with different concentration of NaCl (25-1000 mM). All the values are means ± SD. ANOVA post hoc DMRT was done to check the variation between the variables. Identical letter denotes no significant difference ($p \leq 0.05$)

Treatments NaCl (mM)	***C. vulgaris***		
	TBARS	**EL**	**H_2O_2**
C. vulgaris			
Control (0)	99.78^{a} ±3.18	86.49^{a} ±1.31	176.48^{a} ±1.87
25	108.82^{a} ±2.40	93.34^{b} ±1.49	227.95^{b} ±3.93
50	157.63^{b} ±5.36	170.16^{e} ±1.39	265.64^{c} ±5.92
100	287.53^{c} ±23.72	97.08^{c} ±1.49	277.93^{d} ±4.38
500	336.34^{d} ±22.57	100.93^{d} ±1.69	312.50^{e} ±2.48
1000	354.84^{d} ±2.00	85.11^{a} ±1.99	319.85^{f} ±2.89
C. humicola			
Control	104.30^{a} ±2.67	70.36^{a} ±1.23	198.37^{a} ±2.28
25	103.23^{a} ±9.99	97.07^{d} ±1.13	230.58^{b} ±4.87
50	108.60^{a} ±10.72	90.31^{c} ±1.93	251.91^{c} ±5.71
100	230.11^{b} ±49.49	90.85^{c} ±1.21	267.82^{d} ±6.87
500	288.82^{c} ±29.48	97.14^{d} ±1.83	272.62^{de} ±4.01
1000	369.89^{d} ±5.24	82.56^{b} ±1.23	280.19^{e} ±7.75

4.10.4 Effect on cysteine, proline and ascorbic acid

Cysteine is –SH containing amino acids which contributes significantly to both enzymatic and non-enzymatic antioxidative defense system in the cells. The cysteine content in algae *C. vulgaris* and *C. humicola* treated with various concentration of NaCl was enhanced in proportion to concentration of NaCl (Fig. 4.6A). Maximum increase in cysteine content in both algae *C. vulgaris* and *C. humicola* was observed at 1000 mM concentration of NaCl i.e., 33.41% and 62.81% respectively as compared to control. The results indicated higher salt tolerance response in *C. humicola* against the salinity stress. Similarly, both the algal strains *C. vulgaris* and *C. humicola* treated with 25 mM concentration of NaCl showed minimal increase in the –SH content (cysteine) which was about 15.6% and 14.06%

respectively. Cysteine works as a precursor of different thiols containing antioxidants produced under oxidative stress and thus it is an indicator of stress in algae and plant cells (Wirtz et al., 2010). High cysteine content in both the algal strains exhibited tolerance response of the algae against stresses (Upadhyay et al., 2016). Proline contents in microalgae treated with different concentration of NaCl was also increased with increasing salinity (Fig. 4.6B). *C. humicola* treated with 25 mM NaCl showed no significant change in the proline content as compared to control ($p \leq 0.05$). Maximum increase (>3 fold) in the proline content was observed in *C. vulgaris* at 500 mM concentration of NaCl, while in *C. humicola*, proline was maximum at 1000 mM concentration of NaCl. Proline is an important osmolyte and antioxidant in algae in response to osmotic and other stress. An increase in the production of proline is direct indicator of its protective role in scavenging of free radical, stabilizing subcellular structure, maintains osmotic imbalance and homeostasis of the cell (Zouari et al., 2016).

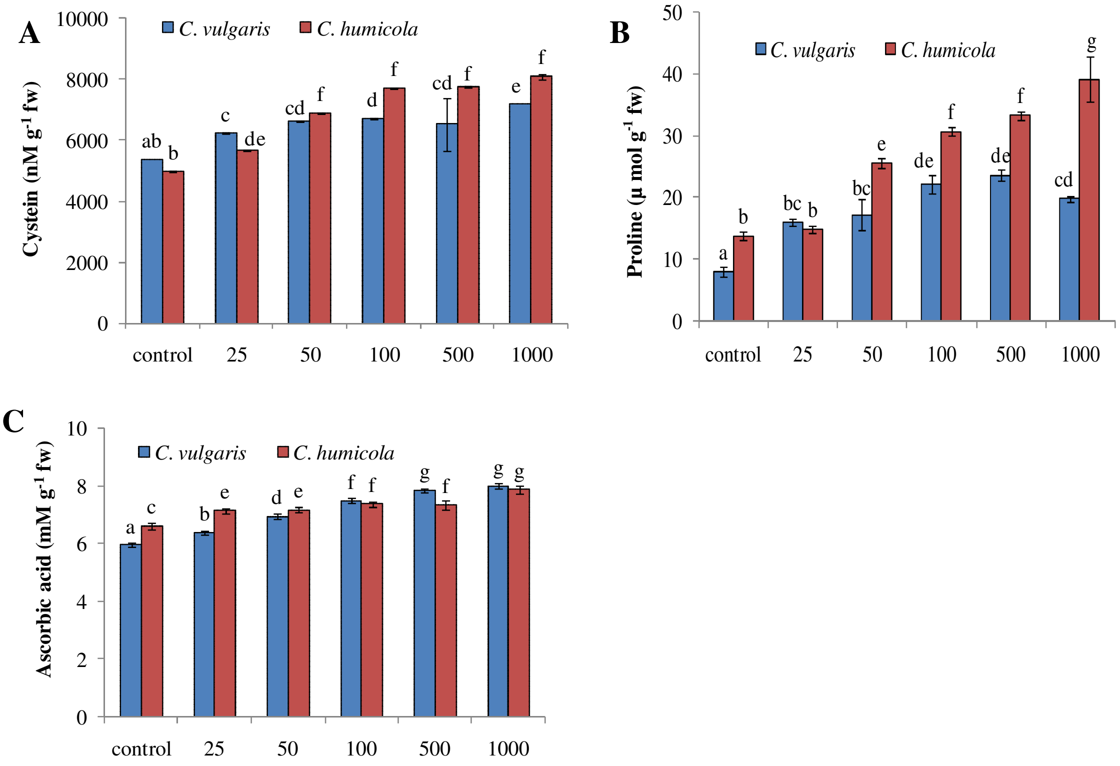


Fig. 4.6: Effect on cysteine (A), proline (B), and ascorbic acid (C) content in algae *C. vulgaris* and *C. humicola* treated with different concentrations of NaCl (0-1000 mM). All the values are means ± S.D. ANOVA significant at level of ($p \leq 0.05$) according to one- way ANOVA test. Identical superscripts denote no significant difference between means in column according to DMRT ($p \leq 0.05$)

Ascorbic acid (AsA) functions as an antioxidant in plant cell under stress condition. In the present study, the concentration of ascorbate in the case of algae *C. vulgaris* increased in proportion to increasing concentration of NaCl (50-1000 mM) (Fig. 4.6C). Algae treated with 25 mM concentration of NaCl exhibited slight increase in the ascorbate content of both *C. vulgaris* and *C humicola* (7.3% and 7.8%, respectively) when compared to their respective control.

However, at 1000 mM concentration of NaCl the ascorbate content was enhanced by 34.4% in *C. vulgaris* and 19.0% in *C humicola*. Overall results indicated that *C. humicola* was better adopted to salinity stress than *C. vulgaris*. Ascorbic acid is the most abundant antioxidant of intracellular origin, maintains the cellular redox balance produced due to oxidative burst of reactive oxygen molecules (Vidal-Meireles et al., 2017). The NaCl induces accumulation of high level of ascorbate is counter balance of algal cells against NaCl induced oxidative injury. Ascorbate induced protection might be attributed to enzyme cascade involved in scavenging of oxyradicals conversion of H_2O_2 and ascorbic acid viz., MDHA, DHA etc. in step-by-step reaction (Shereefa et al; 2016).

The overall increase in the level of non-enzymatic antioxidants viz., proline, cysteine and ascorbic acid in response to NaCl stress presented an interesting observation on intracellular antioxidative defense system in both the microalgae.

4.10.5 Effect on antioxidant enzyme activities

Salt induced oxidative stress in plants is also counteracted by the production of various antioxidants and enzymes including, SOD, catalase, peroxidases and GR (Hossain and Dietz, 2016). Effect of salt on the activity of different antioxidative enzymes are depicted in Fig. 4.7. Microalgae *C. vulgaris* and C. *humicola* treated with different concentration of NaCl showed a significant enhancement in the SOD activity (56.79% and 46.8%), respectively at 500 mM concentration of NaCl when compared to their respective untreated control (Fig. 4.7A). The catalase activity was increased according to the concentration gradient (Fig. 4.7B). *C. humicola* cells exhibited no significant change at 25 mM concentration of NaCl (1.51 Ug^{-1} fw) and 50 mM concentration of NaCl (1.57 Ug^{-1} fw). However, catalase activity in *C. vulgaris* was increased by 2.52% at 50mM concentration of NaCl as compared to catalase activity at 25 mM concentration of NaCl and control. Maximum catalase activity was observed at 1000 mM concentration of NaCl in both the *C. vulgaris* (2.87 Ug^{-1} fw) and *C. humicola* (2.74 Ug^{-1} fw) cells as compared to their respective controls (0.65 and 0.49 Ug^{-1} fw, respectively). Glutathione reductase (GR) activity in algae C. *vulgaris* and *C. humicola* under salinity stress was also increased with the increasing concentration of NaCl (Fig. 4.7C). Maximum activity in *C. vulgaris* of GR was observed at 500 mM concentration of NaCl (0.228 mmol mg^{-1} fw) was more or less the same as in *C. humicola* (0.252 mmol mg^{-1} fw).

The SOD is an important metallozymes which is an effective scavenger of reactive oxygen species and dismutates the superoxide ion (O_2^-) into peroxide (H_2O_2) and water (Upadhyay et al., 2016). The catalase enzyme is known to scavenges the H_2O_2 and transform it into H_2O and O_2 in peroxisome. High catalase activity in the algae represents their high toxicity ameliorating potential under oxidative stress (Rai et al., 2013). Glutathione reductase (GR), reduces the level of GSSG to GSH in the cytoplasm produced as a consequence of H_2O_2 formation during the stress and thus, maintains the redox balance in the cell (Ding et al., 2016). An increase in the GR activity represents stress tolerance responses of algae (Cheng et al., 2016).

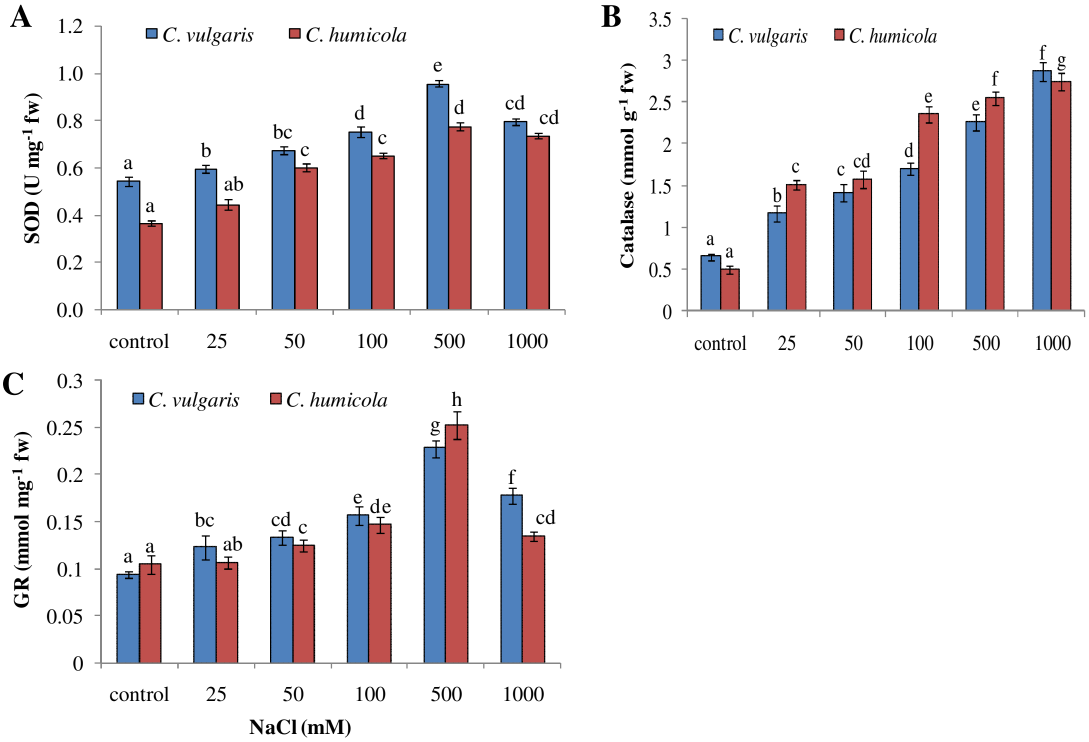


Fig. 4.7: Effect on SOD (A), catalase (B) and GR(C) activity in algae *C. vulgaris* and *C. humicola* treated with different concentration of NaCl. All the values are means ± S.D. ANOVA significant at level of ($p \leq 0.05$) according to one- way ANOVA test. Identical superscripts denote no significant difference between means in column according to DMRT ($p \leq 0.05$)

4.11 Conclusions

The present findings concluded that microalgae *C. humicola* is better adopted to NaCl stress than *C. vulgaris*. Results concluded that algae *C. humicola* and *C. vulgaris* can tolerate NaCl to a concentration of 100 mM. The activities of superoxide dismutase, catalase and glutathione reductase in *C. humicola* and *C. vulgaris* in response to NaCl stress were increased by 2.5-5 fold as compared to their respective control. Further, SEM showed distorted cell morphology and increased cell size by 33.52% (*C. humicola*) and 27.79% (*C. vulgaris*) at 100 mM NaCl. The FTIR analysis revealed that *C. humicola* exhibited higher lipid content than *C. vulgaris* under NaCl stress.

CHAPTER 5

Effect of UV-B on photosynthetic performance, nutrient status and lipid yield in microalgae *Chlorella vulgaris* and *Chlorococcum humicola*

5.0 Effect of UV-B on photosynthetic performance, nutrient status and lipid yield in microalgae *Chlorella vulgaris* and *Chlorococcum humicola*

5.0 Introduction

Since the time immemorial, energy is a fundamental pre-requisite for the existence of lives on the earth. The major source of energy is fossil fuel which is on the verge of extinction due to over exploitation by human beings (Gasparatos et al., 2017) and has caused a paradigm shift towards a search for renewable energy resource to meet the growing demand of energy. The renewable energy sources include solar energy, agricultural crops (Maize, Sunflower), non-agricultural crops (*Jatropha* sp.) and algae (Voloshin et al., 2016). Over the past few years, microalgae- a third generation biofuel, have worked as a bridge in the production of bio-energy against limited water, land and environmental stress (Stockenreiter et al., 2016). The cultivation of microalgae is more ecofriendly and economical as they can be cultivated on waste water and unfertile land. Amongst the various environmental stressors, solar ultraviolet radiation (UVR) emission has profound influence on the chemical composition of the flora, fauna and the environment also (Madronich et al., 2018). It has also been reported that UVR plays significant role in the generation of various environmental problems such as photochemical smog, photo damage, change in precipitation pattern, coral bleaching, vegetation loss, agricultural escalation and changes in the ecosystem services etc. (Jansen et al., 1998; Danovaro et al., 2008; Comont et al., 2013; Kataria et al., 2014; Williamson et al., 2014).

UV-B irradiance (5% of total UVR) by the sun is a serious hazard for living beings on the earth (Herndon et al., 2018). Algae, being dominant photosynthetic microorganisms in upper layer of aquatic system are more prone to high UVR exposure leading to photo bleaching of algae coupled with reduced upwelling of nutrients and oxidative damage (Williamson et al., 2019). In addition, UVR causes inhibition of photosynthesis in algae, damage the efficiency of the biological pumps, fixation of CO_2 and their subsequent transformation into the organic matter (Mata et al., 2010). However, microalgae have shown great tendency to acquire high degree of adaptation against UVR during the course of evolution (Holzinger et al., 2018). The high adaptability of microalgae against UVR is not only helpful in lipid production but is also beneficial for accumulation of nutrients, carbon sequestration, environmental purification, nutrient cycling and production of oxygen in the environment (Brennan and Owende, 2010; Mata et al., 2010).

UV- B (280-320 nm) is reported to have both detrimental and beneficial effects on plants, animals and microalgae (Hockberger, 2002). In animals, UV-B causes skin diseases, dermatitis, DNA damage, mutation, and formation of dimmers (cyclobutane pyrimidine) (Martinez et al., 2015; Zhang et al., 2015). In algae UV-B induces lipid peroxidation, biochemical alteration and photosystem II performance by modulating quantum efficiency, reaction centre (RC) inactivation, by net closing of PS II reaction centre, overall photosynthetic performance index (algal cell vitality) and transfer of electron from RC to electron acceptor etc (Strasser et al., 2004; Markou et al., 2017). To cope with the harmful effect of UV-B, algae are endowed with certain physical and physiological responses including migration, development of protective cell wall, increased synthesis of carotenoids, compatible solute (compound with low molecular weight organic compounds) and adaptation (Seckbach 2007; Zubia et al., 2010; Rastogi et al., 2013).

Microalgae *C. vulgaris* and *C. humicola* were selected to study the effect of various stressors due to their several proficient characteristics viz., fast growth rate, high adaptive response and ability to withstand variety of adverse environmental conditions. It is an ideal tool due to smaller size, short generation time and faster growth with minimal nutritional requirement such as light CO_2 and water.

Various studies have been reported that low dose of UVR stimulate algal growth, while higher doses show growth inhibitory effect on microalgae (Tossi et al., 2009; Singh et al., 2015; Cheng et al., 2016). The present study was aimed to examine the sustainability of algae as next generation biofuel feed stock under high UV-B radiation. As incidence of UV radiation exposure is continuously increasing, the response of microalgae under such stress was studied to the selected algal strain for fulfilling the future demand of high biomass and energy. The present work includes the study of the UV-B irradiance induced changes in the growth, morphology, biomass productivity, tolerance responses and lipid production in microalgae *C. vulgaris* and *C. humicola*.

5.1 Materials and methods

5.1.1Collection of algal strains

Microalgae *C. humicola* was procured from the School of biosciences, MG University, Kerala, India, while, C. *vulgaris* was isolated in the laboratory of DES, BBA, University, Lucknow. The mass culture of both microalgae was obtained under the controlled culture room (24±2°C at 120 μmol photo $nm^{-2}s^{-1}$) with photosynthetically active radiation (PAR) using a photoperiod of 16:8 h (Singh et al., 2018b). All the cultures were maintained in Erlenmeyer flasks (1000 ml) containing 200 mlBG11media.The pH of the medium was adjusted to 6.5-7.5 for the proper growth of the algal strains. The cultures were manually shaken 3-4 times/day for proper aeration as well as for avoiding the cells setting/striking on the walls in the flask.

5.1.2 Experimental setup

All the experiments were performed in the laboratory of Department of Environmental Science, Babasaheb Bhimrao Ambedkar University, Lucknow, India to examine the long term impact of UV-B+PAR (400-700 nm). Pure algal cultures of *C. humicola* and *C. vulgaris* were inoculated in 500 ml Erlenmeyer flasks containing 200 ml BG11 media for growth and acclimatization in the culture room. The inoculated cultures were acclimatized for 24 h under controlled culture conditions. The 72 h old cultures were exposed to UV-B radiations of intensity 15 Wm^{-2} for different duration of 30 min, 1 h, 2 h, 3 h and 4 h with time 24 h interval for a period of 15 d (saturation phase) in a closed UV chamber. The UV-B exposure was provided to cell suspension kept in a sterile, wide mouth plastic container to ensure the homogeneous exposure of cells to UV-B. The closed UV chamber was fitted with three UV-B tubes (Philips TL; 40W) with a wavelength range from 280 to 320 nm. After exposure of the cells to UV-B, the treated cell suspensions were transferred to culture room equipped with PAR. A control set of cell suspension without UV-B treatment was placed under PAR light. All the experiments were performed in triplicates. The cells harvested during the stationary phase of their growth (15 d) and the collected cell samples were refrigerated after centrifugation and stored at 4°C for physiological and biochemical analysis.

5.2 Pigment estimation

5.2.1 Estimation of chlorophyll

After centrifugation, 100 mg cells (fresh weight) were crushed in 3 ml of 80% acetone (w/v) with the help of pestle and mortar thereafter, the sample was placed under dark cold (4^oC) condition for 10 h. Then the suspension was centrifuged at 8000×g for 5 minutes to collect the cell suspension (Arnon, 1949). The supernatant was used for the estimation of chlorophyll content with the help of UV-visible double beam spectrophotometer at the wavelength of 663 and 645nm. The carotenoids content in the extract was analyzed at the wavelength of 480 and 510 nm using the formula described by Yentsch and Duxbury (1956).

5.2.2 Photosynthetic performance

The photosynthetic performance viz., chlorophyll-a fluorescence induction (OJIP) curve, photosynthetic performance index (Pi_{ABS}), maximum quantum yield (Fv/Fm), Net closing rate of reaction centre (Mo), Trapping flux (TRo/RC), effective antenna size expressed in terms of absorbance per reaction centre (ABS/RC) and percent inhibition of RC in microalgae *C. humicola* and *C. vulgaris* were measured in both UV-B treated and untreated (control) cell by using PAM fluorimeter Systems Instruments, reaction centre Aqua Pen-C, Czech, Republic.

The Aqua Pen-C was set to maximum pulse intensity of 3000 μmol photon $m^{-2}s^{-1}$ of red light for 10s, while rapid chlorophyll fluorescence induction curve was measured in the dark adapted cells at room temperature.

5.3 Biochemical characterization

5.3.1 Estimation of protein

100 mg freshly prepared algal cells centrifuged pellet was crushed in 5 ml 10% chilled trichloroacetic acid (TCA) and centrifuged at 10,000×g for 10 min. After decanting the supernatant, the cell pellet was washed and re-suspended in 5 ml of 1N NaOH and heated for 15 min, cooled and then centrifuged at 10,000×g for 10 min. The cell protein was estimated by the methods of Lowry et al. (1951), using bovine serum albumin (Sigma Aldrich) as a standard protein.

5.3.2 Estimation of carbohydrate and total lipid

25 mg fresh algal cells were homogenized in 3 ml of 80% ethanol and centrifuged at 3000×g for 10 min. 1.0 ml of 1.0% phenol solution (v/v) and 5.0 ml of concentrated H_2SO_4 were added to 1.0 ml supernatant and mixed thoroughly. The mixture was kept at room temperature for 30 min and the absorption was recorded at the wavelength of 480 nm as described by Dubois et al. (1956). Total lipid of algal pellets was estimated by using the vanillin method as described by Ben-Amotz et al. (1985).

5.3.3 Estimation of lipid peroxidation, electrolyte leakage and H_2O_2

Lipid peroxidation in the form of TBARS (thiobarbituric acid reactive substance) content in algae was estimated by the method of Heath and Packer (1968). 100 mg of algal sample was crushed in 3 ml 0.1% TCA and centrifuged at 10,000×g for 10 min. 1 ml of aliquot of the supernatant was mixed with 4 ml of TBA reagent (%) and the mixture was heated at 95ºC for 30 min, followed by cooling in an ice bath. The reaction mixture was centrifuged at 10,000×g for 10 min. The absorbance of supernatant was taken at two different wavelengths of 532 and 600 nm, respectively for correction of nonspecific turbidity.

Electrolyte leakage in algal cell was estimated by the methods of Dionisio-Sese and Tobita (1998). H_2O_2 was estimated following the method of Velikova et al. (2000). For the estimation of H_2O_2, 100 mg algal biomass was extracted in 2 ml chilled TCA (0.1% w/v) under cold conditions and thereafter, centrifuged at 12000×g for 10 min. The reaction mixture containing 0.5 ml phosphate buffer (10 mM, pH 7.0) and 0.5 ml supernatant was allowed to react 1ml KI solution. The absorbance of reaction mixture was recorded at 390 nm after 5 min of incubation. H_2O_2 content was determined by using known standard curve of H_2O_2.

5.3.4 Estimation of ascorbic acid, proline and cysteine

Ascorbic acid was estimated by following the methods of Kampfenkel et al. (1995). Proline content in algal cell was determined by the methods of Bates et al. (1973). Cysteine content was determined by the protocol given by Gaitonde (1967).

5.4 Sample preparation and analysis of SEM coupled with EDS

The algal strains were fixed in 2.5% of glutaraldehyde for 4 h at 4^{o}C and then washed with phosphate buffer. Subsequently dehydration of cells was done by ethanol (10%, 30%, 50%, 70% and 95%) for 5 min at each concentration (Prakash and Nawani, 2014). The final dehydration was carried at 100% ethanol for 30 min. The dehydrated samples were mounted on aluminum stub with the help of carbon tape. SEM coupled with energy dispersive X-ray spectrometer (EDS) detector was used to obtain the X-ray spectra, using EDS in conjunction with scanning electron microscopy (SEM) (JEOL, Japan; model JSM-6490LV).

5.5 Fourier transform infrared spectroscopy (FTIR) analysis

To study FTIR spectrum in algae, the dried algal samples were oven dried at 60^{o}C for 2 hr and the dried cell biomass was grinded and powdered. The cell powder was mixed with IR grade potassium bromide (KBr, 1:10 ratio) and to form pellet, using hydraulic press (150 lbs). The pellet was furhter positioned and fixed into socket of a dislocated in FTIR machine for the determination of IR spectra. The absorption spectra were recorded using FTIR (Thermo-Scientific Nicole 6700, USA) in the range of 400-4000 cm^{-1} with 5-7 cm^{-1} resolution and 32 scan number for each spectrum (Singh et al., 2018b). The background absorbance given by KBr was subtracted by using omies software.

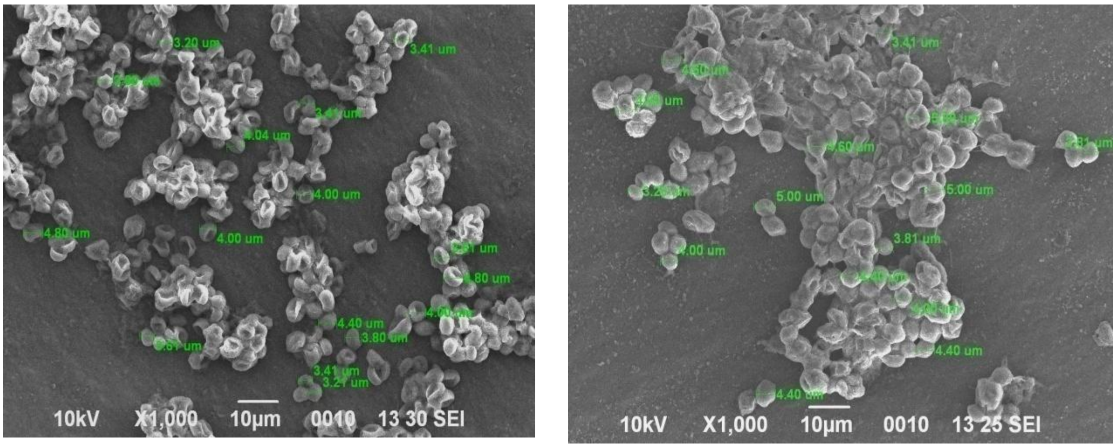
5.6 Data analysis

All the experiments were performed in triplicates. To compare the validity and variability between groups and among each other, on-way analysis of variance (ANOVA) was performed, followed by Duncan’s multiple range test (DMRT, $P \leq 0.05$). All the statistical analysis was carried out using SPSS software.

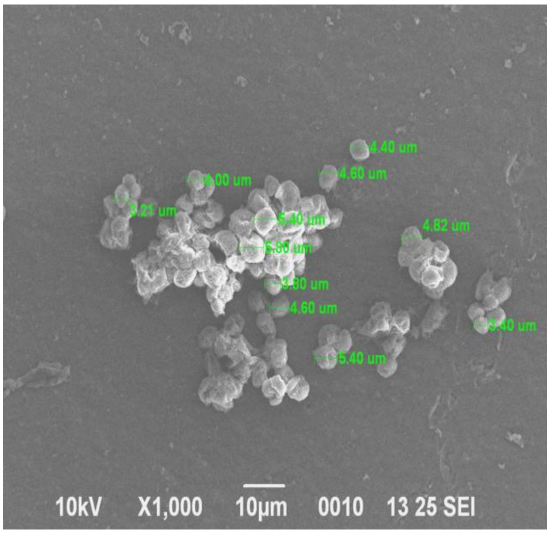
5.7 Results and discussion

5.7.1 Effect on morphology, ultra structure and growth

The structural changes in the algae *C. vulgaris* and *C. humicola* were determined in terms of shape and size by using SEM (JEOL, Japan, Model No. JSM-6490 LV) (Fig. 5.1 and 5.2). The cells of *C. vulgaris* and *C. humicola* were exposed to different duration of UV-B treatment (½-4h) exhibited increased cell size in comparison to control (without UV-B treatment). The size of cells was measured by taking average of 15 cells through SEM software (control user interface version 3.8). SEM images showed distorted cell (from round to oval in appearance), structured, sticking with each other, which was more distinct in the case of *C. humicola* (Fig. 5.2) as compared to *C. vulgaris* (Fig. 5.1) at 4 h after UV-B exposure.



***C. vulgaris* control** ***C. vulgaris* UV-B treated (2 h)**

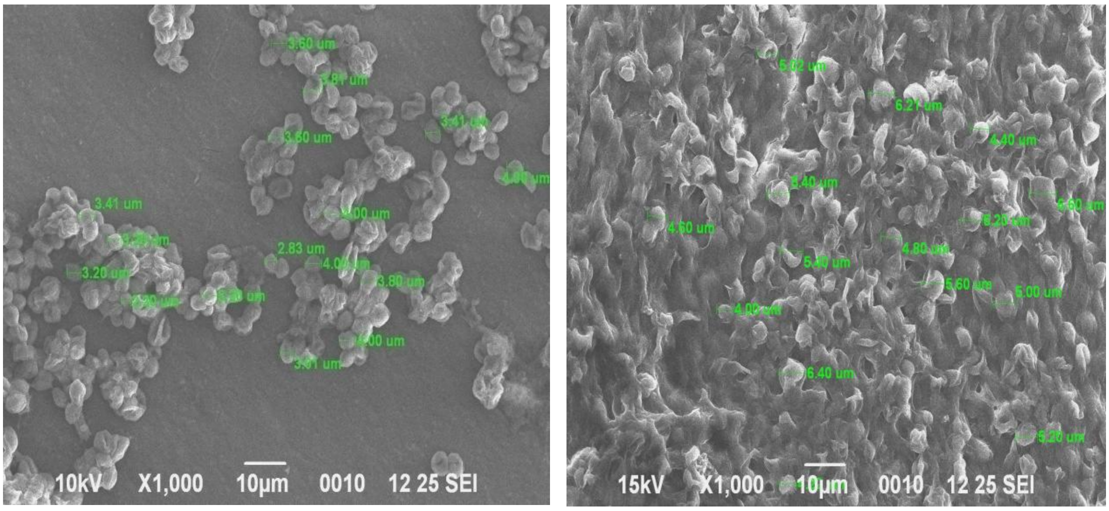


***C. vulgaris* UV-B treated (4 h)**

Fig. 5.1A: Scanning electron micrograph in control and UV-B exposed algae *C. vulgaris*

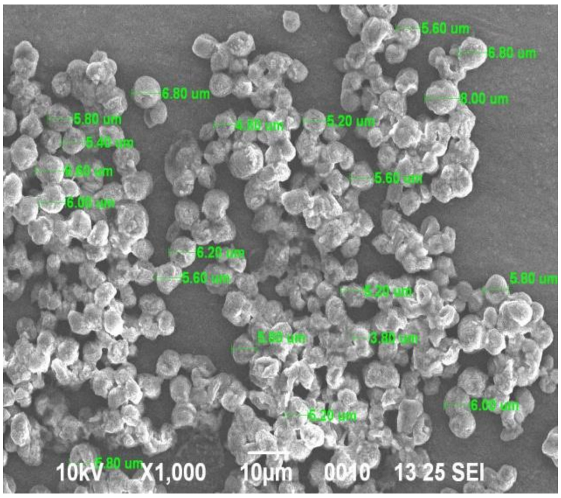
The average cell size of *C. vulgaris* in untreated culture was 3.87 µm but it increased to 4.46 µm after 4 h of UV-B exposure (Fig. 5.1A). However, the cells of *C. vulgaris* treated for 2 h showed average size of 4.06 µm as compared to untreated control. In the case of *C. humicola*, the cell size was significantly increased by 64.6% in comparison to control after 4h of UV-B exposure (Fig. 5.1B). SEM images of *C.*

vulgaris and *C. humicola* exposed to different duration of UV-B showed increase in cell size depending upon the exposure time. The UV-B induced increase in cell size may be ascribed to the production of different secondary metabolites and compatible solutes (Singh et al., 2018a). Fahy et al. (2017) reported that under stress condition the process of photosynthesis and cell division is hampered in algal cells which lead to an increase in the cell size with no distinct cell division. An increase in cell size is directly linked to accumulation of secondary metabolites as reported in earlier studies (Singh et al., 2018b). EDX analysis of high metals is related to occurrence and UV-B induced modulation of different functional group which may either chelate with metals on the surface due to transport of metals inside the cell (Singh et al., 2018).



***C. humicola* control**

***C. humicola*UV-B treated (2 h)**

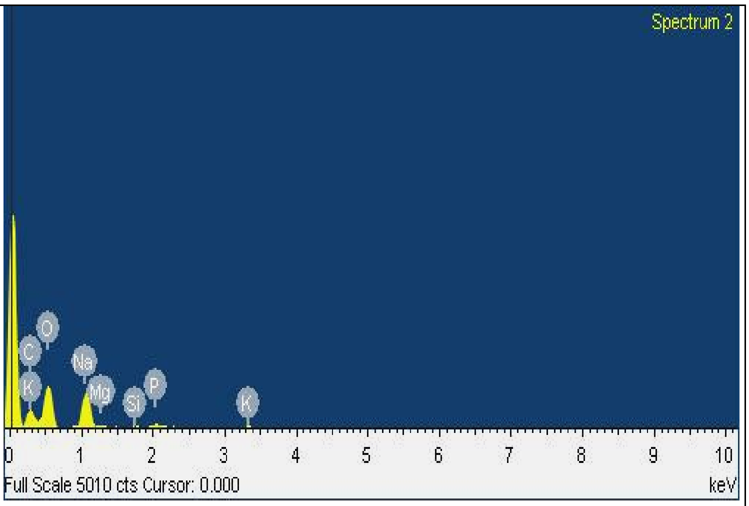


***C. humicola* UV-B treated (4 h)**

Fig. 5.1B: Scanning electron micrograph in control and UV-B exposed algae *C. humicola*

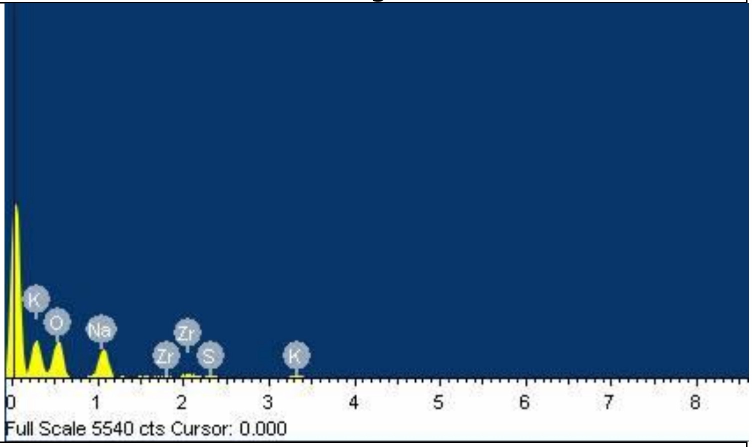
The data on energy dispersive x-ray spectroscopy revealed alteration in the concentration of micro and macro elements of the cells exposed to different duration of UV-B. The elemental constituents in the cell were measured by EDS in terms of atomic number (% atomic) and atomic weight (% weight) of elements based on energy dependent localization of different elements in their respective shells (K, L, M and N) around the nucleus of the atoms (elements). The EDS data exhibited reduction in mineral contents in *C. vulgaris* and *C. humicola* cells exposed to UV-B radiation for 4 hr, when compared with their respective untreated control (Fig. 5.2A and 5.2B).

Element	Weight %	Atomic %
C K	19.59	26.51
O K	55.63	56.52
Na K	21.22	15
Mg K	1.14	0.76
Si K	0.44	0.26
P K	1.18	0.62
K K	0.79	0.33
Totals	100	



***C. vulgaris* control**

Element	Weight %	Atomic %
O K	62.56	73.28
Na K	29.16	23.77
S K	1.67	0.98
K K	2.23	1.07
Zr L	4.38	0.9
Totals	100	



***C. vulgaris* UV-B treated**

Fig. 5.2A: EDS spectra of control and UV-B exposed alga *C. vulgaris.* The EDS data is shown in term of percent atomic and percent weight of different elements present in the alga

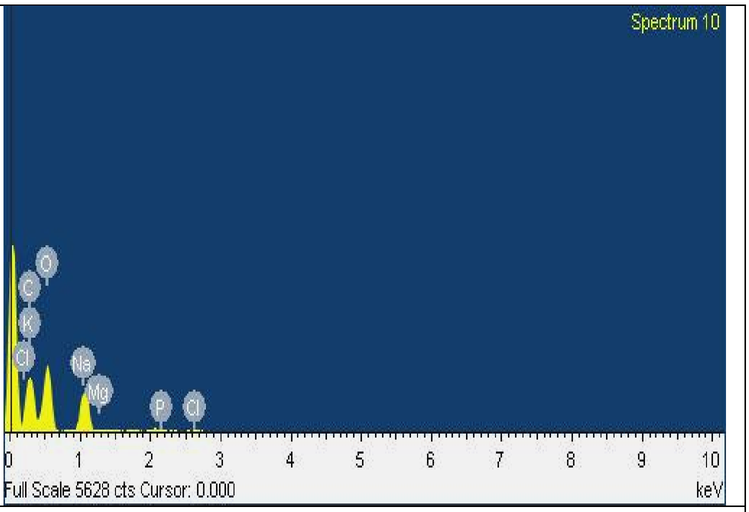
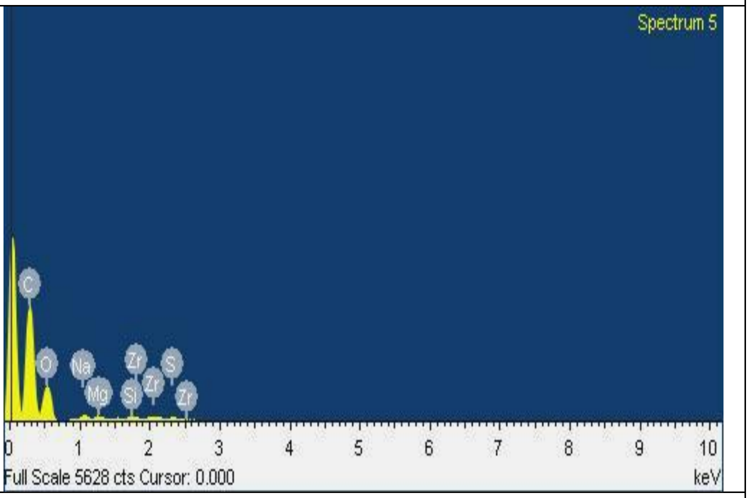
<table>
<tr><td>Element</td><td>Weight %</td><td>Atomic %</td><td rowspan="9">
</td></tr>
<tr><td>C K</td><td>25.81</td><td>33.37</td></tr>
<tr><td>O K</td><td>57.09</td><td>55.41</td></tr>
<tr><td>Na K</td><td>15.08</td><td>10.19</td></tr>
<tr><td>Mg K</td><td>0.65</td><td>0.41</td></tr>
<tr><td>P K</td><td>0.57</td><td>0.28</td></tr>
<tr><td>Cl K</td><td>0.48</td><td>0.21</td></tr>
<tr><td>K K</td><td>0.32</td><td>0.13</td></tr>
<tr><td>Totals</td><td>100</td><td></td></tr>
<tr><td colspan="3"></td><td><i>C. humicola</i> control</td></tr>
<tr><td>Element</td><td>Weight %</td><td>Atomic %</td><td rowspan="9">
</td></tr>
<tr><td>C K</td><td>59.92</td><td>67.33</td></tr>
<tr><td>O K</td><td>37.32</td><td>31.48</td></tr>
<tr><td>Na K</td><td>0.89</td><td>0.52</td></tr>
<tr><td>Mg K</td><td>0.46</td><td>0.26</td></tr>
<tr><td>Si K</td><td>0.31</td><td>0.15</td></tr>
<tr><td>S K</td><td>0.33</td><td>0.14</td></tr>
<tr><td>Zr L</td><td>0.76</td><td>0.11</td></tr>
<tr><td>Totals</td><td>100</td><td></td></tr>
<tr><td colspan="3"></td><td><i>C. humicola</i> UV-B treated (4 h)</td></tr>
</table>

Fig. 5.2.B: EDS spectra of control and UV-B exposed alga *C. humicola*. The EDS data is shown in term of percent atomic and percent weight of different elements present in the alga

In the case of *C. vulgaris*, the percent weight of elements viz., C, O, Na, Mg, Si, P and K localized in K shell were 19.59, 55.63, 21.22, 1.14, 0.44, 1.18 and 0.79, respectively whereas different elements in UV-B exposed cells increased in the order of 62.56 (O), 29.16 (Na) and 2.23 (K). However, other elements were diminished significantly (Fig. 5.2A). In the case of *C. humicola*, the percent weight of O, Na and Mg were reduced by 34.62%, 94.09% and 29.23%, respectively as compared to control (Fig. 5.2B). However, the content of carbon (C) was significantly increased by about 2.5 fold in comparison to control. The percent weight of elements Si (0.31) and sulphur (0.33) were observed in UV-B exposed *C. humicola*, but not detected in untreated control cell (Fig. 5.2B). Decreased mineral content under UV-B exposure may be ascribed to alteration in the stomatal conductance, membrane composition, permeability and cell deformation which ultimately limit the uptake and sorption of important minerals in algae (Zouari et al., 2016).

5.7.2 Effect on photosynthetic pigment

The chlorophyll content in microalgae *C. vulgaris* and *C. humicola* exposed to UV-B stress showed exposure time dependent decline in chlorophyll content (Table 5.1) except ½ h UV-B exposed cells in both the algae. The chlorophyll concentration in *C. vulgaris* and *C. humicola* given ½ h UV-B exposure showed increase in the chlorophyll content by 16.21% and 21.12%, respectively when compared with untreated control. The decreasing trend in chlorophyll content was directly correlates to exposure time. Maximum decrease in the chlorophyll content of *C. vulgaris* and *C. humicola* i.e., 60.81% and 60.56%, respectively was observed after 4 h of UV-B exposure. However, after 3 h of UV-B exposure, both the algae showed about 33.78% (*C. vulgaris*) and 25.35% (*C. humicola*) reduction in chlorophyll level. The results suggested that 4h of UV-B exposure to both the strains expressed more toxicity as compared to other treatments. Perhaps UV-B induced degradation of pigments was responsible for decrease in pigment content.

The accessory pigment carotenoid showed enhanced level of this pigment with the increase in time of UV-B exposure upto 1 h in both the algae (Table 5.1). The carotenoid content in *C. humicola* at 1 h UV-B exposure was found to be increased by 33.82%, while it was increased by 20.25% in the *C. vulgaris* cells when compared with their respective control. Similarly, at ½ h, 2 h, 3 h and 4 h UV-B exposure to algal cells, the carotenoid level was increase in the order of 20.82%, 17.80% 3.88% and 1.02%, respectively, in *C. vulgaris* and 14.38%, 20.36%, 16.59% and 8.22%, respectively in *C. humicola*. The overall results emphasized that decrease in the chlorophyll level and increase in carotenoid content in both microalgae under the UV-B flux indicating the physiological stress status of the cells was due to disruption of various metabolic process viz., electron transport system, photosystem II, ROS production and pigment bleaching (Pathak et al., 2019). Various authors have also reported UV-B induced decrease in the chlorophyll content of microalgae exposed to UV-B radiation (Gao and Ma, 2008; Prasad and Zeeshan, 2005; Tian and Yu, 2009; Zeeshan and Prasad, 2009; Zhang et al., 2015). However, at ½ h UV-B exposed cells showed enhanced chlorophyll content in algae described the additive response in the algae as suggested by Núñez-Pons et al. (2018). Figueroa et al. (2009) also reported the positive response of UV-B in algae *Zygnemopsis decussate* subjected to short duration of UV exposure. On the other hand, Carotenoid/ Chl a ratio were calculated to evaluate the intensity of stress condition of the algal cells (Table 5.1).

Table 5.1: Effect of UV-B on the chlorophyll (mg g^{-1}fw) and carotenoid (mg g^{-1}fw) content in algae *C. vulgaris* and *C. humicola*. All values are in means ±SD. ANOVA significant at level of ($p \leq 0.05$) according to one- way ANOVA test. Identical superscripts denote no significant difference between means in column according to DMRT ($p \leq 0.05$)

Duration of UV-B treatment	Chl a	Chl b	Total chl	Carotenoid	Carotenoid/ chl a
			C. vulgaris		
Control	0.0195^{bc}±0.0017	0.0541^{c}±0.0008	0.074^{cd}±0.001	0.984^{a}±0.028	0.00
1/2 h	0.0327^{ef}±0.0016	0.0532^{c}±0.0025	0.086^{d}±0.003	1.184^{cd}±0.022	36.20
1 h	0.0295^{de}±0.0024	0.0446^{bc} ±0.0011	0.074^{d}±0.009	1.183^{cd}±0.003	40.10
2 h	0.0228^{bc}±0.0044	0.0435^{bc} ±0.0097	0.066^{bcd}±0.014	1.159^{c}±0.002	50.83
3 h	0.0172^{b}±0.0034	0.0322^{ab}±0.0076	0.049^{b}±0.011	1.022^{a}±0.002	60.11
4 h	0.0103^{a}±0.0053	0.0188^{a}±0.0001	0.029^{a}±0.001	0.994^{a}±0.002	76.46
			C. humicola		
Control	0.0190^{b}±0.0014	0.0521^{c}±0.0122	0.071^{cd}±0.011	1.017^{a}±0.032	0.00
1/2 h	0.0348^{f}±0.0061	0.0515^{c}±0.0016	0.086^{d}±0.004	1.164^{c}±0.071	33.44
1 h	0.0265^{cd}±0.0043	0.0422^{bc}±0.0095	0.069^{bcd}±0.014	1.361^{e}±0.001	51.35
2 h	0.0244^{bc}±0.0051	0.0410^{bc}±0.0093	0.065^{bcd}±0.015	1.225^{d}±0.003	50.20
3 h	0.0188^{b}±0.0051	0.0343^{b}±0.0093	0.053^{bc}±0.014	1.186^{cd}±0.002	63.08
4 h	0.0100^{a}±0.0057	0.0182^{a}±0.0099	0.028^{a}±0.001	1.101^{b}±0.002	110.1

The results showed higher Carotenoid/ Chl a ratio with increase in the duration of UV-B exposure which could be due to photoprotection response of carotenoid in algal cells. The highest Carotenoid/ Chl a ratio was observed in the cells exposed to UV-B for 4h in the both algae as compared to their respective control. Carotenoids protect the algal cells from photo damage by acting as antioxidants (Pick et al., 2019) by quenching high amount of energy liberation during excitation and released in the form of heat (Takahashi et al., 2018). In the present study, an increase in carotenoid level indicated better UV tolerance in both the algae. The stress condition of the algae produced due to UV-B radiation can also evaluated by the ratio of carotenoid to Chl a and change in the ratio clearly demonstrated the self photoprotective response of the cells. Under stress, production of secondary metabolites enhances the cell size, synthesis of carotenoid and lipid content in the cells as reported by various authors (Czerpaket al., 2002; Singh et al., 2018b).

5.8 Impact on photosynthetic performance

O-J-I-P curve describes the performance of photosystem II in a cell. The OJIP fluorescence curve is directly related to reduction of plastoquinone (PQ) pool mediated by transfer of electrons. A short fluorescence transient were analyzed for a short duration of O (50µs), J (2 ms), I (60 ms) and P (250-400 ms) under UV-B radiation in both the algae. The specific photosynthetic parameter based on OJIP fluorescence such as Fv/Fo, Fv/Fm (photosynthetic fluorescence ratio), Mo, Pi_{Abs}, ABS/RC and TRo/RC etc. are associated with photochemistry and functioning of photosystem II (Table 5.2). The OJIP results showed that the important photosynthetic parameters viz., Fv/Fo, Fv/Fm and Pi_{Abs} were decreased with the increased duration of UV-B exposure. However, photosynthetic parameters Mo, ABS/RC and TRo/RC after UV-B exposure showed higher values in comparison to untreated control. The quantum efficiency of Fv/Fo and Fv/Fm of photosystem II declined significantly after 4h of UV-B exposure by 63.38% and 50.42% respectively in *C. vulgaris* and 27.52% and 26.80% respectively in *C. humicola* as compared to control. Mo represents the net closing rate of reaction centre (RC) and exhibited faster closing of reaction centre with the increased UV-B exposure time which was maximum in the case of *C. vulgaris* (about 3 fold) after 4 h UV-B exposure, when compared with control. In the case of ABS/RC, which indicate the apparent antennae size of RC, showed increase in the value of photon absorbance per reaction centre with increase in duration of UV-B exposure in both the algae. In the case of ½ h of UV-B exposure, both algae *C. vulgaris* and *C. humicola* exhibited 14.43% and 10.92 %, increase in the ABS/RC ratio respectively. However, a further increase in the exposure period of UV-B treatment, no significant change was observed in the ABS/RC values in both the algae. The opposite response of the ABS/RC value (increase) after UV-B exposure could be due to enhanced inactivation of reaction centres, not because of increase in pigment antenna size. The photosynthetic performance index (Pi_{abs}) exhibited reduction with the increase in the duration of UV-B exposure by 91.12% in the *C. vulgaris* and 84.4% in *C. humicola* after 4h UV-B exposure. The results suggested that *C. vulgaris* was more susceptible to toxicity than *C. humicola*. Further, the trapping flux (TR_0/RC) was also increased due to UV-B exposure in both the algae. The level of trapping flux (TR_0/RC) under UV-B exposure was enhanced by 11.94-57.7% and 14.03-67.2% in *C. vulgaris* and *C. humicola*, respectively. The percent inhibition of RCs in both algae under UV-B exposure might be responsible for respective increase in the ABS/RC and TR_0/RC. These results concluded that *C. humicola* experienced comparatively minimum inhibition of RC under UV-B stress than *C. vulgaris.*

Photosystem II (PSII) is more susceptible towards UV-B than photosystem I (PSI). The measurement of chlorophyll fluorescence is sufficiently well accepted tool to reveal the changes in the photochemistry of PSII affected by UV-B radiation (Beardall et al., 2002; Jiang and Qiu, 2011). Chlorophyll fluorescence analysis is one of the most popular techniques in the form of OJIP curve used to find out state of photosystem II (PSII) and based on relative distribution of energy dissipation processes (Markou et al., 2017). The Chl fluorescence produced in dark adapted algal cells by strong light pulse showed a polyphasic transient (OJIP curve) between minimum fluorescence (Fo) to maximum fluorescence (Fm) (Markou et al., 2017). Fv/Fm acts as a key indicator in photosynthetic performance, measured the efficiency of light energy absorbed by the PSII in the process of photosynthesis (Qiao et al., 2015). Decreased maximum quantum yield (Fv/Fm) in both algae *C. vulgaris* and *C. humicola* under UV-B exposure may be ascribed to UV-B induced damage to photosynthetic apparatus followed by decrease in the light energy conversion efficiency and finally inhibition of photosystem II and photosynthesis (Vadivelooet al., 2016; Chen et al., 2017). Zhao et al (2017) also reported that inhibition of PS II causes incapability to maintain the reduced states of Q_A, impairing electron transfer between photosystems. The other important parameters viz., ABS/RC and TRo/RC is considered to be increased according to the duration of UV-B exposure. The ABS/RC is considered as indicator of apparent pigment antenna size and increase in the level of inactivation of reaction centres (Strasser et al., 2004). An increase in the trapping flux per reaction centre (TR_0/RC) in the present study is the results of closure of RCs and high dissipation of excitation energy in the form of fluorescence or heat which inhibits Q_A reduction and electron transport (Markou et al., 2017). The physiological parameter, photosynthetic performance index (Pi_{abs}) has been used to express the vitality of photosynthetic system in the algal cells (Gururani et al., 2018). A higher Pi_{abs} reflects higher photosynthetic efficiency; however, decrease in the value of Pi_{Abs} under UV-B stress in the present study may be ascribed to decreased vitality of the algae and UV-B toxicity (Jiang et al., 2012; Negi et al., 2016).

Table 5.2: Photosynthetic performance of algae *C. vulgaris* and *C. humicola* under different duration of UV-B exposure. The parameters were derived from OJIP curve measured by PAM fluorometer

UV-B exposure time	**Photosynthetic parameters**					
	Fv/Fo	**Fv/Fm**	**Mo**	**Pi_{Abs}**	**ABS/RC**	**TRo/RC**
			C. vulgaris			
Control	3.564	0.781	0.274	6.714	1.039	0.812
1/2 h	3.249	0.765	0.306	5.386	1.189	0.909
1 h	2.772	0.731	0.331	4.058	1.387	1.014
2 h	1.978	0.664	0.405	2.090	1.716	1.139
3 h	1.446	0.591	0.552	0.850	2.074	1.226
4 h	1.305	0.566	0.630	0.596	2.262	1. 281
			C. humicola			
Control	1.765	0.638	0.080	11.986	1.153	0.634
1/2 h	1.300	0.565	0.083	07.797	1.279	0.723
1 h	1.225	0.551	0.085	07.347	1.390	0.854
2 h	1.221	0.550	0.115	05.136	1.585	0.873
3 h	0.991	0.498	0.136	02.129	1.800	0.887
4 h	0.875	0.467	0.154	01.585	2.129	1.060

In the case of Mo, net closing rate of RC was increased under UV-B exposure in the present study. An increase in the level of Mo may be interpreted as faster closing of RCs and inhibition of PSII (Markou et al., 2017) which was correlated with higher trapping flux (TR_0/RC) in the study. In addition, an enhanced inhibition of RCs indicates UV-B toxicity which leads to closure of RC and the photosystem II

(Jiangand Qiu, 2005). Minimum % inhibition of RC in *C. humicola* might due to high adaptation strategies in algal system, efficient repairing system and photoprotective response in both algae towards UV-B exposure enable these algae to give differential response of photosynthetic machinery (Castenholz and Garcia-Pichel, 2000).

5.9 Effect of UV-B on cellular toxicity biomarkers

H_2O_2, electrolyte leakage (EL) and TBARS (thiobarbituric acid reactive substances) are the direct indicator of toxicity and acts as biomarker. Microalgae *C. vulgaris* and *C. humicola* treated with UV-B exposure exhibited increased level of H_2O_2 (Table 5.3). Maximum increase in the H_2O_2 content was observed in both the algae exposed to 4h of UV-B radiation. Similar results were observed in the case of electrolyte leakage and TBARS content in *C. vulgaris* and *C. humicola*. In the case of EL, no significant change in EL was observed in both *C. vulgaris* and *C. humicola* exposed to 1 h and 2h UV-B, respectively (Table 3, $p \leq 0.05$). However, EL content showed maximum increase in the cells exposed to 3h (UV-B) in both algae as compared to control. TBARS, H_2O_2 and electrolyte leakage are directly associated with the cellular toxicity and act as a bio-indicators and biomarkers in plants and algae (Singh et al., 2018a). Algae *C. vulgaris* and *C. humicola* treated with UV-B for different period of time revealed UV-B induced the time dependent increase in toxicity. High level of TBARS in algae might be ascribed to UV induced production of fatty acid radicals by the reaction with ROS (hydroxyl and peroxyl radicals) followed by hydrogenation and production of intermediate compound (TBARS) (Rastogi et al., 2014). High TBARS content in algae emphasizes severe oxidative damage to membrane lipid as reported by various other authors (Upadhyay et al., 2016: Singh et al., 2018b). H_2O_2 plays duel role of stress causing agent as well as signaling molecules, electrolyte leakage indicated damage to membrane and cell lysis (Upadhyay et al., 2016; Singh et al., 2018a).

Table 5.3: TBARS (mmol g^{-1} fw) electrolyte leakage (µs cm^{-1}) and H_2O_2 (µmol g^{-1} fw) content of *C. vulgaris* and *C. humicola* treated with different exposure of UV-B All the values are means ± SD. ANOVA post hoc DMRT was done to check the variation between the variables. Identical letter denotes no significant difference ($p \leq 0.05$)

UV-B exposure time	**Parameters**		
	TBARS	**Electrolyte leakage**	**H_2O_2**
	C. vulgaris		
Control	$33.98^{a} \pm 2.18$	$86.42^{c} \pm 0.67$	$54.54^{a} \pm 1.68$
½ h	$40.43^{a} \pm 2.40$	$93.56^{e} \pm 1.15$	$130.84^{bc} \pm 4.38$
1h	$113.76^{b} \pm 2.33$	$95.77^{f} \pm 2.08$	$135.79^{c} \pm 5.70$
2 h	$163.23^{d} \pm 2.99$	$97.38^{f} \pm 0.63$	$181.85^{f} \pm 4.70$
3 h	$246.67^{f} \pm 4.97$	$100.91^{g} \pm 1.07$	$237.78^{g} \pm 10.19$
4 h	$281.72^{f} \pm 5.92$	$85.11^{c} \pm 0.99$	$363.25^{h} \pm 10.67$
	C. humicola		
Control	$30.54^{a} \pm 5.24$	$70.53^{a} \pm 0.81$	$60.12^{a} \pm 1.92$
½ h	$39.14^{a} \pm 6.34$	$97.09^{f} \pm 1.00$	$115.92^{b} \pm 6.03$
1h	$47.31^{a} \pm 11.08$	$90.27^{d} \pm 1.14$	$146.72^{cd} \pm 6.29$
2 h	$109.46^{b} \pm 11.45$	$90.91^{d} \pm 1.06$	$148.80^{d} \pm 3.53$
3 h	$146.02^{c} \pm 19.36$	$97.16^{f} \pm 0.98$	$170.89^{e} \pm 6.65$
4 h	$223.22^{e} \pm 11.84$	$82.72^{b} \pm 0.89$	$261.76^{g} \pm 8.21$

5.10 Biochemical characterization

5.10.1 Effect on protein, carbohydrate and total lipid

Microalgae exposed to UV-B radiation for different duration showed decrease in the protein content in a reciprocal manner. Maximum decrease in protein concentration in the both algae was observed after 4 h of UV-B exposure as compared to their respective controls (Fig. 5.3). However, no significant change in the protein content was observed in *C. vulgaris* cells exposed to UV-B upto 2h (0.85-5.80%) and in the case of *C. humicola*, the change in protein content was observed in the range of 36.88-73.09% as compared to control. In the case of carbohydrate and total lipid, both the algae exhibited increasing trend upto 1h of the UV-B exposure followed by gradual decrease in the carbohydrate and lipid content with the increasing UV-B exposure. Minimum level of carbohydrate (Fig. 5.3) and lipid content (Fig. 5.3) were observed in algae *C. vulgaris* i.e., 17.68±0.11 and 25.60±0.70 mgL^{-1}, respectively and in *C. humicola*, it was 0.097±0.003 and 0.106±0.010 mgL^{-1}, respectively after 4 h of UV-B exposure when compared with their respective untreated control. The carbohydrate content in *C. vulgaris* and *C. humicola*, was found to be 19.92±0.24 and 36.50±0.31 mgL^{-1}, respectively after 1 h UV-B exposure as compared to their respective control (19.52±0.14 and 34.14±0.54 mgL^{-1}, respectively). UV-B irradiation preferably targets protein and induces the photo-oxidation of aromatic amino acids leading to changes in the polypeptide chain which ultimately destroy the structure of protein. A decrease in the concentration of protein in algal cells exposed to UV-B radiation was an indicator of UV-B toxicity in both the algae. In case of effect of UV-B in both the algae an increase in lipid and carbohydrate content is the result of higher production and accumulation of metabolites such as lipid and carbohydrates which results into an increase in the cell size (Shuba Kifle, 2018). Pancha et al. (2014) also reported that cultivation of algae under different stress conditions can enhance the accumulation of carbohydrate and lipid, but also inhibits their growth rate and nutrient status.

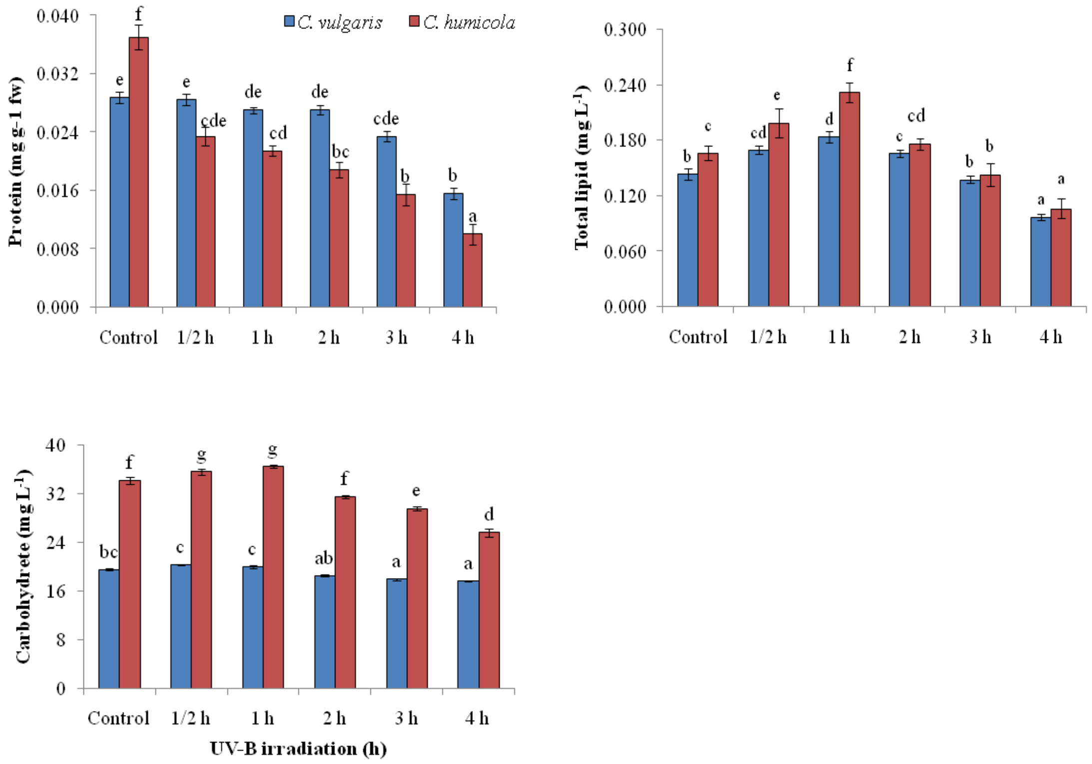


Fig. 5.3: Effect of UV-B on the protein, lipid and carbohydrate content in *C. vulgaris* and *C. humicola* treated with different exposure of UV-B. All the values are means ± S.D. ANOVA significant at level of ($p \leq 0.05$) according to one- way ANOVA test. Identical superscripts denote no significant difference between means in column according to DMRT ($p \leq 0.05$)

5.11 Effect on non-enzymatic antioxidants defense

5.11.1 Effect on ascorbic acid, cysteine and proline

The concentration of cysteine and ascorbate in both the algae was measured for different radiation time of UV-B exposure (Fig. 5.4). The result showed maximum content of cysteine in both the alga *C. vulgaris* and *C. humicola* was 41.87% and 35.05% after 4 h of UV-B exposure. However, the level of cysteine was more pronounced in *C. humicola* than *C. vulgaris* (Fig. 5.4). In the case of *C. vulgaris* no significant alteration in the cysteine content was observed after ½ h and 1 h of UV-B exposure, but both the strains exhibited 2-3 fold increase in the cysteine after 4 h of UV-B exposure. In the case of proline, the concentration increased with increase in the exposure time of UV-B (Fig. 5.4). Maximum increase in the proline content was observed after 4 h of UV-B exposure in both the algae. However, no significant change in proline content was observed in *C. vulgaris* after ½ h of UV-B exposure ($p \leq 0.05$). In the case of *C. humicola*, the proline content was increased by 21.8% as compared to control. Similarly, the ascorbate content in both the algae was also increased by ~4-5 fold as compared to control (Fig. 5.4).

Microalgae *C. vulgaris* and *C. humiocola* are endowed with different type of of UV-B tolerance responses including adaption, production of antioxidant molecules (ascorbic acid, cysteine, proline and glutathione etc) and enzymes (SOD, peroxidase and catalase) to protect themselves against the damaging effects of environmental stresses (Chokshi et al., 2017; Singh et al., 2018a). The antioxidants protect the algal cells by maintaining the structure of photosynthetic membrane, RC, protein integrity, inhibition of xanthophylls cycle and absorption of substantial amount of UV-B (200-300 nm) radiation (Tyagi et al., 2003; Xue et al., 2005).

Rai and Agrawal (2017) reported that ascorbic acid scavenges the nascent oxygen produced during UV-B exposure and diminishes the oxidative damage caused by ROS. Rastogi et al. (2011) also suggested that presence of various reducing agents within the living cell play an important role in maintaining the integrity and function of different cellular constituents and they are probably involved in mitigating the damaging effects of UV-B. The amino acid, cysteine acts as a precursor of thiol group antioxidants (viz., glutathione) which assist in stress tolerance in algae (Wirtz et al., 2010). In the present study, increase in the cysteine and proline content of algae indicates enhanced tolerance due to scavenging of the reactive oxygen species produced by UV-B radiation (Hayat et al., 2012; Upadhyay et al., 2014, 2016; Singh et al., 2018b).

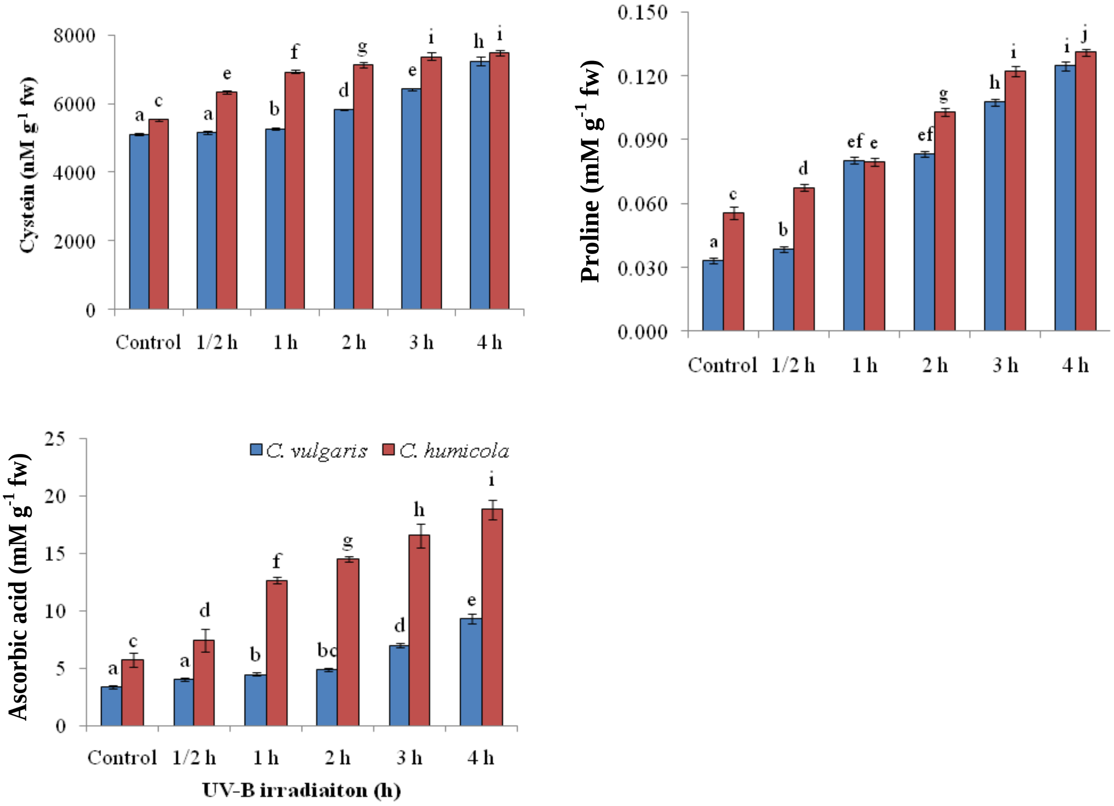


Fig. 5.4: Effect on cysteine, proline, ascorbic acid content in algae *C. vulgaris* and *C. humicola* treated with different exposure of UV-B. All the values are means ± S.D. ANOVA significant at level of ($p \leq 0.05$) according to one- way ANOVA test. Identical superscripts denote no significant difference between means in column according to DMRT ($p \leq 0.05$)

5.12 Effect of UV-B on the FTIR spectra

The IR absorption peak (wavenumer) of macromoecules viz., protein (amide I and amide II) (1540-1650 cm^{-1}), lipid(carbonyl, methylated and phosphodiester) (1650-1750 cm^{-1}) and carbohydrates (between 1200-900 cm^{-1}) were recorded in *C. vulgaris* and *C. humicola* cells exposed to UV-B radiation. The peak intensity and ratio of different IR peaks associated with macromolecules were used to interpret the amount of particular macromolecules.

FTIR spectra of *C. vulgaris* and *C. humicola* cells obtained after under UV-B exposure for different duration (Fig. 5.5 and 5.6) exhibited alteration in the characteristic peak of macromolecules as compared to control (Fig. 5.5 and 5.6). The FTIR spectra showing different IR absorption peaks of macromolecules viz., carbohydrate, proteins, lipids in the microalgae *C. vulgaris* and *C humicola* exhibited variation in the intensity of IR peaks associated with functional group of proteins, carbohydrate and lipids. The cells of *C. vulgaris* after exposure to UV-B showed changes in peak height of NH-amide which was increased by 4.25% after 2 h and 2.12% increased after 4 h of UV-B exposure (Fig. 5.5). Further, increased peak height at 1040 cm^{-1} associated with carbohydrate in *C. vulgaris* increased after 2 h and 4 h of UV-B exposure (7.14% and 10.71%, respectively) as compared to control. The peak height of lipid at 1740 cm^{-1} increased from 1.7 to 2.1 cm after 2 h of UV-B exposure. The ratio of different macromolecules viz., protein/lipid (P/L) and lipid/carbohydrate (L/C) were also evaluated to assess the change in the concentration of macromolecules due to UV-B toxicity (Table 5.5). The P/L ratio (1650/1740 cm^{-1}) decreased in *C. vulgaris* after UV-B exposure. Similarly, L/C ratio (1740/1040 cm^{-1}) showed decreasing trend in both the strains with increase in UV-B exposure time. Maximum decrease in L/C ratio was observed after 4 h of UV-B exposure, while the P/L ratio was reduced after 2 h of UV-B exposure (Fig. 5.5).

In the case of *C. humicola*, no change in the IR peak intensity of protein (1650 cm^{-1}) was observed after UV-B exposure when compared with control (Fig. 5.6). But the IR peaks for lipid was enhanced in *C. vulgaris* and *C. humicola* for 10.5 and 5.26%, respectively and carbohydrate, it was 9.09% and 18.18%, respectively. The ratio of P/L in *C. humicola* exposed to UV-B also decrease from 2.0 to 1.8 after 2h of UV-B exposure and 2.0 to 1.9 after 4h of UV-B exposure. In the case of L/C ratio, it was marginally increased by 1.7% after 2h of UV-B exposure and the ratio decreased by 11.04% after 4h of UV-B exposure (Fig. 5.6). FTIR spectra are fast analytical tool for seeing varititative characterization of lipid, carbohydrates, protein and phosphorylated molecules based on specific absorption frequency by fucntional group of the molecule (Harz et al., 2009). Thus, FTIR allows to elucidate the relative abundance of macromolecules under different environmental conditions (Singh et al., 2018). The spectrum obtained through FTIR represents the molecular fingerprint of the specific molecules and varies according to the structure of the molecules (Sene et al., 1994; Gorgulu et al., 2007). Increase in the IR peak for protein after 2 h UV-B exposure may be ascribed to synthesis of some proteins as self-defense strategy of cells which may be involved in the repair of UV-B induced damage to the cells (Xue et al., 2005). However, decrease in the protein after long duration of UV-B exposure may be due to UV-B induced inhibition of photosynthesis. Forfang et al. (2017) also reported that functional group deformation and bond stretching in macromolecules of protein [C=O (symmetric), N-H; asymmetric deformation and C-N; symmetric deformation] significantly contributes to reduction in the protein content in the algae. In the case of decrease in carbohydrate peak height may be due to UV-B induced inhibition of photosynthesis coupled with cell injury leading increased accumulation of storage compound such as lipid to avoid damage (Singh et al., 2018b). This observation supports our present findings on increase in lipid content after UV-B exposure. Overall, increase in the protein and lipid content as a part of cellular defense strategy against UV-B stress at low dose UV-B signifies the high production efficiency of lipid and protein.

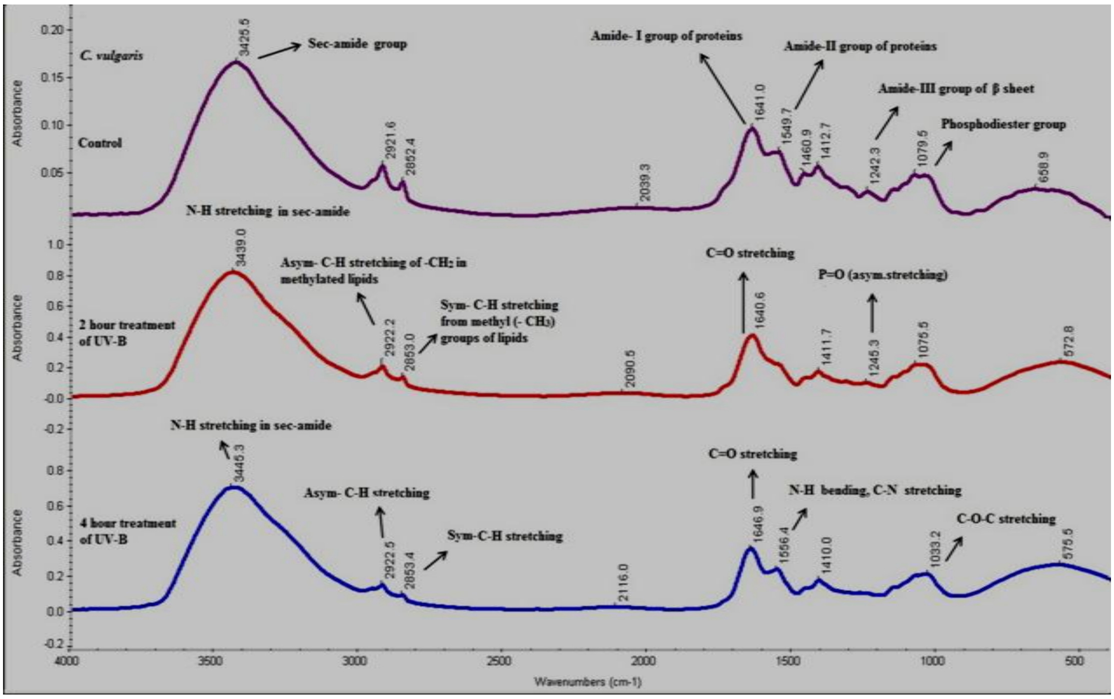


Fig. 5.5: FTIR spectra of *C. vulgaris* treated with different exposure of UV-B

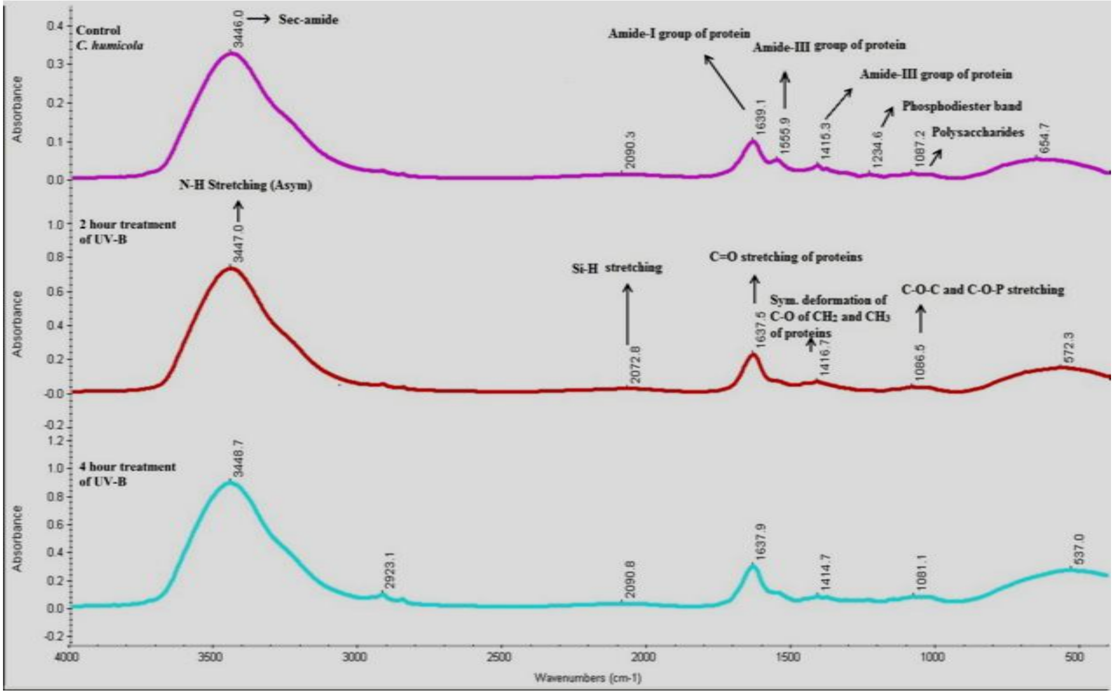


Fig. 5.6: FTIR spectra of *C. humicola* treated with different exposure of UV-B

5.13 Conclusions

The present study concludes that UV-B exposure at 4 h causes severe injury in the both algae *C. humicola* and *C vulgaris* whereas UV-B (2 h) exposure in algae induces higher lipid yield, photosynthetic performance and improved antioxidant tolerance.

The transient chlorophyll fluorescence study in both algae reflects closure of photosystem II, diminished reaction centre and reduced flow of electron after 4h of UV-B exposure which signifies the threshold level of UV-B tolerance through down regulation of PS II. In addition, FTIR spectra also reveal increased peak height of protein and lipids at lower doses of UV-B as a part of adaptation strategy of cells. SEM exhibited variegated structure and increased cell size by ~15-65% which was more pronounced in the case of *C. humicola* under UV-B exposure (2 h and 4 h).

CHAPTER 6

UV-B intensity induced photochemical and biochemical alterations in microalgae *Chlorella vulgaris* and *Chlorococcum humicola*

6.0 UV-B intensity induced photochemical and biochemical alterations in microalgae *Chlorella vulgaris* and *Chlorococcum humicola*

6.0 Introduction

With the advent of modernization, algae have gained attention due to their role as promising source of renewable energy (Hosseini et al., 2016). Microalgae display great adaptability to different abiotic stresses such as temperature, light, salinity, radiation and nutrient deprivation. They also produce high-valued metabolites such as triacylglycerides (TAGs), polyunsaturated fatty acids (PUFAs), sterols, carbohydrates, proteins, polysaccharides, terpenoids, ethanol and carotenoids etc. (Chew et al., 2017; Paliwal et al., 2017; Rizwan et al., 2018). Amongst varying the abiotic stresses, ultraviolet radiation is also important stress on the earth surface mainly due to high anthropogenic activity and release of toxic chemicals. An increase in UV radiation is mainly caused by the reduction of stratospheric ozone layer (Bashri et al., 2018) .UV-B radiation is a part of UVR which constitute about 1.5% of total UVR reaching on the earth surface. UV-B can directly damage the both terrestrial as well as aquatic ecosystem and alters the food chain and its structure at trophic level (Bais et al., 2018).

Microalgae are considered superior over different type of plants because of their high photosynthetic efficiency, short life span, easy cultivation on waste land and variety of habitat i.e., marine, fresh water, salt water, waste water etc. (Varshney et al., 2015; Pathak et al., 2018). Algae have potential to accumulate significant amount of lipid in their cells which make them a suitable candidate for future generation fuel. Several reports are available which suggested that microalgae such as *Chlorella* sp., *Nannochloropsis* sp., *Scenedesmus* sp., *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* can store lipid upto 20-80% of the dry cell weight (DCW) (Sijtsma et al. 2010; Xue et al., 2017). Upon exposure to UVR, the algal cells experiences stress which ultimately causes cell injury mediated by cascade of signaling molecules. Banerjee et al. (2018) have reported that algal cell under UV-B stress develop pyrimidine dimer and causes mutation at gene level leading to alteration in the structure of protein, DNA and RNA. The DNA and protein molecule losses the properties of repair and proof reading activities which push the cells towards mutation, apoptosis and cell death (Kasuba et al., 2015).

Exposure of algae to UVR results into ROS formation which is counter balanced by different mechanisms present in the algae like production of metabolites, division of photosynthetic process towards synthesis of neutral lipids in the form TAG and enzymatic and non-enzymatic antioxidant etc. (Upadhyay et al., 2016).

Present study was conducted to examine the impact of UV-B radiation on growth, biomass productivity, lipid yield and cellular defense in algae *C. vulgaris* and *C. humicola* efforts have been made to understand UV-B tolerance mechanism and biofuel production ability in *C. humicola* and *C. vulgaris*.

6.1 Materials and methods

6.1.1 Experimental setup

All the experiments were conducted in the laboratory of Department of Environmental Science, Babasaheb Bhimrao Ambedkar University, Lucknow, India. Pure algal cultures of *C. humicola* and *C. vulgaris* were inoculated in 1000 ml Erlenmeyer flasks containing 200 ml BG-11 media. The inoculated cultures were kept in culture room for growth and acclimatization under controlled condition (28±2^{o}C) PAR of 150 μmolphotonm^{-2} s^{-1} with photoperiod of 16:8 h (light: dark). After 72 h of growth, algal cultures were transferred to wide mouth PVC beaker as depicted in figure 3.1 in Materials and Methods section and transferred to close UV-B chamber fitted with UV-B lamp. Different intensities of UVR i.e., 5, 10, 15 and 20 Wm^{-2} were provided to algal cells by adjusting the distance from UV-B source. The UV-B radiation was provided with the help of three Philips TL UV-B tubes (40W) with a spectral range between 280 to 315 nm. After the exposure of cells to UV-B for a period of 4 h, the treated algal cultures were shifted under PAR light in culture room. All the UV-B treatments were given to the cells suspended in BG-11 media so as to avoid water loss. All the experiments were carried out in triplicates. The harvesting was done at the end of stationary phase of the growth. The cell biomass was collected and kept refrigerated for further analysis.

6.2 Measurements of photosynthetic performance

The photosynthetic performance of microalgae viz., chlorophyll-a fluorescence induction (OJIP) curve, photosynthetic performance index (Pi$_{ABS}$), maximum quantum yield (Fv/Fm), Net closing rate of reaction centre (Mo), Trapping flux (TRo/RC), effective antenna size expressed as absorbance per reaction centre (ABS/RC) and percentage inhibition of reaction Centre (RC) in microalgae *C. humicola* and *C. vulgaris* were evaluated under different intensity of UV-B exposure for a period of 4 h. The fast chlorophyll fluorescence induction was measured by using Aqua Pen-C portable fluorometer (Photon Systems Instruments), Czech Republic).

The Aqua Pen-C was set to maximum pulse intensity of 3000 μmol photon m^{-2} s^{-1} of red light for 10s, while rapid light/dark induction adopted curves for Chla irradiance levels ranging from 0 to 480 μmol photon m^{-2} s^{-1}. The microalgae cells were adopted in dark (10 to 15 min) at room temperature prior to analysis.

6.3 Biochemical analysis

The chlorophyll estimated by the method of Arnon (1949), carotenoids content by the formula of Duxbury and Yentsch (1956). The protein content was estimated by the method of Lowry et al., 1951) using bovine serum albumin (Sigma) as a standard. Carbohydrate and lipid content in algal cell were estimated by the methods as described by Ben-Amotz et al. (1985) and Dubois et al. (1956), respectively.

6.3.1 Analysis of cellular toxicity

Cellular toxicity in the form of MDA, H_2O_2 and EL was measure. The lipid peroxidation (MDA) in algae was measured by the method of Heath and Packer, (1968). H_2O_2 was estimated following the method of Velikova et al. (2000). Electrolyte leakage in algal cell was estimated by the methods of Dionisio-Sese and Tobita (1998).

6.3.2 Estimation of proline, cysteine and ascorbic acid

Proline, cysteine and ascorbic acid content in algal cell were analyzed using the method of Bates et al. (1973); Gaitonde (1967) and Kampfenkel et al. (1995), respectively.

6.4 Enzyme assay

The antioxidant enzymes activity viz., SOD and CAT were done by the method of Aebi (1984) and Nishikimi and Rao (1972), respectively.

6.5 Sample preparation and analysis of SEM coupled with EDS

SEM study was done with the help of SEM coupled with energy dispersive X-ray spectrometer (EDS) (SEM) (JEOL, Japan; model JSM-6490LV).

6.6 Fourier transforms infrared spectroscopy (FTIR) analysis

To study FTIR spectrum in microalgae, the dried microalgae cell samples were grinded with potassium bromide (KBr, 1:10 ratio) and thereafter the pellet was formed using hydraulic press (150 lbs). Further the pellets were used to elucidate the FTIR spectra (4000-400 cm^{-1}) of cells using FTIR (Thermo- Scientific Nicole 6700, USA).

6.7 Data analysis

All the experiments were conducted in three replicates. One-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT, $P \leq 0.05$) were performed to evaluate the validity and variability of the data. All examines were carried out with the SPSS software.

6.8 Results and discussion

6.8.1 Effect on photosynthetic pigment

Microalgae *C. vulgaris* and *C. humicola* exposed to different intensities of UV-B exhibited decrease in the level of chlorophyll content with the increasing doses of UV-B. Maximum increase in the chlorophyll content was observed at lower UV-B intensity (5 Wm^{-2}) in *C. vulgaris*, cells while minimum level of pigment content was observed at 15 Wm^{-2} (Fig. 6.1). The concentration of chlorophyll in algae *C. vulgaris* and *C. humicola* exposed to higher intensity (15 Wm^{-2}) of UV-B was .069±0.009 and 0.058± 0.009 mgg^{-1} fw, respectively as compared to their respective control (0.082±0.009 and 0.079±0.018 mgg^{-1} fw respectively). Similarly, the carotenoid content in *C. vulgaris* and *C. humicola* cells exposed to 15 Wm^{-2} UV-B found to be 1.168±0.015 and 1.163±0.070 mgg^{-1} fw respectively, in comparison to control i.e., 1.287±0.027 and 1.32± 0.03 mgg^{-1} fw, respectively. Pigments present in algae and plants act as important component in the photosynthesis process. The content of chlorophyll also indicates the growth and overall health of photosynthetic organism (Czerpaket al., 2002). UV-B radiation causes significant reduction in the chlorophyll contents of both microalgae *C. humicola* and *C. vulgaris*. A decrease in the chlorophyll and carotenoids pigments may leads to inhibition of photosynthesis and growth. In the present investigation, the results suggested that chlorophyll and carotenoids are sensitive to UV-B stress. Earlier reports have indicated that increase in UV-B radiation during growth results into significant reduction in the pigment content due to inhibition of chlorophyll biosynthesis (Czerpaket al., 2002; Singh et al., 2018b). Loss of photosynthetic pigment is quite evident during exposure of cells to enhanced UV-B radiation. In most of the crop species as well as aquatic microorganism, there is UV-B induced loss of pigments (Singh et al., 2018b). Straid and Porra (1992) suggested that main reason of UV-B induced decline in chlorophyll level might be due to inhibition of cab gene, which codes for chlorophyll protein. Can and Bornman (1990) have also reported about 20% decrease in the chlorophyll content in *Phaseolus vulgaris* cells exposed to UV-B and low light stress.

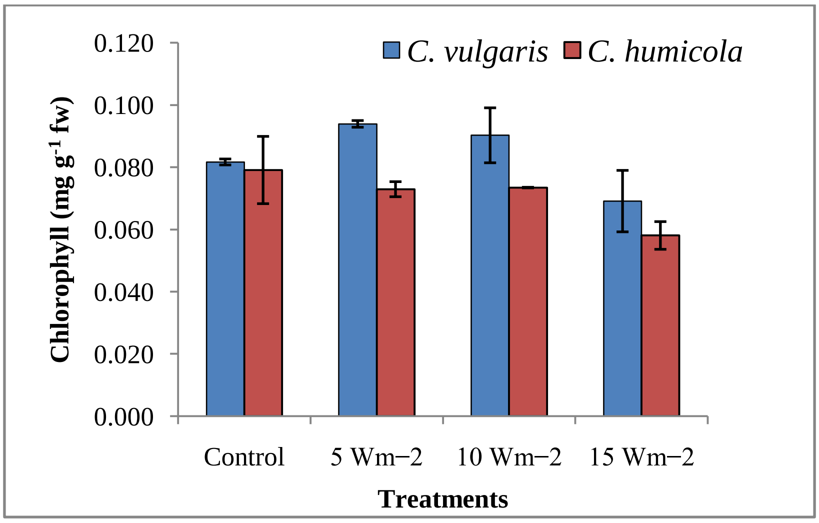


Fig. 6.1: Effect of different UV-B intensities in the chlorophyll content of microalgae *C. vulgaris* and *C. humicola*. All the values are means± SD (n=3)

Carotenoids play very important role against UV-B stress. Carotenoids are scavengers of singlet oxygen formed during UV exposure. The carotenoids have dual role in photosynthesis; as accessory pigments transferring excitation energy to chlorophyll a and also they protect the cells against damaging effect of UV-B (Pick et al., 2019). Besides, their photoprotective nature, carotenoids have also been implicated in antioxidant action in chloroplast due to their capability of scavenging reactive oxygen species (i.e. superoxide radical, hydroxyl radical ($^{\bullet}OH$) hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), H_2O_2 and ($^{\cdot}OH$) radical which are the most destructive agents naturally produced during the process (Pathak et al., 2019). The remarkable reduction in the carotenoid can be indicator of photoxidative damage as evident from chlorophyll degradation and loss of integrity (Takahashi et al., 2018).

Carotenoids play a key role against UV-B. Carotenoids, scavenges singlet oxygen species formed during UV exposure (Fig. 6.2). The carotenoids have dual rolein photosynthesis: as accessory pigments transfer excitation energy to chlorophyll a and to protect against damaging effect of UV-B (Czerpaket al., 2002; Singh et al., 2018b). The remarkable reduction in carotenoid level can lead to oxidative damage due to chlorophyll degradation and thylakoid membrane breakdown (Tian and Yu, 2009).

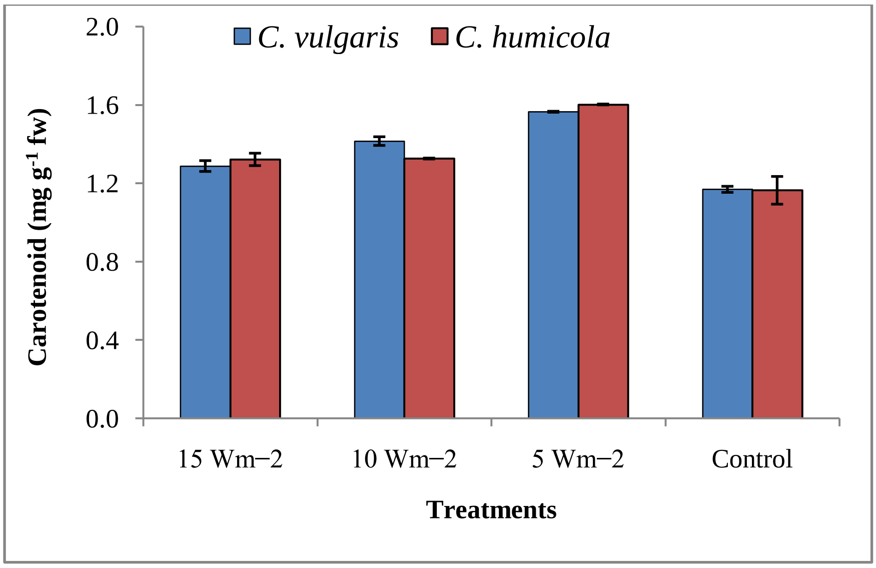


Fig. 6.2: Effect of different UV-B intensities in the carotenoid content of microalgae *C. vulgaris* and *C. humicola*. All the values are means± SD (n=3)

6.8.2 Effect on photosynthetic performance

The photosynthesis performance efficiency of *C. vulgaris* and *C. humicola* under different intensity of UV-B radiation has been depicted in Table 6.1. Result showed that the photosynthetic parameter viz., ABS/RC and TRo/RC in *C. vulgaris* exhibited increasing trend with increase intensity of UV-B as compared to control. The ABS/RC ratio in *C. vulgaris* at 5, 10, 15, and 20 Wm^{-2} was in the order of 1.613, 1.927, 1998, and 2.342 m, respectively, which was higher with respect to control (1.564). The ratio of Fv/Fm (quantum yield) decreased with increase in UV-B intensity. Maximum decrease (18.56%) in quantum yield in *C. vulgaris* was observed at 20 Wm^{-2} intensity. However, the Fv/Fm was increased by 2.56% at 5 Wm^{-2} of UV-

B exposure. The photosynthetic parameter Mo (denoting net closing rate of RC) was found to be as per other reports indicating stress induces closing of RC and decline in Mos. The Mo was maximum at 20 Wm^{-2} (0.220) in comparison to control (0.319). In the case of Pi_{ABS}, it was initially increased at 5 Wm^{-2} intensity but further decrease in UV-B intensity led to decrease in the photosynthetic performance of both.

The inhibition of RC was evident from the ratio of ABS/RC and TPo/RC when compared to control. The ABS/RC value was increased with dose of UV-B treatment. The maximum value of ABS/RC was at 20 Wm^{-2} (66.7%) and minimum at 5 Wm^{-2} (9.6%). In case of *C. humicola,* all the studied parameter declined except ABS/RC and TRo/RC which increased with increase in the intensity of UV-B. The ratio of Fv/Fo and Fv/Fm showed the highest value in *C. vulgaris* and *C. humicola* at 5 Wm^{-2} i.e., 1.351 (Fv/Fo) to 1.434 (Fv/Fm) with *C. vulgaris*, respectively and 0.260 (Fv/Fo) to 0.303 (Fv/Fm), respectively, and lowest value in control sets. Maximum decrease in Fv/Fo and Fv/Fm ratio was observed at 20 Wm^{-2} (15.24% and 36.53%, respectively) in both the alga. In the case of exposure of lower doses of UV-B (10 and 15 Wm^{-2}), no significant change was observed in both the parameters i.e. Fv/Fm & Fv/Fo (Fv/Fo; 1.279 & 1.270 & Fv/Fm; 0.218 & 0.213, respectively, in both the algal. The net closing rate of reaction centre (Mo) was slightly changed as observed in *C. humicola* at 5 and 10 Wm^{-2} intensity of UV-B when compared with control. The photosynthetic parameters PI_{ABS} showed decreasing trend with increase in the UV-B intensity. The photosynthetic parameters ABS/RC & TRo/RC increased with increase in the intensity of UV-B and were found to be maximum increased at 20 Wm^{-2} (57.83% and 29.29%), respectively. Among all the target sites, PSII was possibly the most important site for UV-B action affecting its electron transport and subsequent damage to D1 and D2 protein (Jiang and Qiu, 2011). Nedunchezhian and Kulandaivelu (1997) have reported that UV-B induced loss of PSII coupled electron transport activity and O_2 evolving capacity was mainly due to the depletion of polypeptides during UV-B treatment. These polypeptide stabilize the structural integrity of PSII complex (Tyr-Z, Tyr-D redox active tyrosines and QA, QB quinone electron acceptors) and control the catalytic efficiency throughout the sequential oxidation of Mn clusters atoms via the PSII reaction centre (Markou et al., 2017). In addition, high intensity of UV-B induces closing of reaction centre due to closure of pigment antennae system and excitation of electron, which consequently result into inhibition of photosynthesis in the algae (Strasser et al., 2004). Inhibition of Fv/Fo, fv/Fm, Mo and inhibition of RC under different stress have been reported earlier by various workers in plants as well as in algae (Gururani et al., 2018; Jiang et al., 2012).

Table 6.1: Photosynthetic performance of algae *C. vulgaris* and *C. humicola* under different intensity (5, 10, 15 and 20 Wm^{-2}) of UV-B exposure. The parameters were derived from OJIP curve measured by PAM fluorometer

UV-B intensity (Wm^{-2})	**Photosynthetic parameters**					
	Fv/Fo	**Fv/Fm**	**Mo**	**Pi_{Abs}**	**ABS/RC**	**TRo/RC**
			C. vulgaris			
Control	1.663	0.625	0.319	2.225	1.564	1.003
5	1.788	0.641	0.310	4.081	1.613	1.008
10	1.352	0.582	0.289	2.379	1.927	1.122
15	1.393	0.575	0.261	2.342	1.998	1.142
20	1.036	0.509	0.220	2.013	2.342	1.192
			C. humicola			
Control	1.351	0.260	0.361	0.179	4.779	0.777
5	1.434	0.303	0.356	0.176	5.566	1.241
10	1.279	0.218	0.355	0.128	6.353	1.387
15	1.270	0.213	0.271	0.112	7.269	1.550
20	1.145	0.165	0.156	0.102	7.543	1.781

6.8.3 Effect on TBARS, electrolyte leakage and H_2O_2

TBARS content was measured in the algal strains under different intensities of UV-B. The UV-B toxicity is represented by enhances production of TBARS as a result of lipid peroxidation (Rastogi et al., 2014). The TBARS content in *C. vulgaris* treated with 5 Wm^{-2} intensity was increased by 22.66% while at 10, and 15 Wm^{-2} UV-B, the MDA content was increased by 32.62% and 39.69%, respectively. In the case of *C. humicola* it was 26.35%, 67.79% and 83.70%, respectively at 5, 10 and 20 Wm^{-2} of UV-B intensity, respectively. The level of electrolyte leakage in algal cells due to stress represents cellular damage to membrane system. Microalgae *C. vulgaris* and *C. humicola* exposed to varying intensity of UV-B radiation exhibited increase in the level of EL which was maximum at the intensity of 15 Wm^{-2} in comparison to control (untreated). Algae treated with 5 Wm^{-2} of UV-B exposure showed enhanced value of EL from 83.15±2.25 to 98.66±3.49 in *C. vulgaris* and 82.97±3.72 to 102.71±2.95 in *C. humicola.* Similarly, H_2O_2 content in microalgae *C. vulgaris* and *C. humicola* also represented increasing trend with the increase intensity (0-15 Wm^{-2}) of UV-B. Results showed the algae treated with higher intensity (15 Wm^{-2}) of UV-B were found to be increased by 47.09% (*C. vulgaris*) and 67.90% (*C. humicola*) as compared to control. MDA, electrolyte leakage and H_2O_2 in algae represent toxicity induced by environmental stress (Upadhyay et al., 2016). Low level of cell damage in microalgae under 5 Wm^{-2} UV-B intensity may be explained by the enhance storage and accumulation of phenolics, flavonoids and other metabolites (Forzan Lozano et al., 2007).Liang et al. (2006) suggested that under UV-B exposure, a non-specific signaling pathway take place inside the cell which enhances the production of hydrogen peroxide and lipid peroxidation. High content of electrolyte leakage is the result of membrane damage and extracellular release of electrolytes exterior from the cells. Under severe stress condition, peroxidation of lipid oxidizes the lipid molecules present in the membrane triggering the rupture of wall and leakage of ions as evident from peroxide damage to the membrane (Singh et al., 2018; Upadhyay et al., 2016).

Table 6.2: Effect of TBARS (mM g^{-1} fw), H_2O_2 (µmole g^{-1} fw) and EL (µs cm^{-1}) content in microalgae C. *vulgaris* (CV) and *C. humicola* (CH) treated with different intensities of UV-B. All the values are means ±SD (n=3)

UV-B intensity (Wm^{-2})	**TBARS**		**H_2O_2**		**EL**	
	CV	**CH**	**CV**	**CH**	**CV**	**CH**
Control	67.76± 5.95	42.72± 4.32	141.43± 15.21	184.51± 17.57	83.15± 2.25	82.97± 3.72
5	83.12± 1.16	53.98± 5.24	219.69± 28.75	217.75± 24.07	98.66± 3.49	102.71± 2.95
10	89.87± 3.75	71.68± 4.04	269.37± 26.86	249.30± 40.15	116.16 ± 7.98	131.58± 9.34
15	94.66± 3.56	78.48± 2.58	349.47± 40.31	309.81± 31.32	146.46 ± 6.13	141.68± 14.33

6.8.4 Effect on protein, total lipid and carbohydrate content

Protein content in *C. vulgaris* and *C. humicola* exposed under different intensity of UV-B was found to be decreased with all the intensity except 10 Wm^{-2} (Table 6.3). The concentration of protein in *C. vulgaris* exposed to 15 Wm^{-2} UV-B intensity was reduced by 20.22% as compared to control, while in *C. humicola* by 17.91%. However, protein content in algal cell exposed to lower UV-B dose (5 Wm^{-2}) no significant change in the protein content was observed. Protein is one of the main target of UV-B radiation due to presence of aromatic amino acids which strongly absorb in the UV-B region (280-320 nm). Under UV-B exposure, proteins undergo a several modifications including photodegradation, membrane permeability and oxidation of peptide chains (Phull et al., 201). These changes are associated with bond breaking and protein denaturation (Singh et al., 2018). In the case of lipid, maximum lipid content in both alga *C. vulgaris* and *C. humicola* was 2.059±0.022 mgL^{-1} and 1.961±0.032 mgL^{-1}, respectively at 10 Wm^{-2} (Table 6.3). Minimum concentration of lipid in both algae after exposure to UV-B stress supported the earlier findings on stress induced enhancement of lipid content in algae. Decreased protein content in microalgae exposed under UV stress may be ascribed to shifting of metabolic cycle towards production of storage compound such as lipid to minimize the impact of toxicity (Pancha et al., 2014). Similar results were also reported by various other workers who suggested that protein content under stress condition undergo carboxylation, oxidation, which reduces the membrane fluidity and overall protein content within the cells (Shuba Kifle, 2018).

The carbohydrate content in algae under different intensity of UV-B was decreased with the increase in the UV-B intensity (Table 6.3). The present results showed that algae *C. vulgaris* and *C. humicola* exposed to higher level of UV-B, the carbohydrate content declined to the level of 9.96±0.28 and 9.18±0.35 mgL^{-1}, respectively in comparison to their respective untreated control (18.44± 0.35 and 23.44±0.52 mgL^{-1}, respectively).

Table 6.3: Effect of UV-B on protein (mgg^{-1} fw), total lipid (mgL^{-1}) and carbohydrate (mgL^{-1}) content of microalgae C. *vulgaris* (CV) and *C. humicola* (CH) treated with different intensities of UV-B. All the values are means ±SD (n=3)

UV-B intensity (Wm^{-2})	**Protein**		**Total lipid**		**Carbohydrate**	
	CV	**CH**	**CV**	**CH**	**CV**	**CH**
Control	2.012± 0.428	1.732 ±0.049	0.533 ±0.012	1.538 ±0.036	18.44 ±0.35	23.45 ±0.52
5	2.047 ±0.378	1.728 ±0.167	1.319 ±0.039	1.366 ±0.021	12.84 ±0.521	10.053 ±0.228
10	1.701 ±0.246	1.425 ±0.145	2.059 ±0.022	1.961 ±0.033	18.57 ±0.384	19.333 ±0.280
15	1.605 ±0.119	1.397 ±0.136	0.999 ±0.070	1.814 ±0.025	9.96 ±0.283	9.182 ±0.35

6.8.5 Effect on non-enzymatic antioxidants

Ascorbic acid, proline and cysteine content in the algal cells are considered to be important antioxidants which protect the algae from oxidative injury. Microalgae *C. vulgaris* and *C. humicola* exposed to different intensity of UV-B radiation showed increase in the level of antioxidants with the increased intensity of UV-B (Table 6.4). The ascorbate content in the cells of *C. vulgaris* treated with 5, 10 and 15 Wm^{-2} of UV-B radiation increase in the order of 1.18±0.44, 3.99±0.35 and 5.37±0.28 mmolg^{-1} fw, respectively than the control (Table 6.4). In *C. humicola*, it was 1.33±0.27, 3.63±0.60 and 6.26±0.13 mmolg^{-1} fw, respectively, as compared to control. In case of *C. vulgaris* the changes in proline content was 0.249±0.002, 0.393±0.004 and 0.470±0.005 µmolg^{-1} fw, respectively while in *C. humicola* it was 0.357±0.01, 0.377±0.005 and 0.383±0.002 µmolg^{-1} fw, respectively in comparison to control (0.110±0.004 and 0.258±0.002 µmolg^{-1} fw, respectively). Ascorbate is the strong and water soluble antioxidant acts to control and minimizing the damage caused by ROS in algae (Chokshi et al., 2017). Ascorbate is measured as the most powerful ROS scavenger because of its capacity to donate electrons in a number of enzymatic and no-enzymatic reactions. It can provide defense to membranes through directly scavenging of $O_2^{\cdot -}$and $^{\bullet}OH$ and by regenerating α-tocopherol from tocoperoxyl radical (Chokshi et al., 2017; Singh et al., 2018a). Further as an osmolyte, proline is measured as a strong antioxidant and potential inhibitor of algal and plant cell death (Xue et al., 2005). Increase proline content may be the results of increased synthesis or reduced degradation in algae and plants (Tyagi et al., 2003).

Table 6.4: Effect of ascorbic acid (mmol g^{-1} fw), proline (µmol g^{-1} fw) and cysteine (nmol g^{-1} fw) content in microalgae C. *vulgaris* (CV) and *C. humicola* (CH) treated with different intensities of UV-B. All the values are means ±SD (n=3)

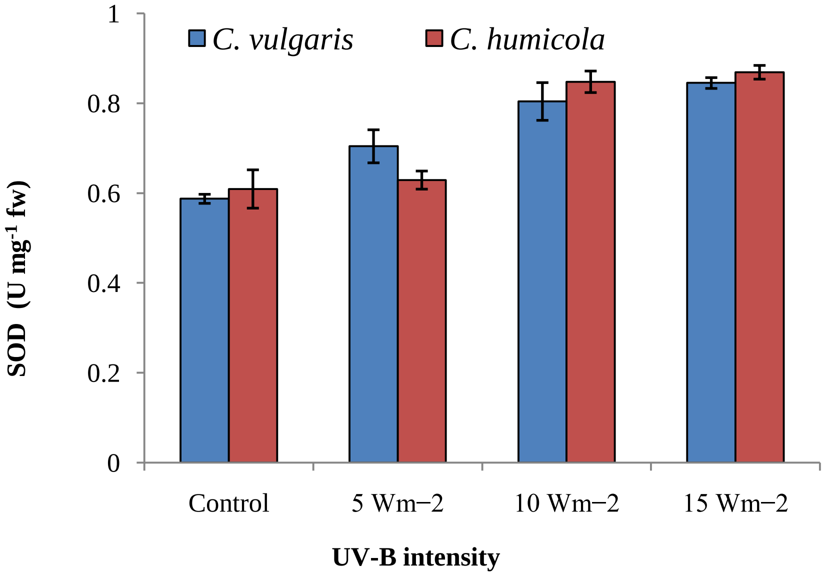
UV-B intensity (Wm^{-2})	**Ascorbic acid**		**Proline**		**Cysteine**	
	CV	**CH**	**CV**	**CH**	**CV**	**CH**
Control	0.57± 0.26	0.95± 0.50	0.110± 0.02	0.258± 0.002	2492.0± 88.8	2552.0± 85.9
5	1.18± 0.44	1.33± 0.27	0.249± 0.002	0.357± 0.010	2714.4± 108.5	3284.0± 98.8
10	3.99± 0.36	3.63± 0.60	0.393± 0.004	0.377± 0.006	3740.0± 78.9	3800.8± 96.2
15	5.37± 0.29	6.26± 0.13	0.470± 0.005	0.383± 0.002	3997.0± 66.2	4204.0± 84.9

Cysteine content in microalgae *C. vulgaris* and *C. humicola* exposed to different exposure intensity of UV-B also showed similar trends as in the case of ascorbic acid and proline. The level of ascorbic acid increased with the increase intensity of UV-B. Cysteine content in *C. vulgaris* at 5, 10 and 15 Wm^{-2} intensity of Uv-B was in the order of 2714.4±108.50, 3740.0±78.9 and 3997.0±66.2 $nmolg^{-1}$ fw, respectively as compared to control. In the case of *C. humicola*, it was 3284±98.84, 3800.8±96.2 and 4204.0±84.9 $nmolg^{-1}$ fw, respectively in comparison to control. Cysteine also acts as photoprotectant against oxidative stress and assist in the initiation of ascorbate cycle to reduce the peroxide level in cell (Wirtz et al., 2010; Upadhyay et al., 2014). High production of cycteine against stress has been reported by many researchers (Wirtz et al., 2010).

6.8.6 Effect on antioxidant enzymes

SOD acts as a primary antioxidant enzyme which dismutates the superoxide radical formed during stress into peroxide and water. Microalgae *C. vulgaris* and *C. humicola* exposed to different intensity of UV-B exhibited increasing enzymatic activity with the increase in the UV-B intensity. SOD activity in *C. vulgaris* and *C. humicola* was found to be increased by 43.95% and 42.69%, respectively at 15 Wm^{-2} of UV-B radiation as compared to their respective control (Fig. 6.3). The catalase activity in *C. vulgaris* and *C. humicola* exposed to UV-B 5 Wm^{-2} was increased by 37.98% and 41.10%, respectively as compared to control. However, after 15 Wm^{-2} UV-B exposure, the activity of catalase was increased by 2~3 fold in both the algae. In the case of 10 Wm^{-2} both algae exhibited >50-57% enhancement in the catalase activity i.e., 57.69% in case of *C. vulgaris* and 51.22 in case of *C. humicola*.

The formation of ROS is prevented by the antioxidative enzymes.SOD acts as first line of defense against oxidative stress and scavenge the formation of excess superoxide radicals through dismutation and formation of peroxide which are subsequently degraded by the catalase enzyme into water and oxygen (Upadhyay et al., 2016). The present findings showed increase in the level of antioxidant enzymes in algae *C. vulgaris* and *C. humicola* exposed to UV-B radiation. Both the strain demonstrated antioxidative defense against the ROS. However, *C. humicola* exhibited better antioxidaive defense response than the *C. vulgaris.*



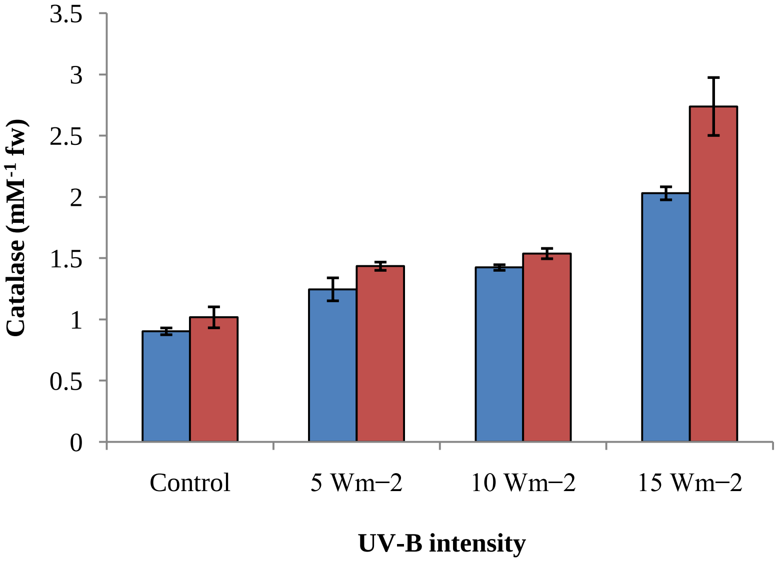


Fig. 6.3: Effect of SOD (U mg^{-1}fw) and CAT (mM^{-1}fw) activity in microalgae *C. vulgaris* and *C. humicoal* treated with different UV-B intensities. All value are means ±SD (n=3)

6.9 Conclusions

The overall results concluded that impact of UV-B intensity at 15 Wm^{-2} was highly toxic for both algae while lower intensity i.e., 5 Wm^{-2} was least effective in term of chlorophyll, biomass, lipid and antioxidant. UV-B elicited dose dependent photo-oxidative response in both the strains. It was observed that photosynthetic efficiency was severely impaired after exposure to UV-B.

CHAPTER 7

Combined effect of UV-B and sodium chloride stress on cellular response of microalgae *Chlorococcum humicola* and *Chlorella vulgaris*: Evaluation of photosynthetic performance, photo-oxidative injury and antioxidant status

7.0 Combined effect of UV-B and sodium chloride stress on cellular response of microalgae *Chlorococcum humicola* and *Chlorella vulgaris*: Evaluation of photosynthetic performance, photo-oxidative injury and antioxidant status

7.0 Introduction

Energy is the vital component for the existence of world and its growing demand can be accomplished by renewable source of energy such as solar energy, wind energy and biodiesel etc (Shahsavari and Akbari, 2018). The non renewable energy resources are on the verge of exhaust due to increased anthropogenic interventions and over growth of population (Kellogg, 2019), which might develop in serious energy crises in the near future (Kellogg, 2019). The other sources of energy like hydro-electric nuclear power and thermal power industries significantly compete with fossil fuels reserves (Oh et al., 2018). However, due to their destructive nature, energetic issues and considerable risk on human being and ecosystem, our present concern is shifting towards renewable, affordable, sustainable and low cost alternatives of energy.

Microalgae being the dominant photosynthetic autotrophs could be a powerful alternative of fossil fuel because of the fact of its adaptability to diverse habitat (waste water, saline water, arable land, dumping sites and water reservoir etc.), fast doubling time, limited land area requirement and ability to grow under harsh environment (Korosi et al., 2017). Singh et al. (2018) reported that the cultivation and management of microalgae are more ecofriendly and cheaper as it can be cultivated in waste water as well as in unproductive land. Microalgae are the dominant group of photoautotrophic organisms and essential biomass producer in aquatic ecosystems. Algal biomass holds a great potential as a substitute for conventional energy production and their applied application (Singh et al., 2018). The high photosynthetic capability of microalgae is not only valuable for lipid production but also beneficial for accumulation of nutrients, carbon sequestration and production of oxygen and balanced N-cycling in the thrust of environment (Wang et al., 2018). Over the past few years, research has focused on metabolic engineering of algae to produce fourth generation biofuel stock against limited water, land and environmental stresses (Abdullah et al., 2019).

Salinity and UVR are the major environmental obstacles for the present world and the environment (Wang et al., 2003). Salinity is introduced in the environment by the natural sources, anthropogenic activities and modern agricultural practices (Ismail et al., 2017) and UV radiation is a consequence of ozone depletion (Herndon et al., 2018). UV radiation reaching on the earth surface due to environmental pollution has increased due to various natural as well as anthropogenic activities.

Microalgae grown in the saline habitat in the presence of undesirable UV-B radiation may result into variety of complications for the plant and algae including osmotic stress, upwelling of nutrients and generation of reactive oxygen species (ROS) which causes physiological alterations, ionic disturbances photobleaching and oxidative damage (Jahnke et al., 2019; Williamson et al., 2019). However, microalgae have shown tendency to adapt to extreme conditions such as UV-B and salinity stress during the course of evolution (Singh et al., 2018). The high photosynthetic attributes of microalgae under various stresses is not only beneficial for lipid production, carbon sequestration, accumulation of nutrients, but also phycoremediation of waste water and environmental cycling of nutrients in the ecosystem (Mata et al., 2010).

The ROS produced inside the algal cell is a byproduct of cell metabolism which serves as a signal cascade to induce oxidative stress and provokes toxicity. The ROS damage the redox homeostasis and cellular functioning of the plant and algae (Daschoudhary and Roy, 2014). In microalgae, high NaCl activates ex-osmosis driven plasmolysis, shifting of water potential of the cell which restricts the entry of water and essential mineral contents (Singh et al., 2015). ROS induced oxidative damage in microalgae is counter attacked by the radical scavengers (enzymatic and non-enzymatic antioxidants) present in the algal system (Singh et al., 2018). Under high salinity, the photo synthetically generated energy is diminished and enhanced in the production of storage compound in the form of neutral lipids (Waltz, 2009; Upadhyay et al., 2016). However, UV- B is known to have both beneficial and toxic effects on the plants and algae (Kataria et al., 2014; Mata et al., 2010). UV-B induced alteration in the photosystem II (PSII) performance are due to changes in the quantum efficiency, RC inactivation, net closing rate of RC, trapping flux, performance index (cell vitality) and transfer of electron from RC to electron acceptor etc. (Strasser et al., 2004; Markou et al., 2017). But a long term exposure to UV-B radiation can result into inhibition in growth, lipid peroxidation, DNA damage, development of dimer (cyclobutane and pyrimidine) and death in algae as well as in plants (Zhang et al., 2015). To escape the harmful effect of UV-B, algae are endowed with certain cellular responses such as migration, development of protective cell wall, increase in the synthesis of carotenoids, compatible solute (compound with low molecular weight organic compounds) and the physiological and biochemical adaptations such as synthesis of various antioxidants (Zubia et al., 2010; Rastogi et al., 2013).

The objectives of this investigation was aimed to examined the synergistic effect of salinity and UV-B (time dependent) stresses on changes in the nutrient status, physiological and biochemical changes along with the lipid production potential of the microalgae *C. humicola* and *C. vulgaris*.

7.1 Methods and materials

7.1.1 Experimental setup

Pure algal cultures of *C. humicola* and *C. vulgaris* were inoculated in 1000 ml Erlenmeyer flasks containing 200 ml BG-11 media. The inoculated cultures were kept in culture room were acclimatized under laboratory condition (28 ± 2^{o}C under PAR of 150 µmol photo nm^{-2} s^{-1} and photoperiod of 16:8 h (light: dark). After 72 h of growth, 100 mM NaCl (Sigma-Aldrich, ACS reagent, >99.0% purity) was added separately in culture flasks. The UV-B radiation exposure was provided with the help of three Philips TL UV-B tubes (40W) with a spectral range from 280 to 315 nm. The average intensity of the UV-B radiation was 15 Wm^{-2}. The treatment was provided in following manner; algae + UV-B (2 h) + NaCl (100 mM) and algae + UV-B (4 h) + NaCl (100 mM). Two sets of control were used; (1) untreated control (2) UV-B treated (without NaCl). After the exposure of UV-B i.e., 2 h and 4 h, algal cultures were shifted under PAR light in culture room. All the treatments were maintained in BG-11 media. All the experiments were setup in triplicates. The harvesting of the biomass was carried out during the stationary phase of the growth (15d after NaCl and UV-B exposure) with help of refrigerated centrifuge for physiological as well as biochemical analysis. The whole experiment was set according to random block designing (RBD).

7.2 Measurements of photosynthetic performance

The photosynthetic performance viz., chlorophyll-a fluorescence induction (OJIP) curve, photosynthetic performance index (Pi_{ABS}), maximum quantum yield (Fv/Fm), Net closing rate of reaction centre (Mo), Trapping flux (TRo/RC), effective antenna size expressed as absorbance per reaction centre (ABS/RC) and percentage inhibition of reaction centre (RC) in microalgae *C. humicola* and *C. vulgaris* cells exposed to NaCl+UV-B were examined using Aqua Pen-C portable fluorometer (Photon Systems Instruments) Czech Republic).

7.3 Estimation of pigments

7.3.1 Estimation of chlorophyll and carotenoid

The chlorophyll estimation was done by the method of Arnon (1949), Carotenoids content in the extract was calculated at the wavelength of 480 and 510 nm, using the formula given by Duxbury and Yentsch (1956).

7.4 Biochemical parameters

7.4.1 Estimation protein content

The estimation of protein content was done by the protocol of Lowry et al. (1951) using bovine serum albumin (Sigma) as a standard.

7.4.2 Estimation of carbohydrate and total lipid content

25 mg freshly centrifuged algal cell was homogenized in 3 ml of 80% ethanol and then centrifuged at 3000×g for 10 min. 1.0 ml of supernatant was mixed with 1.0% phenol solution containing 5.0 ml of concentrated H_2SO_4 and the mixture was kept at room temperature for 30 min. The absorption of reaction mixture was recorded at the wavelength of 480 nm. Total lipid content of algal pellet was estimated by vanillin reagent prepared by ortho-phosphoric acid. The absorption of reaction mixture was recorded at 600 nm by following the method described by Ben-Amotz et al. (1985).

7.4.3 Estimation of MDA, H_2O_2 and electrolyte leakage

The lipid peroxidation (MDA) in algae was measured by the method of Heath and Packer, (1968). H_2O_2 was estimated following the method of Velikova et al.

(2000). Electrolyte leakage in algal cell was estimated by the methods of Dionisio-Sese and Tobita (1998).

7.4.4 Estimation of proline, cysteine and ascorbic acid

Proline content in algal cell was determined by using the methods of Bates et al. (1973). Cysteine content was determined by the method of Gaitonde (1967). Ascorbic acid was estimated by following the methods of Kampfenkel et al. (1995).

7.5 Preparation of enzyme extract

100 mg algal cells were homogenized in 3 ml of 100 mM potassium phosphate buffer (pH 7.5), containing polyvinyl polypyrrolidone (PVP) and 1 mM of EDTA followed by centrifugation at 10,000×g for 10 min at 4^{o}C. The preparation of enzyme extracts was carried out in cold room at 4^{o}C. The crude enzymes extracts used for the assay of different enzymes.

7.5.1 Enzymes assay

Catalase (CAT) activity was performed by the method of Aebi (1984) Superoxide dismutase (SOD) activity was assayed by the method of Nishikimi and Rao (1972), using the crude enzyme extracts. Glutathione reductase (GR) activity was assayed by the method of Smith et al. (1988).

7.6 Sample preparation and analysis of SEM coupled with EDS

The microalgal cells were fixed in 2.5% of glutaraldehyde solution for 4 h at 4^{o}C, washed with phosphate buffer and subsequently dehydrated by ethanol (10%, 30%, 50%, 70% and 95%) for 5 min at each concentration (Singh et al., 2018). SEM coupled with energy dispersive X-ray spectrophotometer (EDS) detector was used for the X-ray spectra. X-ray plotting was done using EDS in conjunction with scanning electron microscopy (SEM) (JEOL, Japan; model JSM-6490LV).

7.7 Fourier transforms infrared spectroscopy (FTIR) analysis

For measurment of FTIR spectrum in microalgae, the dried microalgae cell samples were grinded with potassium bromide (KBr, 1:10 ratio) and pressed to form pellets using hydraulic press (150 lbs). Further the pillet were used to elucidate the spectra of biomolecules using FTIR (Thermo-Scientific Nicole 6700, USA). The background absorbance of KBr was corrected by using the softwere.

7.8 Data analysis

All determinations were done in three replicates. To compare validity and variability between groups and among each other, one-way analysis of variance (ANOVA) was used, followed by Duncan's multiple range test (DMRT, P≤0.05). All statistical calculations were carried out using the SPSS software.

7.9 Results and discussion

7.9.1 Effect on growth

The growth characteristics of microalgae *C. humicola* and *C. vulgaris* under different varieties i.e. 100 mM NaCl, UV-B exposure (2 h and 4 h) and both 100 mM NaCl + 2 h UV-B and 100 mM NaCl+4 h UV-B showed variation in phases of growth (Fig. 7.1). The exponential phase in all the microalgae strains started from 3rd day and continued up to 15-16th day under control lab condition. However, NaCl + 4 h UV-B exposure in both algal strains resulted into reduction in the total span of exponential phase i.e., from 9 to 12 day. At 2 h UV-B + 100 mM NaCl, the exponential phase in both *C. humicola* and *C. vulgaris* was increased as compare to 4 h UV-B + 100 mM NaCl. However, growths of microalgal were decreased as compared to untreated control indicating toxic effect of both NaCl and UV-B.

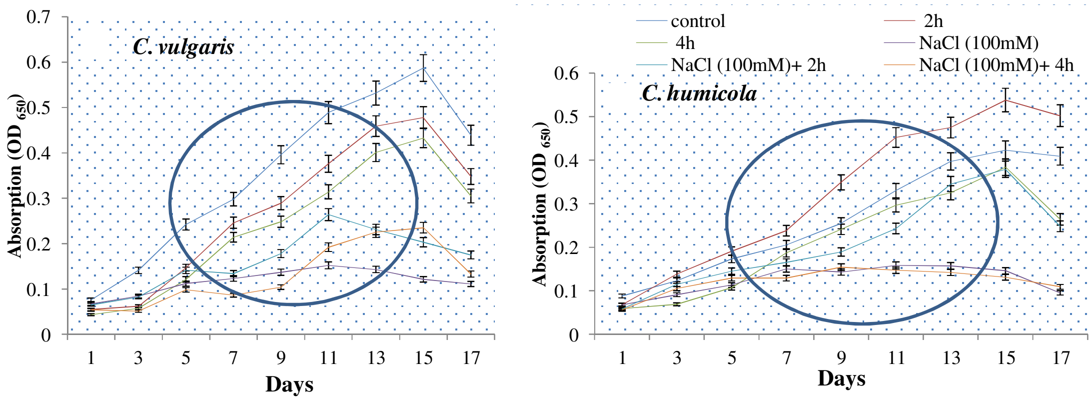


Fig. 7.1: Growth response of algae *C. vulgaris* and *C. humicola* exposed to combined treatment of NaCl (100 mM) and UV-B (h). All the values are means ± S.D.

7.9.2 Effect on photosynthetic pigment

The chlorophyll content in algae *C. vulgaris* exposed to UV-B alone at 2 h and 4 h experienced severe toxicity as evident by significant decreased by 32.87% and 60.27%, respectively (Fig. 7.2). *C. vulgaris* treated with 100 mM NaCl alone exhibited increase in the chlorophyll content by 16.4%. But the combined effect of 100 mM NaCl+UV-B (2 h and 4 h) exhibited reduced effect of UV-B exposure. However, exposure of both the strains to UV-B alone for duration of 2 h and 4 h showed maximum inhibition of chlorophyll pigment. Algae being a potential renewable source for sustainable world, its impact were evaluated and results emphasize the decreasing chlorophyll level. Decreased chlorophyll concentration under UV-B and salt might be due to disruption of various photosynthetic process viz., electron transport system, photosystem II and pigment bleaching (Pathak et al., 2019). Various authors have been reported the decreased chlorophyll content in microalgae *Dunaliella salina, Nostoc muscorum, Plectonema boryanum, Aphanothece* sp. and *Arthrospira (Spirulina) platensis*, exposed to UV-B radiation (Prasad and Zeeshan, 2005; Gao and Ma, 2008; Tian and Yu, 2009; Zeeshan and Prasad, 2009; Zhang et al., 2015). However, UV-B exposed algae supplemented with 100 mM salt and UV-B (NaCl+UV-B) exhibited slight increase in chlorophyll level at 2 h (1.36%) as compared to their respective control (untreated) in microalgae *C. vulgaris* Further, *C. vulgaris* treated with NaCl (100mM)+UV-B (4h), 60.27% decrease in chlorophyll content was observed. In the case of *C. humicola* cells, exposed to UV-B alone for 2 h and 4 h, the chlorophyll content drastically decreased from 0.071±0.010 to 0.053±0.014 and 0.071±0.010 to 0.028±0.002 mg g^{-1} fw, respectively. Increase in the chlorophyll concentration under UV-B and salt might be due to antagonistic effect of salt against UV-B (Pathak et al., 2019). Upadhyay et al. (2016) have also reported a marked and progressive decrease in the chlorophyll content attributed to ROS induced photoreduction, chlorosis and formation of triplet chl which strongly damage the photosystem I and II, followed by photosynthetic machinery and chlorophyll formation. Under combined treatment of NaCl and UV-B, algal cells might be experiencing a cascade of physiological alterations including CO_2 flux, decreasing nutrients uptake, increased NADPH and limited entry of absorbed light into the photosystem, causing a decrease in the chlorophyll biosynthesis and biomass accumulation (Srivastava and Goud, 2017). On the other hand, low dose of UV-B enhances the chlorophyll concentration in the present study may be due to additive response to UV-B flux and PAR by microalgae (Núñez-Pons et al., 2018). Figueroa et al. (2009) have also reported the positive response of UV-B in algae *Zygnemopsis decussate* under short duration of UV exposure.

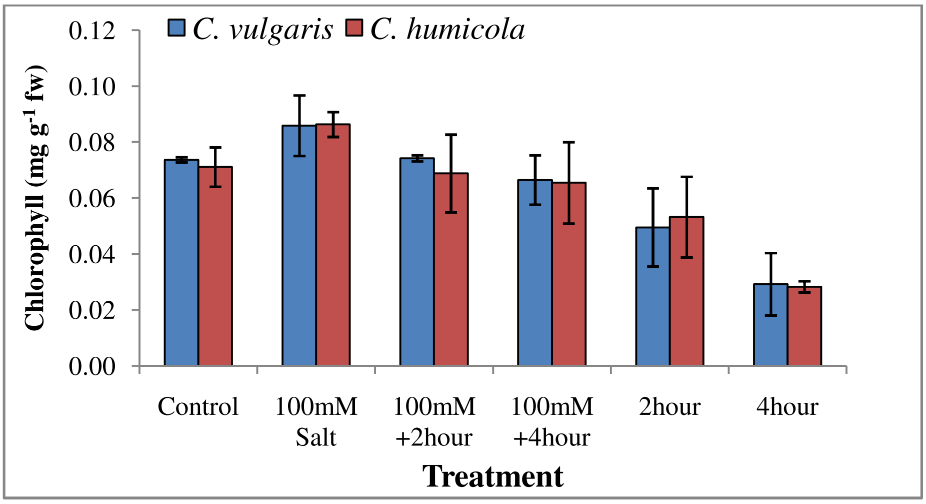


Fig. 7.2: Effect of NaCl (100 mM) and UV-B (2 h and 4 h) on chlorophyll content in microalgae *C. vulgaris* and *C. humicola*. All the values are means ±S.D (n=3)

The accessory pigment carotenoid showed increase in the level with the increasing duration of UV-B exposure to both the algae (Fig. 7.3). In the presence of 100 mM NaCl results showed increasing trend in the carotenoid content in both the algae. The percent increase in the carotenoid in *C. vulgaris* treated with UV-B (2 h), UV-B (4 h), 100 mM + 2 h and 100 mM + 4 h UV-B exposure was in the order of 3.8, 1.01, 20.4 and 17.7%, respectively. In *C. humicola*, the carotenoid content with similar treatment was in order of 16.6, 8.25, 33.82 and 20.35%, respectively. Carotenoids are accessory pigment fundamentally important in light harvesting and protection against oxidative damage (Ramanna, et al., 2017) by acting as antioxidants (Pick et al., 2019). In the present study an increase in the carotenoid level indicated adaptive tolerance responses of the algae against oxidative species to cope with the oxidative damage. The protective role of carotenoid may be ascribed to carotenoid induced deactivation of singlet oxygen and quenching of excited triplet state of chlorophyll and rendering the formation of ${}^1O_2$ species (Rady et al., 2018). Carotenoid protects the algae by quenching high amount of energy during excitation and release, it in the form of heat (Takahashi et al., 2018). The stress condition of the algae produced due to UV-B and salt can also evaluated by the ratio of carotenoid to chl a and change in the ratio clearly indicated the photoprotective response of the cell. Under stress condition, production of secondary metabolites enhances the cell size, carotenoid and lipid content of the cells (Czerpak *et al.,* 2002). An enhanced level of carotenoid under NaCl and UV-B has been reported by various other workers (Singh et al., 2018b, Upadhyay et al., 2016).

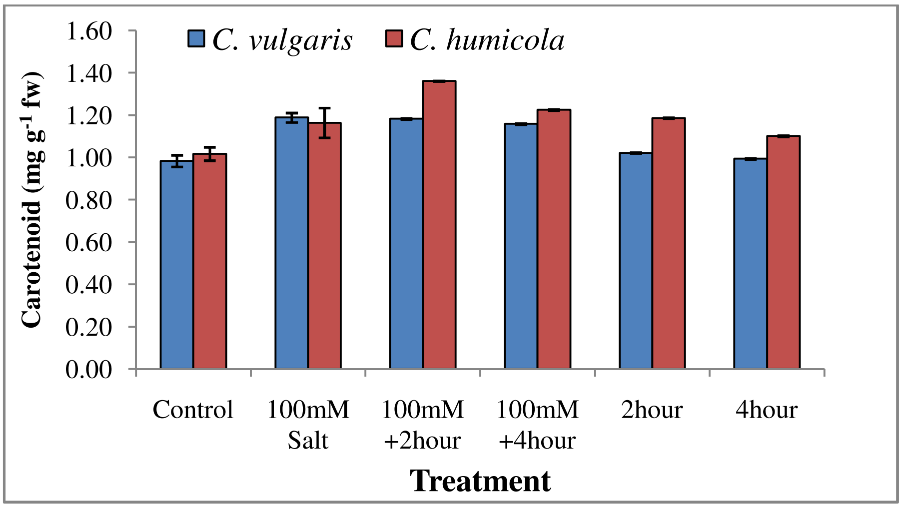


Fig. 7.3: Effect of NaCl (100 mM) and UV-B (2 and 4 h) on carotenoid content in microalgae *C. vulgaris* and *C. humicola*. All the values are means ±S.D. (n=3)

7.9.3 Effect on protein

Microalgae *C. vulgaris* and *C. humicola* treated with NaCl (100 mM) and UV-B exhibited increased in the concentration of protein after 2 h UV-B exposure under the NaCl supplemented condition, which was in the order of 45.6% and 48.95%, respectively, when compared with their respective control (Fig. 7.4). Microalgae separately treated with salt 100 mM and UV-B (4 h) exposure separately showed reduction in protein by 8.56% and 24.09%, respectively in *C. vulgaris*. However, 2 h of UV-B exposure to *C. vulgaris*, the protein content was increased by 7.2% as compared to untreated control. *C. vulgaris* treated with 100 mM NaCl +UV-B (4 h) also exhibited increase in the protein content to 0.934±0.025 mg g^{-1} fw in comparison to control (0.747±0.022 mg g^{-1} fw). In the case of *C. humicola*, protein content in alga treated with NaCl+ UV-B (4 h) was enhanced by 12.52%. Further, *C. humicola* exposed to 4 hr UV-B only, exhibited maximum decrease (26.60%) in protein content as compared to control. However, with 100mM NaCl+UV-B (2h), it was maximum increased by 48.95% as compared to control.

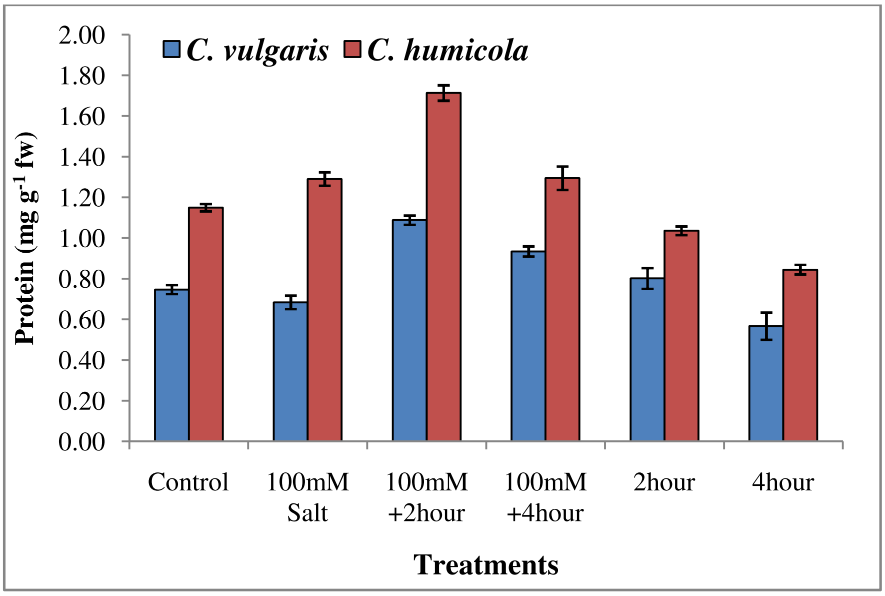


Fig. 7.4: Effect of NaCl (100 mM) and UV-B (2 and 4 h) on protein content in microalgae *C. vulgaris* and *C. humicola*. All the values are means ±S.D. (n=3)

7.9.4 Effect on lipid and carbohydrate

C. vulgaris and *C. humicola* treated with NaCl and UV-B exposure showed increase in the level of lipid. Maximum increase in the lipid content was observed in both the algae given combined treatment of NaCl and UV-B (2 h) (Table 7.1). Lipid content in microalgae *C. vulgaris* exposed to UV-B (2 h) alone was found to be increased by 31.9%. However, maximum increase in the lipid content of *C. humicola* was found 2.120±0.11 mg L^{-1} treated with NaCl+UV-B (2 h) treatment in comparison to control (1.352±0.017 mg L^{-1}) i.e., 56.80%. Maximum decrease in lipid content was observed in both the algae exposed to 4 h of UV-B exposure. On the other hand, *C. vulgaris* and *C. humicola*, treated with NaCl (100 mM) only exhibited increased lipid content by 12.80% and 6.2%, respectively.

Carbohydrate content in microalgae *C. vulgaris* and *C. humicola*, treated with NaCl and different duration of UV-B exposure (2 h) period showed enhanced accumulation of carbohydrate by 8.9 % and 16.36%, respectively as compared to control (Table 7.1). *C. vulgaris* cells exposed to NaCl+UV-B (2 h) showed maximum increase in the level of carbohydrates followed by 2 h , NaCl (100 mM), NaCl+UV-B (4 h) and UV-B (4 h) respectively. In the case of *C. humicola*, decreasing level of carbohydrate was observed at UV-B exposure of 4 h by 18.38% while with other treatment salt, UV-B alone and NaCl+UV-B, the level of carbohydrate showed increasing trend in comparison to control. Carbohydrate and lipid plays an important role in carbon partitioning, photosynthesis, osmotic homeostasis and cell metabolism (Rosa et al., 2009). A variation in the carbohydrate content of algae during various treatments may be ascribed to changes in the photosynthetic rate, inhibition of cell division and osmotic imbalance (Sami et al., 2016). An increase in the lipid and carbohydrate content of algae under salinity and UV-B stress may be due to alteration in the photosynthetic processes (PSII) and synthesis of different compound including glycerol, sugar, osmoprotectant and protein (Paliwal et al., 2017). George et al. (2014) also reported that lipid and carbohydrate content in microalgae can be enhanced by different stress factors viz., nutrient starvation, salt stress, pH, temperature and UV-B. A comparative increase in the lipid content of microalgae in NaCl with UV-B treated *C. humicola* might be due to shift of sugar/ carbohydrate metabolism towards lipid storage form to counter attack the effect of stress in microalgae (Yilancioglu et al., 2014). Pancha et al. (2014) reported that cultivation of algae under different and continuous stress conditions might enhance the accumulation of carbohydrate or lipid but that decreases their growth rate, protein and nutrients status.

Table 7.1: Effect on lipid (mg L^{-1}) and carbohydrate (mg L^{-1}) content in microalgae *C. vulgaris* (CV) and *C. humicola* (CH) treated with NaCl (100 mM) and UV-B. All the values are means ±S.D.

Treatment	**Lipid**		**Carbohydrate**	
	CV	**CH**	**CV**	**CH**
Control	1.421±0.012	1.352±0.017	24.22±0.354	26.56±0.485
NaCl (100 mM)	1.603±0.009	1.437±0.025	22.396±0.303	27.182±0.258
UV-B (2 h)	1.875±0.012	1.529±0.011	26.387±0.309	30.907±0.270
UV-B (4 h)	1.143±0.016	1.038±0.014	20.23±0.857	21.676±0.346
NaCl (100 mM) +UV-B (2 h)	2.094±0.011	2.120±0.021	28.018±0.228	32.080±0.336
NaCl (100 mM) +UV-B (4 h)	1.437±0.020	1.313±0.018	21.511±0.280	28.764±0.378

7.9.5 Effect on thiobarbituric acid reactive substances (TBARS)

Microalgae *C. vulgaris* and *C. humicola* given combined treatment of salt and UV-B increased the MDA content. *C. vulgaris* exposed to NaCl+UV-B (4 h) showed maximum increased in MDA level (Table 7.2). Similar results were observed in case of *C. humicola* treated with NaCl+UV-B (4 h). However, both algae exhibited minimum increase in MDA content under NaCl+UV-B (2 h) treatment as compared to other treatment and exposure time. Overall, results depicted that microalgae given combined treatment of NaCl and UV-B (2 h) expressed least toxicity. Thiobarbituric acid reactive substances (TBARS) a product of lipid peroxidation in algae cause cellular toxicity under NaCl and UV-B stress. TBARS is byproduct of lipid peroxidation act as a bio indicator of cellular toxicity (Yilancioglu et al., 2014). High level of TBARS in algae might be ascribed to UV-B and salt induced production of fatty acid radicals as a result of ROS (hydroxyl and peroxyl radicals) reaction followed by hydrogenation and production of intermediate compound (TBARS) (Rastogi et al., 2014). High TBARS content in algae emphasizes severe oxidative damage to membrane lipid as reported by various authors (Upadhyay et al., 2016; Singh et al., 2018b).

7.9.6 Effect on H_2O_2 and electrolyte leakage

Microalgae *C. vulgaris* and *C. humicola* exposed to UV-B+NaCl exhibits the toxic effect of treatment in the form of increased H_2O_2 content (Table 7.2). The H_2O_2 content was found to be higher in all the treatments. *C. vulgaris* and *C. humicola* exposed to 2 h UV-B, H_2O_2, the content was found to be maximum increased by 60.63% and 48.95%, respectively as compared to control. H_2O_2, a non-radical reactive oxygen species involves in form of dual role of stress causing agent as well as signaling molecules H_2O_2 causes toxicity only at higher concentration (Sofo et al., 2015). The enhanced H_2O_2 concentration in the present study was in accordance with the results of Singh et al. (2018). High H_2O_2 content indicated cell damage mediated by membrane breakage and cell lysis (Upadhyay et al., 2016; Singh et al., 2018a).

The amount of electrolyte leakage (EL) in microalgae exposed to NaCl (100 mM) + UV-B (2 h) showed increase in the EL level by 12.68% in the case of *C. vulgaris*, while with *C. humiocola* EL content was enhanced by 27.98% as compared to control (Table 7.2). In the case of UV-B exposure alone for different duration of (2 h and 4 h), algae *C. vulgaris* and *C. humicola* expressed different degree of EL in the order of 85.11 µs cm^{-1} (2 h) and 82.72 µs cm^{-1} (2 h) as compared to control. However, 4 h exposure of algae to UV-B, the change in EL was 93.56 and 97.09 µs cm^{-1} respectively as compared to their control 86.42 and 70.53 µs cm^{-1}, respectively. In the case of NaCl (100 mM) treated microalgae C. *vulgaris* and *C. humicola*, the level of EL was found to be increased by 16.67% and 57.75%, respectively, in comparison to control. The electrolyte leakage (EL) is the measure of ionic concentration in the solution. EL is associated with the membrane damage and acts as biomarker in plants and algae (Singh et al., 2018a).

Table 7.2: Effect on TBARS (mM g^{-1} fw), H_2O_2 (μmole g^{-1} fw) and electrolyte leakage (μs cm^{-1}) content in microalgae *C. vulgaris* and *C. humicola* treated with NaCl (100 mM) and UV-B. All the values are means ±S.D.

Treatment	**TBARS**		**H_2O_2**		**EL**	
	CV	**CH**	**CV**	**CH**	**CV**	**CH**
Control	107.74± 10.81	90.97± 13.11	103.47± 2.99	124.52± 4.22	86.42± 0.67	70.53± 0.81
100 mM NaCl	125.59± 8.88	106.48± 12.58	109.97± 18.26	136.16± 9.53	100.91 ± 1.08	97.16± 1.06
2 h UV-B	119.68± 11.92	103.22± 5.81	166.21± 5.07	185.48± 14.11	85.11± 0.99	82.72± 0.98
4 h UV-B	147.53± 23.56	143.44± 14.76	145.43± 3.24	171.55± 3.83	93.56± 1.15	97.09± 0.89
2 h UV-B +NaCl (100 mM)	113.69± 5.24	102.80± 5.24	132.99± 8.50	155.31± 8.63	97.38± 0.64	90.27± 1.00
4 h UV-B +NaCl (100 mM)	185.23± 14.04	176.40± 11.13	161.75± 6.08	178.44± 13.93	170.81 ± 2.08	90.91± 1.15

EL is directly linked with injury to the algal cells due to high NaCl and UV-B exposure. High level of NaCl in algal cell induces the efflux of K^+ leakage which ultimately changes the membrane polarization and significantly diminishes the osmotic tolerance response triggered by K^+ present in the cytosol (Demidchik et al., 2014). However an increase in EL content indicates the membrane breakage and cell lysis under various environmental stresses (Upadhyay et al., 2016).

7.10 Effect on antioxidants system

7.10.1 Ascorbic acid

Microalgae treated with NaCl and different UV-B exposure periods showed increase in ascorbic acid content with increase exposure time of UV-B (Fig. 7.5). Maximum ascorbate content in *C. vulgaris* was found at NaCl+UV-B (4 h) i.e., 6.699±0.437mM g^{-1}fw in comparison to control. *C. vulgaris* treated with NaCl (100mM) alone showed approximately 4-5 folds increase in ascorbic acid content while with *C. humicola*, it was 2-3 fold. Ascorbate content in *C. vulgaris* exposed to 2 h UV-B was increased by 25.87% and in *C. humicola* by 56.93%. Microalgae treated with NaCl(100 mM)+ UV-B (2 h) exhibited ~3 fold (*C. vulgaris*) and ~2 fold (*C. humicola*) increased in the level of ascorbate as compared to control. Overall results showed that maximum increased in ascorbate content in both the algae was observed at NaCl(100mM) + UV-B (4 h) and minimum with UV-B (2 h) only. Ascorbic acid (AsA) functions as an antioxidant in the plant cell under stress condition. It is one of the most abundant antioxidant of endogenous origin, maintains the cellular redox imbalance produced due to oxidative burst of reactive oxygen molecules (Vidal-Meireles et al., 2017). High level of AsA clearly reflects the stress tolerance response of algae. Ascorbate induced stress tolerance might be attributed to enzyme cascade conversion of H_2O_2 and ascorbic acid viz., MDHA, DHA etc. in step-by-step reaction (Shereefa et al; 2016) in AsA-GSH cycle. Rai and Agrawal (2017) reported that ascorbic acid binds with nascent oxygen produced during environmental stress condition and diminishes the damage caused by ROS. Rastogi et al. (2011) also suggested that presence of various reducing agents inside living cell play a key role in maintaining the integrity and function of different cellular constituents probably involved in mitigating the damaging effects of UV-B.

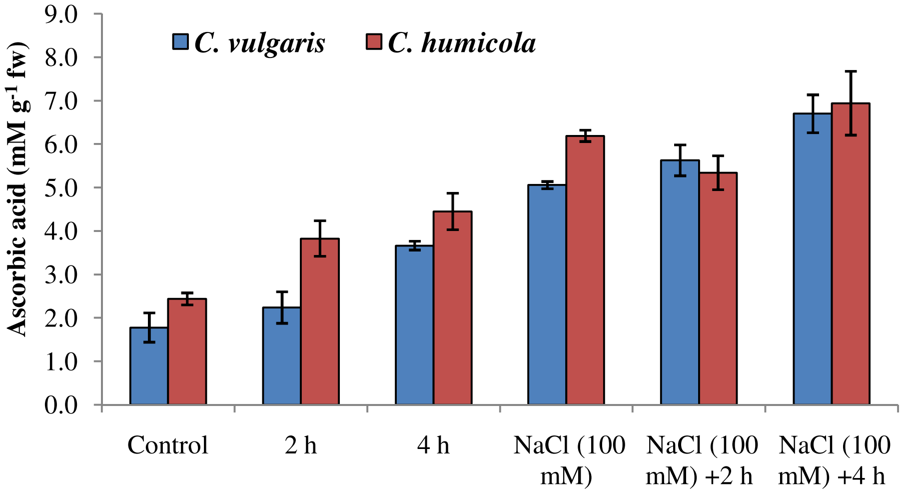


Fig. 7.5: Effect on ascorbate content in microalgae *C. vulgaris* and *C. humicola* treated with NaCl (100 mM) and UV-B (2 and 4 h). All the values are means ±S.D.

7.10.2 Cysteine

Cysteine content in *C. vulgaris* and *C. humicola* treated with salt (NaCl, 100 mM) and UV-B (2 h and 4 h) was found to be increased with their respective control. (Fig. 7.6). *C. vulgaris* and *C. humicola* exposed to NaCl+UV-B (4 h), showed maximum increased in cysteine content by 63.83 and 72.88% respectively, as compared to their respective control. While minimum increase in cysteine content was observed in both algae exposed to UV-B (2 h) in comparison to control. Cysteine is –SH group containing amino acid, which contributes significantly to number of enzymatic and non enzymatic reactions in the cells.

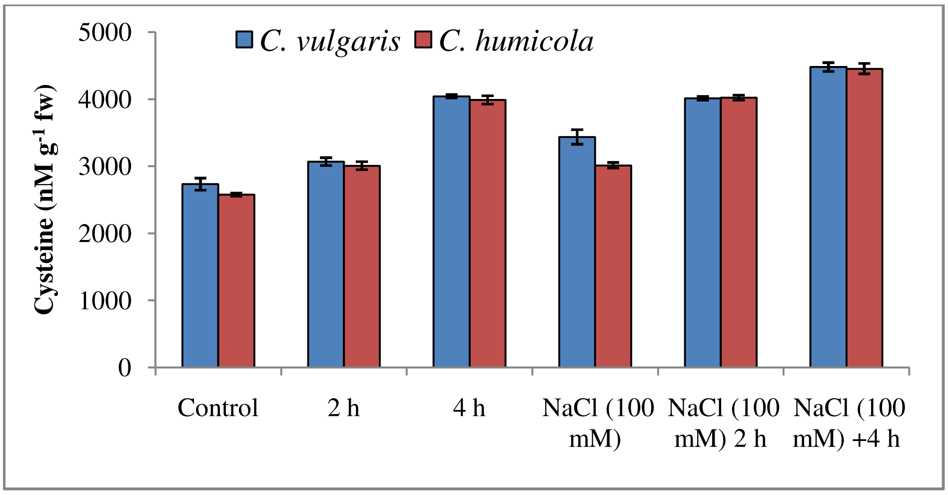


Fig. 7.6: Effect on cysteine content in microalgae *C. vulgaris* and *C. humicola* treated with NaCl (100 mM) and UV-B (2 and 4 h). All the values are means ±S.D.

Cysteine works as a precursor of different thiol containing antioxidants produced under stress, thus cysteine content indicates the extent of stress in algae and plant cell (Wirtz et al., 2010). Increase in the level of cysteine in the present study indicated higher stress tolerance responses in the algae due to scavenging of reactive oxygen species produced due to UV-B exposure and high salt concentration (Hayat et al., 2012; Upadhyay et al., 2014; 2016).

7.10.3 Proline

C. vulgaris exposed with 2 h and 4 h UV-B exposure showed increase in the concentration of proline as compared to control (Fig. 7.7). Proline content in *C. vulgaris* showed maximum value under NaCl+UV B (4 h) treatment. However, no significant change was observed in *C. vulgaris* treated with NaCl (100mM) +UV-B (2h) exposure as compared to 2 h of UV-B exposure. In case of *C. humicola*, more than 3 fold increase in the proline content was observed under NaCl+UV-B (4 h) treatment. However, minimum increase in proline (46.05%) was observed after 2 h UV-B exposure as compared to control. Proline is an important osmolyte and antioxidants in algae produced during stress. High proline content exhibited stress tolerance response of the algae due to its free radical scavenging attributes in subcellular structure. It is also participate in redox imbalance and homeostasis of the cell (Zouari et al., 2016).

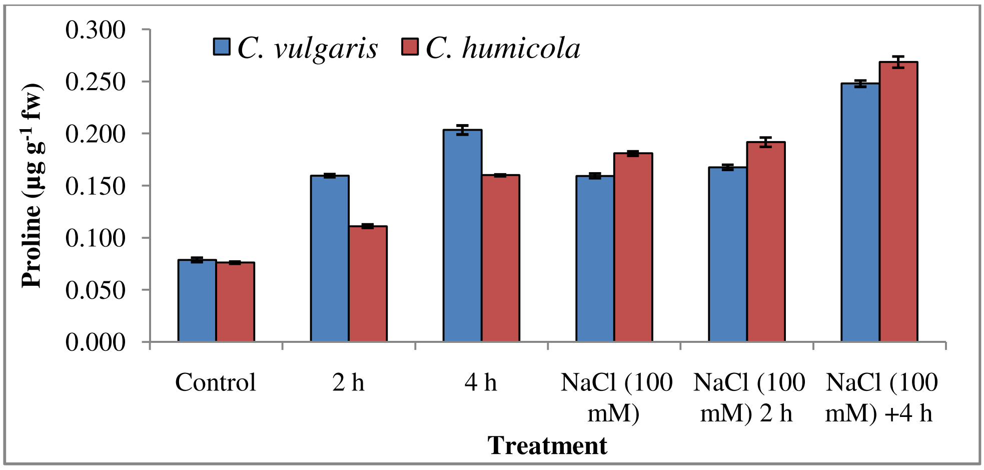


Fig. 7.7: Effect on proline content in microalgae *C. vulgaris* and *C. humicola* treated with NaCl (100 mM) and UV-B (2 and 4 h). All the values are means ±S.D.

7.10.4 Effect on antioxidant enzyme system

The antioxidants protect the algae by maintaining the structure of photosynthetic membrane, RC, protein integrity, inhibition of xanthophylls cycle and absorption of substantial amount of UV-B (200-300 nm) radiation and salt induced injury (Tyagi et al., 2003; Xue et al., 2005). Salt and UV-B induced oxidative stress in algae is counteracted by the production of various antioxidative enzymes including peroxidases, catalase, SOD and GR etc (Hossain and Dietz, 2016).

7.10.5 Superoxide dismutase

SOD acts as first line of defence against the cellular toxicity induced by ROS. Microalgae *C. vulgaris* and *C. humicola*, exposed to different duration of UV-B (2 h) showed no significant change in SOD activity (3.9% and 2.97%, respectively) as compare to their respective control. But exposure of algal cells to 4 h of UV-B treatment, the SOD was increased by 10.99 % and 5.69%, respectively (Fig. 7.8). The SOD activity in microalgae *C. vulgaris* and *C. humicola* treated with 100 mM NaCl, the activity was found to be increased by 41.31% and 39.85% respectively. In the case of combination of UV-B+NaCl, maximum SOD activity was observed at UV-B (4 h)+NaCl (100 mM) which was 58.11% and 45.04% as compared to control. SOD is an effective metallozymes which acts as primary scavenger of reactive oxygen species and dismutates the superoxide ion (O_2^-) into H_2O_2 and water (Singh et al., 2016).

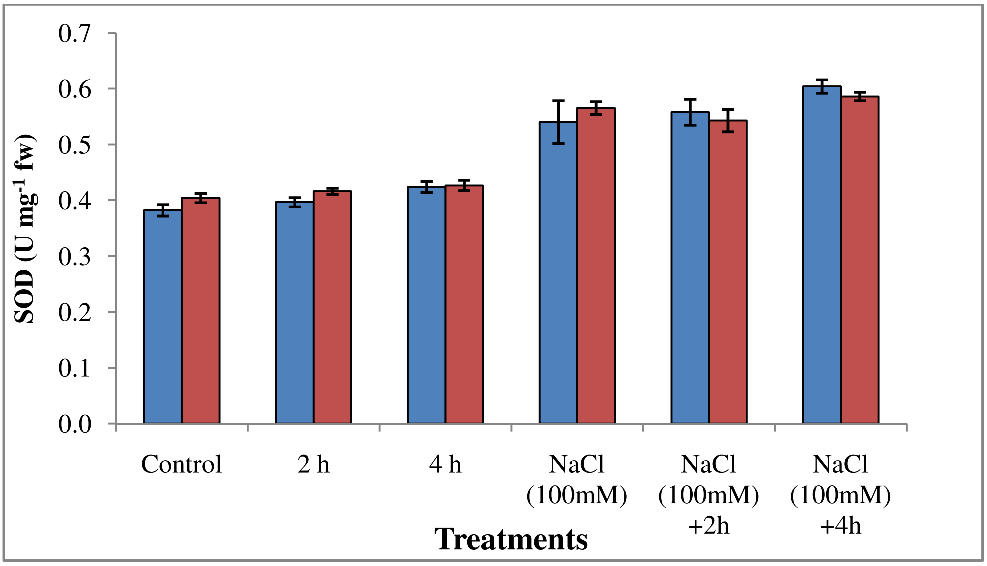


Fig. 7.8: Effect on superoxide dismutase activity in microalgae *C. vulgaris* and *C. humicola* treated with NaCl (100 mM) and UV-B (2 and 4 h). All the values are means ±S.D.

7.10.6 Catalase

Catalase is an important antioxidant enzyme in defense system in plants and algae. Microalgae exposed to different treatment variables of UV-B and NaCl, expressed increase in the catalase activity with respect to control (Fig. 7.9). Catalase activity in *C. vulgaris* cells exposed to 2 h and 4 h of UV-B treatment was increased by 21.9% and 46.1%, respectively, while with *C. humicola* it was enhanced by 24.79% and 47.23%, respectively. In the case of microalgae *C. vulgaris* and *C. humicola* exposed under the combination of NaCl (100 mM)+UV-B (2 h and 4 h), the catalase activity was >2 fold in comparison to control. Catalase significantly scavenges the H_2O_2 into H_2O and O_2 in peroxisomes. High catalase activity represents stress injury and ameliorating potential of algae (Rai et al., 2013).

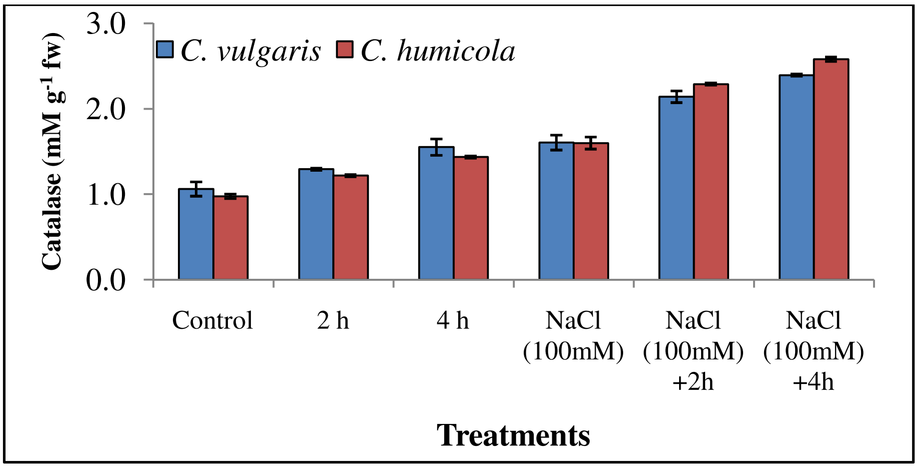


Fig. 7.9: Effect on catalase activity in microalgae *C. vulgaris* and *C. humicola* treated with NaCl (100 mM) and UV-B (2 and 4 h). All the values are means ±S.D.

7.11 Effect on photosynthetic performance

The photosynthetic performance efficiency of *C. vulgaris* and *C. humicola* under different treatment of UV-B, (2 h and 4 h) with NaCl (100 mM) and alone NaCl has been depicted (Table 7.3). The Fv/Fo and Fv/Fm ratio decreased in all the treatments except when microalgae were exposed to 2 h of UV-B radiation. Maximum decrease in Fv/Fo and Fv/Fm ratio was observed in *C. vulgaris* treated with NaCl (100 mM)+4 h UV-B exposures (80.57%) as compared to control, while with *C. humiocla,* the lowest value of Fv/Fm was observed in NaCl (100 mM) treatment cells only. The ABS/RC ratio in *C. vulgaris* at 100 mM, 100 mM+2 h, 100 mM+4 h, 2 h and 4 h was in order of 2.155, 2.344, 2.470, 2.057 and 2.140, respectively with respect to control and with *C. humicola*, it was 2.468, 1.741, 1.565, 1.285 and 1.334, respectively. In the case of TR_0/RC, *C. vulgaris* exhibited maximum level at 2 h UV-B exposure and with *C. humicola* maximum value was observed with 100 mM NaCl treatment. The photosynthetic parameter Mo, denoting net closing rate of reaction centre. The Mo value in *C. vulgaris* was found to be minimum after 2 h exposure of UV-B and 4 h in *C. humicola*, the Mo value was 0.240 after 4 h of UV-B exposure as compared to their respective control (0.334 and 0.322). In case of Pi_{ABS}, the value was increased after 2 h of UV-B exposure, but it decreased with the other treatments in *C. vlugaris*. In the case of *C. humicola*, Pi_{abs} value was increased by approx. 2 fold after 2 h and 4 h of UV-B treatment.

The percent inhibition of RC was deduced by the calculating the ratio of ABS/RC of untreated control to the ABS/RC treated and found maximum inhibition of reaction centre in both the algae, after 2 h and 4 h of UV-B exposure. Minimum inhibition (62%) was observed at 100 mM concentration of NaCl in microalgae *C. humicola*. The closure of RC in *C. vulgaris* was the highest (72%) in cells treated with NaCl (100 mM) +4 h UV-B. The Fv/Fm is an indicator of photosynthetic yield of the PSII for the process of photosynthesis (Maxwell and Johnson, 2000; Oxborough, 2004; Qiao et al., 2015). Decreased maximum quantum yield (Fv/Fm) in both algae *C. vulgaris* and *C. humicola* under NaCl, UV-B and NaCl+UV-B may be ascribed to damage to PSII reaction centre of photosynthetic apparatus (Vadiveloo et al., 2016; Chen et al., 2017). ABS/RC ratio i.e., apparent antenna size and trapping flux per reaction centre (TR_0/RC) in microalgae exposed under different treatment as compared to their control might be due to high absorption which leads to closure of reaction centre and thus photosynthesis (Strasser et al., 2004; Markou et al., 2017).

The photosynthetic performance index (Pi_{abs}) denotes the vitality of algal cells (Gururani et al., 2018). High Pi_{abs} reflects high photosynthetic efficiency. However, decrease Pi_{Abs} may be due to decline in the vitality of the algae photosystem and enhanced toxicity induced by UV-B and NaCl (Jiang et al., 2012; Negi et al., 2016). The net closing rate of RC (Mo) was found to be increased due to various combination of treatment. Markou et al., (2017) reported that faster inhibition of PSII activity enhances the level of Mo in both algae (Castenholz and Garcia-Pichel, 2000).

Table 7.3: Photosynthetic performance of algae *C. vulgaris* and *C. humicola* under different duration of UV-B exposure and NaCl (100 mM). The parameters were derived from OJIP curve measured by PAM fluorometer

Treatments	**Photosynthetic parameters**					
	Fv/Fo	**Fv/Fm**	**Mo**	**Pi_{Abs}**	**ABS/ RC**	**TRo/ RC**
			C. vulgaris			
Control	1.251	0.556	0.334	1.381	1.789	0.994
100 mM	1.108	0.526	0.354	1.130	2.155	1.133
2 h+100 mM	0.818	0.450	0.358	0.679	2.344	1.055
4 h+100 mM	0.811	0.448	0.403	0.579	2.470	1.106
2 h	1.270	0.560	0.321	1.600	2.057	1.151
4 h	1.119	0.528	0.336	1.236	2.140	1. 130
			C. humicola			
Control	2.000	0.667	0.322	2.848	1.547	1.031
100 mM	0.934	0.483	0.402	0.743	2.468	1.192
2 h+100 mM	1.546	0.621	0.336	1.910	1.741	1.057
4 h+100 mM	1.639	0.607	0.297	2.385	1.565	0.922
2 h	2.596	0.722	0.241	5.757	1.285	0.928
4 h	2.502	0.718	0.240	5.687	1.334	0.958

7.12 Effect of UV-B on the FTIR spectra

FTIR has been used to demonstrate the functional group orientation and concentration of macromolecules in plant and algal cells. The spectral peak (wave number) of macromoecules viz., protein (amide I and amide II) (1540-1650 cm^{-1}), lipid (carbonyl, methylated and phosphodiester) (1650-1750 cm^{-1}) and carbohydrates (ranges between 1200-900 cm^{-1}) were observed in *C. vulgaris* and *C. humicola* exposed to UV-B and NaCl. The spectral peak length of different biomolecules were used to examine the alteration in cell constituents caused by NaCl and UV-B treatment of cells.

FTIR spectra of *C. vulgaris* and *C. humicola* under UV-B and NaCl exposure have been depicted in Fig. 7.10. The FTIR spectra showed different absorption peaks of macromolecules viz., carbohydrate, proteins, lipids and phosphorylated molecules.

Microalgae *C. vulgaris* and *C humicola* represented different bond length and associated functional groups according to the level of toxicity. In the case of algae *C. vulgaris*, exposed for 4 h UV-B radiation, bond peak length of NH- amide was increased by 4.25% (Fig. 7.10.A). After 4 h of UV-B, peak of C-H stretching of methylated lipid (wave number =2922.5 cm^{-1}) was observed which was not detectable in untreated control cells. The results indicated the increase in the concentration of lipid biomolecules. The cell of *C. vulgaris* treated with NaCl+UV-B (4 h) also exhibited enhanced peak of lipid (wave number= 2925.5 cm^{-1}). *C. vulgaris* exposed under 4 h UV-B showed enhanced peak of isothiocynate compound as compared to control. The protein peak, it were increased in *C. vulgaris* exposed to UV-B alone, but it was higher in the cells treated with the UV-B+NaCl as compared to control. In case of carbohydrate, altered peak length was observed in *C. vulgaris* exposed under NACl+UV-B, however no significant alteration in carbohydrate peak was observed in algae trated with UV-B alone.

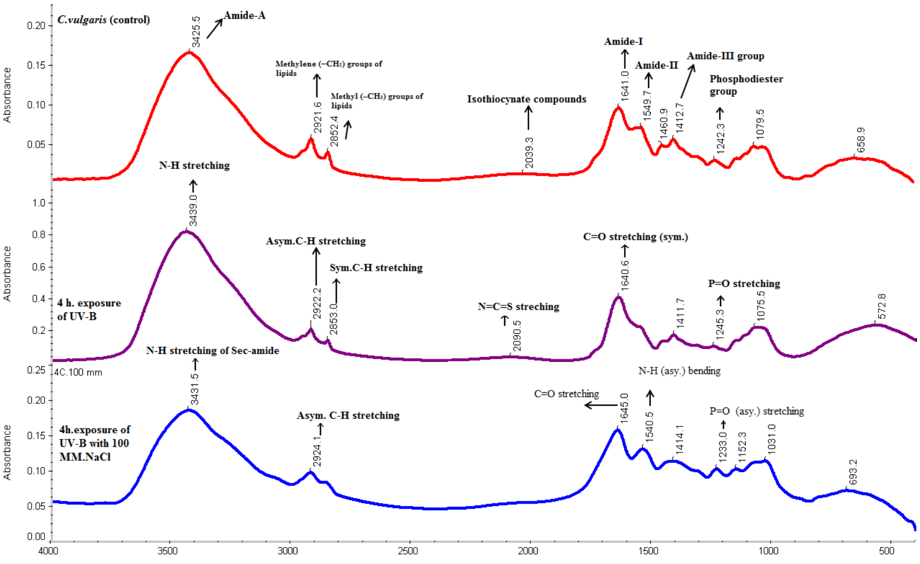


Fig. 7.10.A: FTIR spectral study in microalgae *C. vulgaris* treated with UV-B (4 h) and NaCl (100 mM) + UV-B

In the case of *C. humicola,* spectral peak of amide I showed no change after the exposure to UV-B (4 h). However, algae *C. vulgaris* treated with NaCl+UV-B showed decrease in amide I peak as compared to control (Fig. 7.10.B). In the case of lipid, enhanced spectral peak (1740 cm^{-1}) length was observed in algae exposed to UV-B, but it was more pronounced in the case of algal cells exposed to NaCl+UV-B.

C. humicola exposed to 4 h UV-B showed peak of isothiocynate compound as compared to control which was in significant when compared with NaCl+UV-B treatment. The protein peak (Amide I) was significant increased amide in algae exposed to UV-B and NaCl either singly or in combination. The spectral peak of amide II, amide V, amide III and phospholipid exhibited variations after NaCl and UV-B treatment.

FTIR can be used in deduction of relative concentration of macromolecules fingerprint IR absorption bands in algae under different abiotic stress (Sene et al., 1994; Gorgulu et al., 2007). An increase in the protein peak (amide I) after 4 h of UV-B and UV-B+NaCl exposure may be associated with UV-B radiation protectant molecules, which act as antioxidant adding protection to cell from harmful UV-R (Xue et al., 2005). However, decrease spectral peak represents protein degradation, peptide bond stretching and deformation of N-H functional groups. Similar results were also observed by Forfang et al. (2017). In case of carbohydrate related spectral peak (1040 cm^{-1}) is increase in peaks might be due to stimulated injury demand and increased to photosynthesis lead to the increased production of storage compound such as lipid to avoid damage (Singh et al., 2018b).

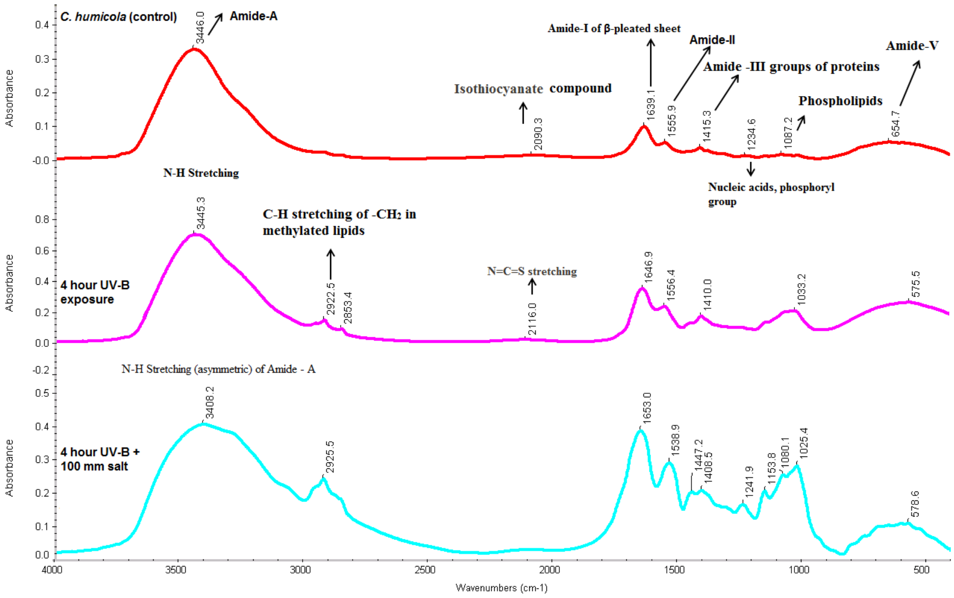
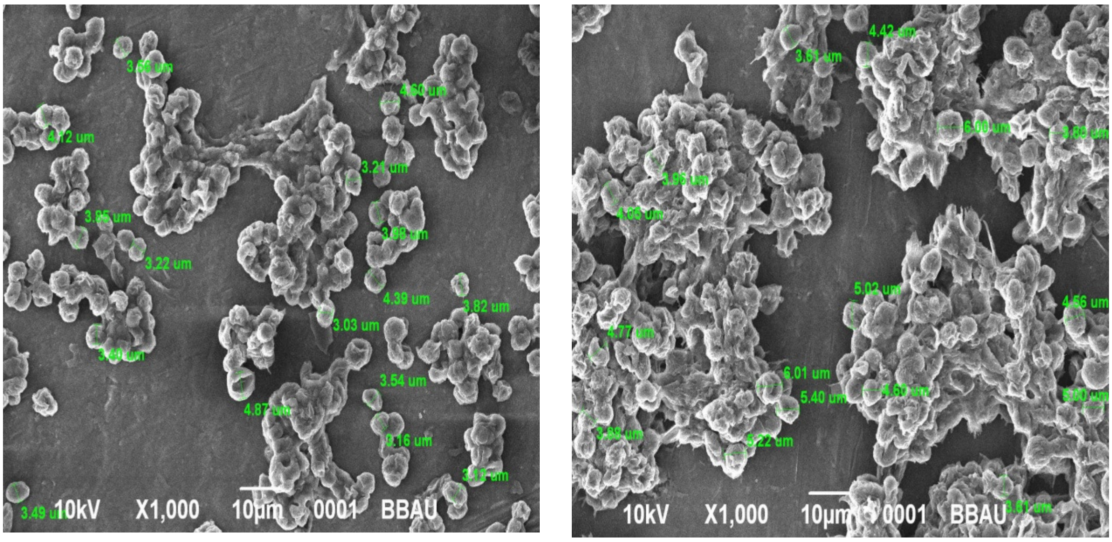


Fig. 7.10.B: FTIR spectral study in microalgae *C. humicola* treated with UV-B (4 h) and NaCl (100 mM)+UV-B

7.13 Effect on morphology, ultra structure and growth

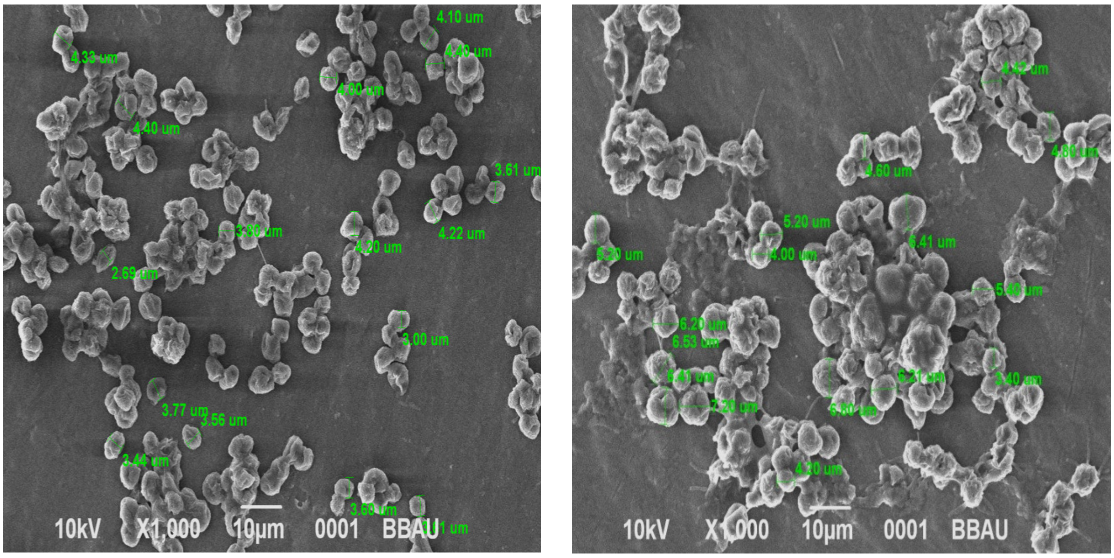
SEM study in microalgae *C. vulgaris* and *C. humicola* treated with UV-B and NaCl+UV-B are depicted in Fig. 7.11. The ultrastructure of *C. vulgaris* treated with

NaCl+UV-B showed distorted morphology and cell clumping. The average size of algal cells treated with NaCl+UV-B was 4.49 µm as compared to control cells (3.49µm). Similarly, altered size was also observed in the case of *C. humicola* however, it showed little clumping of cells as compared to *C. vulgairs*.



C. vulgaris **Control**

C. vulgaris **treated with NaCl (100 mM) + 4 h UV-B**



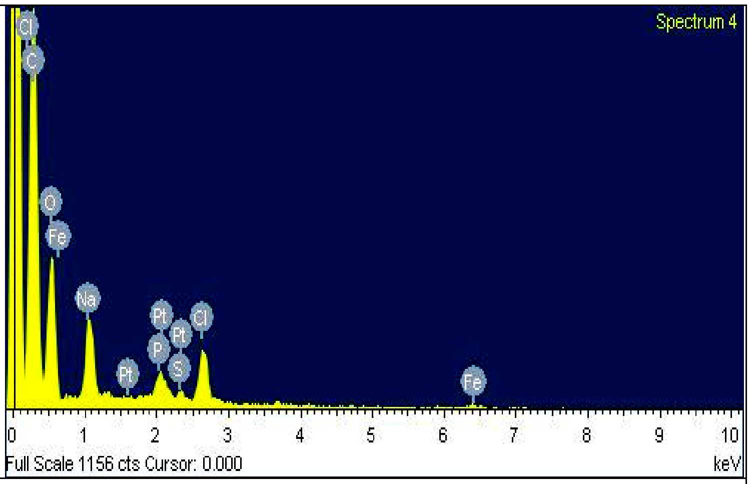
C. humicola **control**

C. humicola **treated with NaCl (100 mM)+ 4 h UV-B**

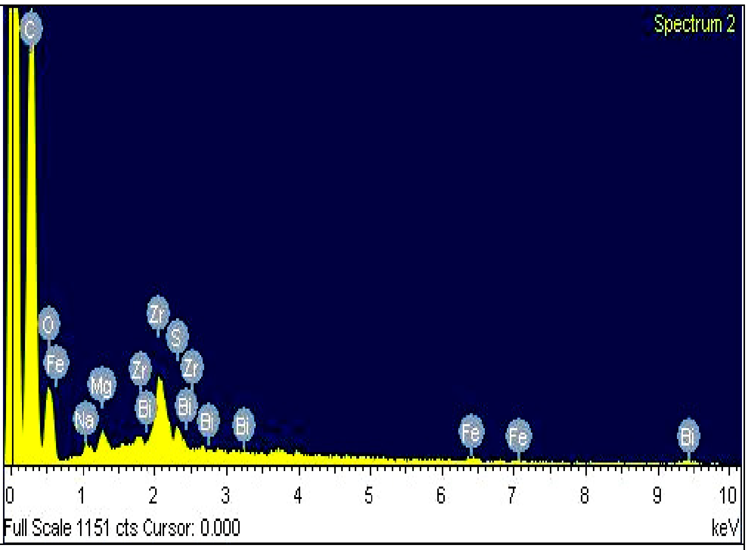
Fig. 7.11: SEM image of *C. vulgaris* and *C. humicola* treated with NaCl+UV-B

The average size was found to be increased (5.34 µm) as compared to untreated control (3.79µm). Increase in the cell size in microalgae might be due to the production of different secondary metabolites, compatible solutes and impaired cell division (Fahy et al., 2017). The binding of metal with cell is related to occurrence of different functional group which is involved in either chelates/ adsorption on the surface or transports them inside the cell (Singh et al., 2018).

The EDS data showed alteration in the concentration of micro and macro elements of the cell exposed to UV-B and NaCl. The EDS measure the elemental constituents by atomic number (% atomic) and atomic weight (% weight). The localization of particular elements is based on energy dependent localization of different elements in shells (K, L, M and N) nucleus of the atoms (elements). In the case of *C. vulgaris* treated with NaCl+UV-B, the percent weight of elements viz., C, O, Na, P, S, Cl and Fe localized in K shell were 60.05, 32.55, 3.56, 0.48, 0.30, 1.83 and 0.33, respectively as compared to the control i.e. 69.90,23.47, 0.54, 064 (S) and Fe (0.62) (Fig. 7.12.A). However, elements P and Cl were found to not be detectable in the control algae. In the case of *C. humicola,* a significant change in elemental localization was observed (Fig. 7.12.B). *C. humicola* treated with NaCl+UV-B, there was reduction in elements viz., Mg, Si, P, Fe and Zn. Results also showed a strong delocalization of carbon (% atomic weight; 30.28) as compared to control (59.03 % atomic weight). Decreased in mineral content after UV-B exposure may be ascribed to alteration in the stomatal conductance, membrane composition, permeability and cell deformation which ultimately limit the uptake and sorption of important minerals by algae (Zouari et al., 2016).

<table>
<tr><td>Element</td><td>Weight %</td><td>Atomic %</td><td rowspan="10"> </td></tr>
<tr><td>C K</td><td>69.9</td><td>78.47</td></tr>
<tr><td>O K</td><td>23.47</td><td>19.78</td></tr>
<tr><td>Na K</td><td>0.54</td><td>0.31</td></tr>
<tr><td>Mg K</td><td>0.64</td><td>0.35</td></tr>
<tr><td>S K</td><td>0.64</td><td>0.27</td></tr>
<tr><td>Fe K</td><td>0.62</td><td>0.15</td></tr>
<tr><td>Zr L</td><td>4.68</td><td>0.69</td></tr>
<tr><td>Bi M</td><td>-0.48</td><td>-0.03</td></tr>
<tr><td>Totals</td><td>100</td><td></td></tr>
<tr><td colspan="3">*C. vulgaris* control</td><td>EDS spectra of control algae</td></tr>
</table>

Element	Weight %	Atomic %
C K	60.05	68.71
O K	32.55	27.96
Na K	3.56	2.13
P K	0.48	0.21
S K	0.3	0.13
Cl K	1.83	0.71
Fe K	0.33	0.08
Pt M	0.91	0.06
Totals	100	

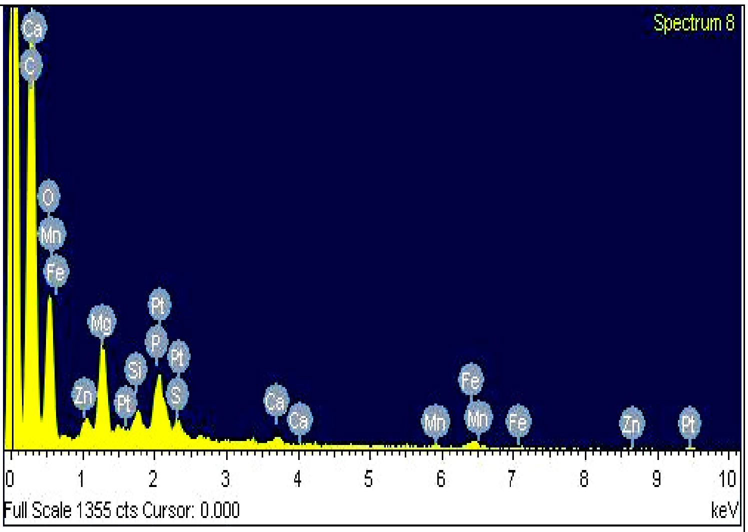


***C. vulgaris* treated**

EDS spectra of treated algae

Fig. 7.12.A: EDS spectra of C. vulgaris exposed under NaCl+UV-B

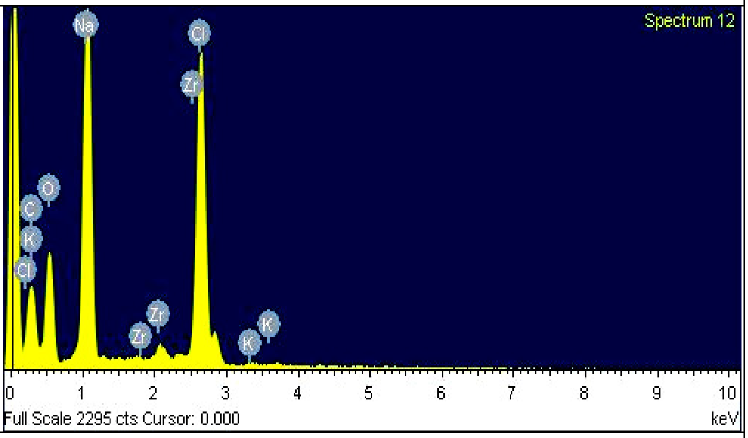
Element	Weight %	Atomic %
C K	59.03	68.33
O K	33.11	28.77
Mg K	2.67	1.53
Si K	0.57	0.28
P K	0.96	0.43
S K	0.45	0.19
Ca K	0.28	0.1
Mn K	0.26	0.07
Fe K	0.59	0.15
Zn K	0.05	0.01
Pt M	2.04	0.15
Totals	100	



***C. humicola* control**

EDS spectra of control algae

Element	Weight %	Atomic %
C K	30.28	43.06
O K	31.46	33.59
Na K	20.49	15.22
Cl K	16.13	7.77
K K	0.17	0.08
Zr L	1.47	0.27
Totals	100	



***C. humicola* treated**

EDS spectra of treated algae

Fig. 7.12.B: EDS spectra of *C. vulgaris* exposed under NaCl+UV-B

7.14 Conclusions

The overall results concluded that the chlorophyll content in *C. vulgaris* and *C. humicola* treated with the combined exposure of NaCl (100 mM) + 2 h UV-B, exhibited antagonistic effect as compared to algae exposed to 4 h UV-B alone. Further, both the algae exposed to NaCl (100 mM) + 2 h UV-B showed comparatively better tolerance and stress responses as evident from the SOD and catalase activity. The lipid content in microalgae *C. humicola* exposed to NaCl (100 mM) + 2 h UV-B was found to be highest as compared to algae exposed under other different variables.

CHAPTER 8

Summary and Conclusions

8. Summary and Conclusions

The deterioration of environment is the results of variety of factors including downfall in soil, air and water quality, ecosystem degradation, habitat loss, species extinction and pollution (Goudie, 2018). With increasing globalization and standardization of livelihood, human interference continuously damages the natural protective environment on earth.

Solar energy is fundamentally important for sustaining life on the earth. The spectrum of sun constitutes number of electromagnetic radiation of different wave length with different degree of potential energy (Shahsavari and Akbari, 2018). The ultraviolet radiation (200-320 nm) emitted from sun is harmful for living environment. The natural system protects the living world from this harmful radiation by absorbing its major fraction in the stratospheric ozone layer (Herndon et al., 2018). However, due to increasing anthropogenic interventions, ozone layer is continuously depleting. Solar UVR is reported to be increasing on the atmosphere surface due to numerous anthropogenic activities and depleting ozone layer which can damage the biological system. Ultraviolet radiation (UVR) is one of the main stressor for all plants and algae (Comont et al., 2013). The level of salinity is continuously increasing and it is estimated that ~50% land would be salinized till 2050 (Ismail et al., 2017). High salinity in the environmental may cause a deleterious effect on living world of each ecosystem (Wang et al., 2003). Thus, salt and UV are together adversely effecting the functioning of ecosystem (Wang et al., 2003). To tackle the combined threat of both UV-R and salinity in a sustainable manner, microalgae could be a potential candidate with multifold benefits including remediation, biomass production in non arable saline land and synthesis of fatty acids, lipid and biofuel.

Microalgae *C. vulgaris* and *C. humicola* grown under different environmental stress individually in combination may exhibit (NaCl and UV-B exposure) different degree of responses towards their toxicity and tolerance. Algae *C. vulgaris* and *C. humicola* treated with different doses of NaCl and UV-R are exposed to elucidate their impact on morphology, lipid synthesis, minerals level and antioxidant responses. Scanning Electron microscopy (SEM) of (100 mM NaCl) treated cells showed distorted cell morphology and increase in cell size by 33.52% (*C. humicola*) and 27.79% (*C. vulgaris*). Energy Dispersive Spectroscopy data revealed reduction in mineral contents (C, S, Fe, Mg, Si, Mn and Zn) by 14-54% in both algae after treatment with 100 mM salt. Further, *C. humicola* accumulated high lipid content than *C. vulgaris* in presence o fNaCl as evident from the FTIR analysis showing increase in the peak length of lipid (1740 cm^{-1}) and altered Lipid/Protein, Lipid/Carbohydrate and Protein/Carbohydrate ratio. Theactivities of superoxide dismutase, catalase and glutathione reductase were increased by 2.5~5 fold in *C. humicola* and *C. vulgaris*as compared to control. The increased level of ascorbic acid, cysteine and proline in both algae exhibited their enhanced tolerance against salt stress which was more pronounced in the case of *C. humicola* than *C. vulgaris*.

Microalgae *C. humicola* and *C. vulgaris* exposed to different durations of UV-B exposure (1/2 h, 1h, 2h, 3h and 4h) revealed that UV-B reduces the photosynthetic performance of photosystem II by altering the light use efficiency and inhibition of RCs as evident from photosynthetic performance index (Pi_{ABS}), maximum quantum yield (Fv/Fm), net closing rate of reaction centre (Mo), Trapping flux (TRo/RC) and effective antenna size (ABS/RC) as compared to their respective control. Scanning Electron microscopy (SEM) exhibited variegated structure and increase in cell size by ~15-65% which was more pronounced in the case of *C. humicola* after 4h of UV-B exposure. The Energy Dispersive Spectroscopy (EDS) data showed that the content of microelements (C, O, Na, and K) in terms of atomic percent weight was significantly increased in *C. vulgaris* while with *C. humicola*, it was restricted to carbon (C) only. Further, the high antioxidant (ascorbic acid, cysteine and proline) potential and carotenoid/chl a photoprotection response reflected protective response of both algae against UV-B. In the case of lipid, an allevation in carbon allocation in *C. humicola* may be the reason for high lipid yield as compare to *C. vulgaris* after 2h of UV-B exposure.

Microalgae *C. vulgaris* and *C. humicola* treated with NaCl and UV-B were used to study the combined effect of NaCl +UV-B on nutrient status, physiological and biochemical changes in the microalgae *C. humicola* and *C. vulgaris*. The chlorophyll content in algae *C. vulgaris* exposed to UV-B alone for 2h and 4h were severely reduced as evident from significant decrease in chlorophyll by 32.87% and 60.27% respectively as compared to respective control. *C. vulgaris* treated with NaCl alone also showed increase in chlorophyll content by 16.4%. However, UV-B exposed algae in the presence of 100mM salt (NaCl+UV-B) exhibited slight increase in chlorophyll level after 2h UV-B exposure (1.36%) as compared to control (untreated) in microalgae *C. vulgaris*. Further, *C. vulgaris* treated with NaCl (100mM)+UV-B (4h), 60.27% decrease in chlorophyll content was observed. In the case of *C. humicola*, UV-B 2h and 4h, resulted into decrease in chlorophyll content from 0.071±0.010-0.053±0.014 and 0.071±0.010-0.028±0.002 mg g^{-1} fw, respectively. *C. humicola* cells treated with100 mM salt and different duration of UV-B (NaCl+UV-B) showed decrease in chlorophyll level i.e., 12.82% (2h) and 8.45% (4h) as compared to untreated control.

Microalgae *C. vulgaris* and *C. humicola* exposed to different intensity of UV-B exhibited decrease in the level of both chlorophyll and carotenoids contents with increasing intensities of UV-B. Maximum increase in the chlorophyll content was observed at lower UV-B intensity (5 Wm^{-2}) in both algae *C. vulgaris* and *C. humicola*, while minimum effect on pigments was observed at 15 Wm^{-2}. The concentration of chlorophyll in algae *C. vulgaris* and *C. humicola* exposed to higher intensity (15 Wm^{-2}) was 0.081±0.009 and 0.079±0.018 mg/g fw, respectively as compared to their respective control (0.069±0.009 and 0.058± 0.009 mg/g fw, respectively). TBARS content in the algae exposed to different intensities of UV-B showed increase in peroxidative damage as represented by enhanced production of MDA as a result of lipid peroxidation. *C. vulgaris*treated with 5 Wm^{-2} intensity showed increase in MDA by 11.21% while at 10, and 15$Wm^{-2}$of UV-B doses resulted into an increase in MDA by 37.91% and 68.86%, respectively. In the case of *C. humicola* treated with (5, 10 and 15 Wm^{-2}) of UV-B showed increase in MDA by 9.38%, 28.67% and 59.80%, respectively.

The present finding concluded that microalgae *C. humicola* was better adapted to NaCl and UV-B stressthan *C. vulgaris*. Growing algae in salt affected arable lands and salt lakes/lagoons may be an economic success for the whole to generate biomass which can be utilized for production of biofuel and other value added products in a sustainable manner. However, optimization of growing condition of algae under various stress condition is essential. Thus, an ecotoxicological assessment of algae and their physiological response could be used in the mass cultivation and production of algae on waste land and water body for production of lipid. Such algae can be helpful for harvesting of algal biomass in salt water and lagoons, which provides a cheap source of lipid and bioenergy in order to fulfill the growing demand for biofuel in a cost effective and sustainable manner.

The present study concludes that low-dose (2h) of UV-B radiation exposure could enhance the photosynthetic performance and lipid yield in microalgae*C. humicola* and *C. vulgaris* due to improved antioxidant status. Further, the transient chlorophyll fluorescence induction study in both algae reflected the closure of photosystem II, diminished reaction centre and reduced flow of electrons in the cells treated with UV-B for 4h. The results suggested that increase in net closure of reaction centres of PSII is an adaptation mechanism called as down regulation of PSII, to protect the cella against photooxidative damage (Jiang and Qiu, 2011). Improved antioxidant status of cells under UV-B exposure for 2h exhibited better accumulation of biomass, lipid yield and synthesis of value added products in both strains in cost effective and sustainable manner:

1. Algae *C. humicola* and *C. vulgaris* could tolerate NaCl upto 100 mM concentration of NaCl.
2. The increase in level of antioxidants such as ascorbic acid, cysteine and proline etc in both algae exhibited enhanced tolerance against salt, which was more pronounced in *C. humicola* than *C. vulgaris*.
3. The activities of superoxide dismutase, catalase and glutathione reductase were found to be higher in NaCl treated (100 mM) in *C. humicola* and *C. vulgaris* as compared to their respective control.
4. SEM showed distorted cell morphology and increase in cell size of both *C. humicola* and *C. vulgaris* by 33.52 % and 27.79% respectively as compared to control.
5. Algae *C. humicola* exhibited higher lipid content than *C. vulgaris* in the presence of NaCl as evident from FTIR results.
6. UV-B exposure for 4 h resulted into severe injury to both algae *C. humicola* and *C vulgaris*, when compared with the results obtained after 2 h of UV-B exposure.
7. UV-B reduces the overall photosynthetic performance of photosystem II by altering the photosynthetic efficiency, however, at the 2 h of the UV-B expposure, PS II showed the adaptive and protective responses.
8. The high antioxidant (ascorbic acid, cysteine and proline) potential and carotenoid/chl a (photoprotection response) reflects protective response of cells against UV-B in the both algae.

9. The photosynthetic pigment content was enhanced in *C. vulgaris* and *C. humicola* provided combined exposure of NaCl (100 mM) + UV-B (2 h) and NaCl (100 mM)+ UV-B (4 h) as compared to algae exposed to UV-B for 2 h and 4 h UV only.
10. Maximum lipid yield and protein content was observed in *C. humicola* exposed to NaCl (100 mM) + UV-B (2 h).
11. UV-B intensity (15 Wm^{-2}) was highly toxic for both algae, while lower intensity i.e., 5 Wm^{-2} could improve the growth responses of the algae.

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List of Publications

List of Publications

A. Research papers:

1. **R. Singh**, A.K. Upadhyay, D.V. Singh, J.S. Singh, D.P. Singh (2019). Photosynthetic performance, nutrient status and lipid yield of microalgae *Chlorella vulgaris* and *Chlorococcumhumicola* under UV-B exposure. Current Research in Biotechnology. In press.
2. **R. Singh**., A.K. Upadhyay, D.P. Singh (2018). Sodium chloride incites reactive oxygen species in green algae *Chlorococcumhumicola*and *Chlorella vulgaris*: Implication on lipid synthesis, mineral nutrients and antioxidant system. Bioresource Technology.270, 489-497.
3. **R. Singh**, A.K. Upadhyay, D.P. Singh (2018). Regulation of oxidative stress and mineral nutrient status by selenium in arsenic treated crop plant *Oryza sativa*. Ecotoxicology and environmental safety. 148,105- 113.
4. P. Chowdhary, A. Yadav, **R. Singh**, Ram Chandra, D.P. Singh, A. Raj, R.N. Bharagava (2018). Stress response of *Triticumaestivum*L. and*Brassica juncea*L.against heavy metals growing at distillery and tannery wastewater contaminated site. Chemosphere. 206, 122-131.
5. A.K. Upadhyay, N.K. Singh, **R. Singh**, U.N. Rai (2016). Amelioration of arsenic toxicity in rice: Comparative effect of inoculation of *Chlorella vulgaris* and *Nannochloropsis sp*. on growth, biochemical changes and arsenic uptake. Ecotoxicology and environmental safety. 124, 68-73.

B. Book chapters

1. A.K. Upadhyay, **R. Singh**, J.S. Singh, D.P. Singh (2019). Microalgae assisted phycoremediation and energy crisis solution: challenges and opportunity In: Microbial Biotechnology in Agro-Environmental Sustainability. Elsevier Science. ISBN 978044441915.
2. A.K. Upadhyay, **R. Singh**, D.P. Singh (2019). Phycotechnological Approaches toward wastewater management. In emerging and eco-friendly approaches for waste management. Springer, Singapore, pp. 423-435.

3. A.K. Upadhyay, **R. Singh,** D.P. Singh (2019). Phytoremediation and sustainable developmental policies and practices. Restoration of wetland ecosystem: A trajectory towards a sustainable environment, pp. 17-29.

C. Book

Restoration of wetland ecosystem: A Trajectory towards a sustainable environment. Edited by Atul K. Upadhyay, **Ranjan Singh**, Devendra Pratap Singh. Publisher Springer Nature Singapore. ISBN. 978-981-13-7664-1

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Photosynthetic performance, nutrient status and lipid yield of microalgae *Chlorella vulgaris* and *Chlorococcum humicola* under UV-B exposure

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ABSTRACT

The present study was conducted to examine the photosynthetic performance, nutrient status and lipid yield in microalgae *Chlorococcum humicola* and *Chlorella vulgaris* under different durations of UV-B exposure (1/2 h, 1 h, 2 h, 3 h and 4 h). Results revealed that UV-B reduces the photosynthetic performance of photosystem II by altering photosynthetic performance index (Pi_{ABS}), maximum quantum yield (Fv/Fm), net closing rate of reaction centre (Mo), trapping flux (TRo/RC) and effective antenna size with their respective control. Scanning Electron Microscopy (SEM) exhibited variegated structure and increased cell size by ~15–65% which was more pronounced in the case of *C. humicola* at 4 h of UV-B. The Energy Dispersive Spectroscopy (EDS) data showed that the content of microelements (C, O, Na, and K) in terms of atomic weight % was found to be significantly increased in *C. vulgaris* while with *C. humicola*, it was restricted to carbon (C) only. Further, the high antioxidant (ascorbic acid, cysteine and proline) potential and carotenoid/chl a photoprotection response reflects protection against UV-B in the both algae. In the case of lipid, comparative greater increase in cell size of *C. humicola* correlated with high lipid yield as compare to *C. vulgaris* at 2 h of UV-B could be employed in the production of biofuel in sustainable manner. Thus, algae *C. humicola* could be a best alternative feed stock of lipid and biofuel production in the area receiving high solar radiation.

1. Introduction

Since time immemorial, energy is the fundamental prerequisite for the existence of lives on the earth. The major source of energy is fossil fuel which continuously dwindling due to overexploitation and anthropogenic interferences (Gasparatos et al., 2017). This might have cause a paradigm shift towards a search for renewable source to meet the demand of energy. The renewable sources includes solar energy, agricultural crops (Maize, Sunflower), non-agricultural crops (*Jatropha* sp.) and algae (Voloshin et al., 2016). The cultivation of microalgae are more ecofriendly and cheaper as it can be cultivated in waste water as well as in non-arable land. Over the past few years, microalgae- a third generation biofuel acts as a bridge in the production of bio-energy against limited water, land and environmental stress (Stockenreiter et al., 2016). Off the environmental stressors, solar ultraviolet radiation (UVR) emission has profound effect on the chemical composition of the atmosphere, flora and fauna (Madronich et al., 2018). It has also been reported that UVR play significant role in the generation of photochemical smog, photo damage, change in precipitation pattern, coral bleaching, vegetation loss and agricultural escalation and altering ecosystem services etc. (Jansen et al., 1998; Danovaro et al., 2008; Comont et al., 2013; Kataria et al., 2014; Williamson et al., 2014).

The UV-B irradiance (5% of total UV light from the sun) supplied by the sun is a serious hazard to lives on the earth (Herndon et al., 2018). Algae being dominant photosynthetic microorganisms in upper layer of aquatic surface are more prone to high UVR exposure leads to bring about photo bleaching coupled reduced upwelling of nutrients and oxidative damage (Williamson et al., 2019). In addition, UVR causes photosynthesis inhibition in algae, damage the efficiency of the biological pumps and fixation of CO_2 for their subsequent transfer into the organic matter (Mata et al., 2010). However, microalgae have shown tendency to acquire high degree of adaptation against UVR during the course of evolution (Holzinger et al., 2018). The high adaptability of microalgae against UVR is not only valuable for lipid production but also beneficial for accumulation of nutrients, carbon sequestration, environmental remediation, nutrient cycling and production of oxygen in the environment (Brennan and Owende, 2010; Mata et al., 2010).

The UV- B (280–320 nm) is known to have both detrimental and beneficial effects on plants animals and microalgae (Hockberger, 2002). In animals, UV-B causes skin diseases, dermatitis, DNA damage, mutation,

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Sodium chloride incites reactive oxygen species in green algae *Chlorococcum humicola* and *Chlorella vulgaris*: Implication on lipid synthesis, mineral nutrients and antioxidant system

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ARTICLE INFO

Keywords:
Algae
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Mineral
Antioxidant
SEM-EDS

ABSTRACT

In the present study, microalgae *Chlorococcum humicola* and *Chlorella vulgaris* were grown in different concentrations of NaCl (25–1000 mM) to elucidate its impact on morphology, lipid synthesis, minerals status and antioxidative responses. Scanning Electron microscopy showed distorted cell morphology and increased cell size by 33.52% (*C. humicola*) and 27.79% (*C. vulgaris*) at 100 mM NaCl. Energy Dispersive Spectroscopy data revealed reduction in mineral contents (C, S, Fe, Mg, Si, Mn and Zn) by 14–54% in both algae. Further, *C. humicola* was found to have high lipid content than *C. vulgaris* under NaCl regime. The activities of superoxide dismutase, catalase and glutathione reductase were increased by 2.5–5 folds in both algae as compared to control. The increased level of ascorbate, cysteine and proline in both algae indicated tolerance against salinity. Thus, *C. humicola* and *C. vulgaris* may exhibit dual benefits viz., high lipid production and reclamation of sodic soil.

1. Introduction

Microalgae are dominant, photosynthetic, biofuel and largest oxygen producing organisms on the earth indispensable for planet function and ecosystem sustainability (Brodie et al., 2017). The sustainable functioning of an environment is largely depends on the nonrenewable energy sources such as oil and coal which continuously dwindles due to increased demand. The existing resources like fossil fuel and coal are contributing to global high CO_2 concentration, green house gases and responsible for changing environmental paradigm causing stern effects on the both living as well as nonliving component of the earth (Bagchi et al., 2018). These factors impelled a shift towards search for renewable energy resources, where microalgae could be a viable and cost effective biofuel depository (Rai et al., 2013). The fast growth rate, high lipid content, high degree of adaptability and capacity to withstand under extreme stressors including waste water, salinity, radiation and heat etc (Korosi et al., 2017) make high success of the algae being employed as potential feed stock.

Salinity is introduced in the environment by the natural processes, anthropogenic activities and modern agricultural practices (Ismail and Horie, 2017). The salt concentration is rising in the world and would cover approx. 50% of the arable land to be salinized by the year 2050 (Ismail and Horie, 2017; Wang et al., 2003). Growth of salt tolerant microalgae in such salinized water and soil system might offer alternative source of renewable energy in a sustainable manner (Costa and de Morais, 2011) without competing with arable land. Salt is easiest and cheapest stressor for the successful large scale production of biomass, lipid and biofuel by algae (Zhang et al., 2018). Yilancioglu et al. (2014) reported that under high salinity stress, the photosynthesis dependent carbon fixation pathway in plants and algae is diverted to synthesis of lipid, particularly the production of storage compounds in the form of neutral lipid. An increased in lipid content of microalgae under salt stress is the result of various metabolic changes as reported by various authors (Benavente-Valdés et al., 2016; Sun et al., 2014). Salt tolerance in microalgae are achieved by a combination of biochemical and molecular pathways including exclusions of ions (K^+ and Ca^{+2}), compartmentalization, synthesis of osmolytes (proline, glycerol and glycin betaine etc), alteration in membrane structure, activation of enzymatic and non enzymatic antioxidant defense system and change in the photosynthesis route (Sun et al., 2014). The ion homeostasis (Na^+, K^+ and Ca^{+2}) inside the cells of the algae play a key role for survival of the cells under NaCl stress since at high salinity, Na^+ over compete with Ca^{+2}, bind with cell wall and decreases K^+ level which renders the shrinkage of cell wall, altered osmoregulation, protein synthesis and

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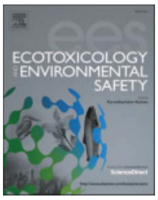

Regulation of oxidative stress and mineral nutrient status by selenium in arsenic treated crop plant *Oryza sativa*

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ARTICLE INFO

Keywords:
Rice
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Antioxidants enzyme
Mineral nutrient

ABSTRACT

The present study was conducted to examine the impact of selenium (Se) on mineral nutrient status and oxidative stress in crop plant *Oryza sativa* treated with arsenic (As). Scanning electron microscopy (SEM) coupled with Energy dispersive x-ray spectroscopy (EDS) study revealed the morphological deformities in leaf veins along with granular deposition on the leaf surface. The EDS analysis exhibited loss of elements (S, Si, Cl, K, Ca, Fe and Cu) in As(III) treatment in rice roots as compared to untreated root. In the case of As(III) treated shoot, changes in elements content in term of percent atomic weight was K (1.17–0.90%), Cl (1.04–24.75%), Na (0.65–3.52%) and S (0.49–2.52%) when compared with untreated shoot. The result of EDS analysis showed that As limits the concentration of important mineral elements present in the rice root and shoot. Rice plant treated with Se (10 µM) and sub lethal dose of As(III) (60 µM) showed better growth responses in term of root, shoot length (11.4% and 10.71%, respectively), biomass (11.7%), reduced malonyldialdehyde content (35.14%) and stimulated antioxidant level indicating better As tolerance potential against As. Further, a selenium dependent significant reduction in As accumulation was also observed in root (14.24%) and shoot (23.78%) of rice plant when compared with plant treated with As alone. This study highlights the potential of Se to ameliorate the ecotoxicological risks associated with the As buildup in agricultural land.

1. Introduction

Inorganic arsenic (iAs) is a pervasive, non-threshold environmental carcinogens that has raised the concern worldwide without any safe exposure limit (Lomax et al., 2012). Arsenic enters in soil through different sources, including ground water irrigation, volcanic activities, mining, fossil fuel burning, fertilizers and pesticide application (Yin et al., 2011). In current scenario, millions of peoples are suffering from As toxicity due to the use of As contaminated ground water for drinking and irrigation purpose (Chatterjee et al., 2017). Studies have shown that rice is the major staple crop in South-East Asian nations and is a dominant source of important mineral nutrients which get dwindled due to As toxicity (Kumar et al., 2016; Sohn, 2014; Zhao et al., 2010). The situation is dreadful in Bangladesh due to its geogenic contamination of groundwater and water logging condition (Sohn, 2014). Rice grown in flooded soil accumulated high As content due to release of bound As by As reducing bacteria and reductive dissolution of iron hydroxides (Hare et al., 2017; Jia et al., 2012). Xu et al. (2008) reported that arsenic is taken up by rice plants might be due to the reductive mobilization of As(III) in anaerobic paddy soils (Rahman et al., 2009; Su et al., 2010) and via silicic acid transport pathway (Ma et al., 2008). The uptake and translocation of As in rice depends on the mineral nutrients of plant and soil (Duan et al., 2013). It has been reported that As accumulation by plants may limit the accumulation of different mineral nutrients (Se, Zn, Ni, Mg etc) which results into retarded growth, infertility, skin diseases and neurobehavioral impairment in human beings (Williams et al., 2009). Therefore, study on the interaction of mineral nutrients with As and a protective measure towards prevention of As accumulation by rice plant seem to be essential.

In human, chronic As exposure is associated with high risk of skin, blood and kidney cancer (Fernández et al., 2012). In plants, it results in oxidative damages to membrane resulting in electrolyte leakage from the cytosol and DNA damage due to reactive oxygen species (ROS) (Carlin et al., 2016). To minimize the toxic impact of ROS, plants are equipped with different types of enzymatic and non enzymatic antioxidative system (Verma et al., 2016). The enzymatic system includes SOD (superoxide dismutase), APX (ascorbate peroxidase), Catalase, GR (glutathione reductase), while non enzymatic antioxidants include ascorbate, reduced glutathione (GSH), carotenoids and tocopherol (Sharma et al., 2014). The malondialdehyde (MDA) is a common

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Stress response of *Triticum aestivum* L. and *Brassica juncea* L. against heavy metals growing at distillery and tannery wastewater contaminated site

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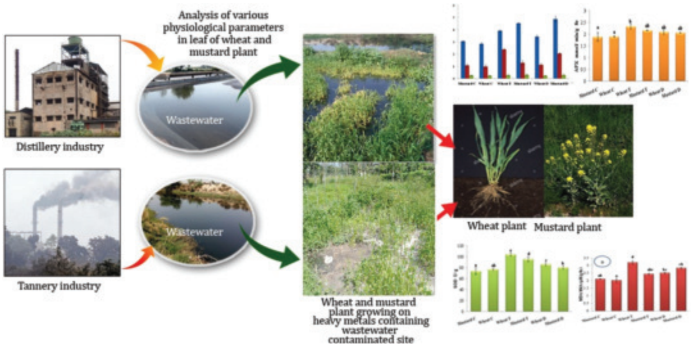
HIGHLIGHTS

- Industrial wastes contains high metals content and BOD, COD values.
- Metals induces antioxidant enzymes SOD, APx, GPX, MDA, H_2O_2, and CAT.
- Mustard plant exhibited high anti-oxidant responses.
- Translocation factor >10 (10.31) of Cr in tannery mustard plants.

GRAPHICAL ABSTRACT



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Wastewater
Potentially toxic elements
Antioxidant enzymes

ABSTRACT

This study aimed to investigate the effects of potentially toxic elements on biochemical parameters in wheat (*Triticum aestivum* L.) and mustard (*Brassica juncea* L.) plants growing at distillery and tannery wastewater contaminated sites. The analysis of plants showed the highest accumulation of Fe (361 $mg\,kg^{-1}$ in wheat root and 359 $mg\,kg^{-1}$ in mustard leaves) followed by Zn, Cr and Mn in leaf>shoot>root. Further, the Chl-a, b, and carotenoids content was also found high in plant samples. Results also showed that photosynthetic content in wheat and mustard growing at tannery wastewater contaminated sites was Chl-a 3.92, 4.53 ($mg\ g^{-1}$ fw), Chl-b 2.39, 1.29 ($mg\ g^{-1}$ fw) and carotenoids 0.28, 0.32 ($mg\ g^{-1}$ fw), respectively. Whereas, photosynthetic content in these plants with distillery waste was as Chl-a 3.43, 4.88 ($mg\ g^{-1}$ fw), Chl-b 1.12, 2.05 ($mg\ g^{-1}$ fw) and carotenoids 0.24, 0.29 ($mg\ g^{-1}$ fw), respectively. In addition, the activity of plant enzymes such as SOD, APx, GPX, MDA, H_2O_2, and CAT was also higher in selected plants in comparison to control plants. Moreover, the high bioconcentration factor of Zn > 1 (1.29) and translocation factor >10 (10.31) of Cr in tannery wastewater affected mustard plants. This study concluded that industrial wastewaters are the primary sources of metal accumulation in agricultural crops and thus, it should not be discharged into the environment before its proper treatment.

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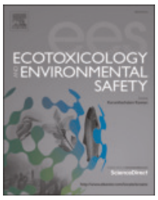

Amelioration of arsenic toxicity in rice: Comparative effect of inoculation of *Chlorella vulgaris* and *Nannochloropsis* sp. on growth, biochemical changes and arsenic uptake

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ABSTRACT

The present study was conducted to assess the responses of rice (*Oryza sativa* L. var. Triguna) by inoculating alga; *Chlorella vulgaris* and *Nannochlropsis* sp. supplemented with As(III) (50 μM) under hydroponics condition. Results showed that reduced growth variables and protein content in rice plant caused by As toxicity were restored in the algae inoculated plants after 7 d of treatment. The rice plant inoculated with *Nannochloropsis* sp. exhibited a better response in terms of increased root, shoot length and biomass than *C. vulgaris* under As(III) treatment. A significant reduction in cellular toxicity (thiobarbituric acid reactive substances) and antioxidant enzyme (SOD, APX and GR) activities were observed in algae inoculated rice plant under As(III) treatment in comparison to uninoculated rice. In addition, rice treated with As(III), accumulated 35.05 mg kg^{-1} dw arsenic in the root and 29.96 mg kg^{-1} dw in the shoot. However, lower accumulation was observed in As(III) treated rice inoculated with *C. vulgaris* (24.09 mg kg^{-1} dw) and *Nannochloropsis* sp. (20.66 mg kg^{-1} dw) in the roots, while in shoot, it was 20.10 mg kg^{-1} dw and 11.67 mg kg^{-1} dw, respectively. Results demonstrated that application of these algal inoculum ameliorates toxicity and improved tolerance in rice through reduced As uptake and modulating antioxidant enzymes. Thus, application of algae could provide a low-cost and eco-friendly mitigation approach to reduce accumulation of arsenic in edible part of rice as well as higher yield in the As contaminated agricultural field.

1. Introduction

Inorganic arsenic (iAs) is a toxic groundwater contaminant of geogenic origin, particularly in large deltas and along major rivers in the deprived regions of South and East-Asia (Sharma et al., 2014). Arsenic is present predominantly as arsenate (As(V)) in the aerobic environment and as arsenite (As(III)) under anaerobic or waterlogged conditions (Lomax et al., 2011). However, different organic species such as methylarsenate [MAs(V)], dimethylarsenate [DMAs(V) or cacodylate] and trimethylarsine oxide [TMAsO (V)] of As have been also reported (Yin et al., 2011). Besides, use of organic As compound like ROX (4-hydroxyl-3-nitrophenylarsenic acid) and p ASA (4-aminophenylarsonic acid) in agricultural application poses toxicity and risk to human health (Zheng et al., 2014). Recent studies have shown that contamination of arsenic (As) in paddy soils is a widespread problem due to irrigation of As-laden groundwater and mining activities or uses of arsenical agrochemicals in other regions of the world (Williams et al., 2007; Zhu et al., 2008; Brammer, 2009). Although the main source of As exposures are drinking water and the food supply (Guha Mazumder et al., 2014), concern is growing over the human exposure of As through dietary consumption of rice (Meharg, 2004; Smith et al., 2009). Rice, being particularly efficient in assimilating arsenic from paddy soils becomes part of the food chain and develops arrays of diseases such as keratosis, cancer, cerebrovascular disease, diabetes mellitus, and kidney disease resulting into slow and painful death (Ma et al., 2008; Jomova et al., 2011; Rossman and Klein, 2011). In addition, the toxicity associated with organoarsenic compounds depends on associated organic functional groups and biological or environmental induced biotransformation (Zheng et al., 2014). The accumulation of As in rice may be attributed to two main factors: the reductive mobilization of As(III) in anaerobic paddy soils (Xu et al., 2008) and uptake of arsenite via the silicic acid pathway in rice (Ma et al., 2008). Plants exposed to As, develop reactive oxygen species (ROS) leading to photosynthetic pigment degradation, lipid peroxidation, electrolyte leakage and DNA damage (Rai et al., 2011). Malondialdehyde, a common oxidation product of polyunsaturated fatty acids, refers

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C H A P T E R

21

Microalgae-Assisted Phyco-Remediation and Energy Crisis Solution: Challenges and Opportunity

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O U T L I N E

21.1 INTRODUCTION

Phycoremediation refers to the removal, degradation, biotransformation, and accumulation of toxic pollutants and xenobiotics from the environment (Olguı´n, 2003). The term phycoremediation was coined by John about two decades ago and is described as a technology that uses microalgae to remove contaminants from water. The phycoremediation implies natural processes to clean organic and inorganic contaminants present in the water (Rawat et al., 2011). The concept of plant-assisted remediation was started around 60 years ago by Oswald and Gotaas (1957) in the United States. The role of algae in phytoremediation came into light with the work of Palmer (1974) in which Palmer identified different algae species that grow successfully in waste pond, viz., *Chlorella, Ankistrodesmus, Scenedesmus, Euglena,*

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Chapter 18

Phycotechnological Approaches Toward Wastewater Management

Atul Kumar Upadhyay, Ranjan Singh, and D. P. Singh

Abstract Phycoremediation technology is a cost-effective and environment-friendly approach, which involves use of microalgae to suck the pollutant present in the soil and water. The accumulation of inorganic and organic pollutants like pesticides, herbicides, insecticide, PCBs, DDT, and metals along with metalloids (Cd, Pb, Se, As) in the aquatic ecosystem can cause deleterious impacts on environment and organisms. Natural and intensive anthropogenic activities are the main factor responsible for the accumulation of these pollutants in plants and animals. These serious problems of pollution can be resolved by the application of phycoremediation technologies using microalgae to remove pollutants from the environment in a sustainable manner. The present chapter focuses on the different techniques used in remediation of pollutants and the mechanism of remediation adopted by micro- and macroalgal species to absorb the organic and inorganic pollutants from wastewater and soil.

Keywords Phycoremediation · Microalgae · Wastewater · Pollutant

1 Introduction

Phycoremediation may be defined as the use of algae for removal and biotransformation of pollutants including xenobiotics from the wastewater. The algae consist of macroalgae, microalgae and marine algae commonly known as the seaweeds. The algae are widely distributed on the earth and are adapted to a variety of habitats. This unique feature of their fast adaptation allows the algae to develop a wide range of tolerance toward different environmental conditions, suited for wastewater treatment and production of biofuel and other valuable products, including food, feed, fertilizer, pharmaceuticals, and lastly biofuel (Menon and Rao 2012; Nigam and Singh 2011; Chowdhary et al. 2017). Algae reduce global warming through photosynthesis

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Chapter 2
Phytoremediation and Sustainable Developmental Policies and Practices

Atul Kumar Upadhyay, Ranjan Singh, and D. P. Singh

Abstract Phytoremediation is a green strategy of environmental decontamination and offers a cost-effective approach for the remediation of variety of pollutants. This is an emerging technology toward sustaining the future of the world and mankind. The phytoremediation technology has been successfully applied in developed and developing nations to achieve the sustainable development goal. The present chapter encompasses the basic strategies, rules, regulation policies, and protective measures for the successful implementation of plant-based waste treatment technology in a cost-effective and sustainable manner.

Keywords Phytoremediation · Sustainable development goal · Environmental pollution · Environmental policies

1 Introduction

Environmental degradation and population burst are two main components which impede the world's sustainability (Carley and Christie 2017). The current world is facing challenges like waste mitigation, water pollution, and access to safe, affordable drinking water. These challenges are produced due to inadequacy of treatment system, awareness, and unlawful policies of the government (Upadhyay et al. 2016). Besides, ignorance is the key factor responsible for pollution, drought, and starvation.

The idea of environmental cleanup through plant-based phytoremediation technology is certainly very old and has been proved as an alternative cost-effective approach in the treatment of different contaminants including organic, inorganic, pathogen, radionuclide, and hydrocarbon (Alkorta and Garbisu 2001; Paz-Alberto and Sigua 2013; Rezania et al. 2015; Salt et al. 1995; Tangahu et al. 2011). The term phytoremediation was coined in the year 1991. Phytoremediation relies on the

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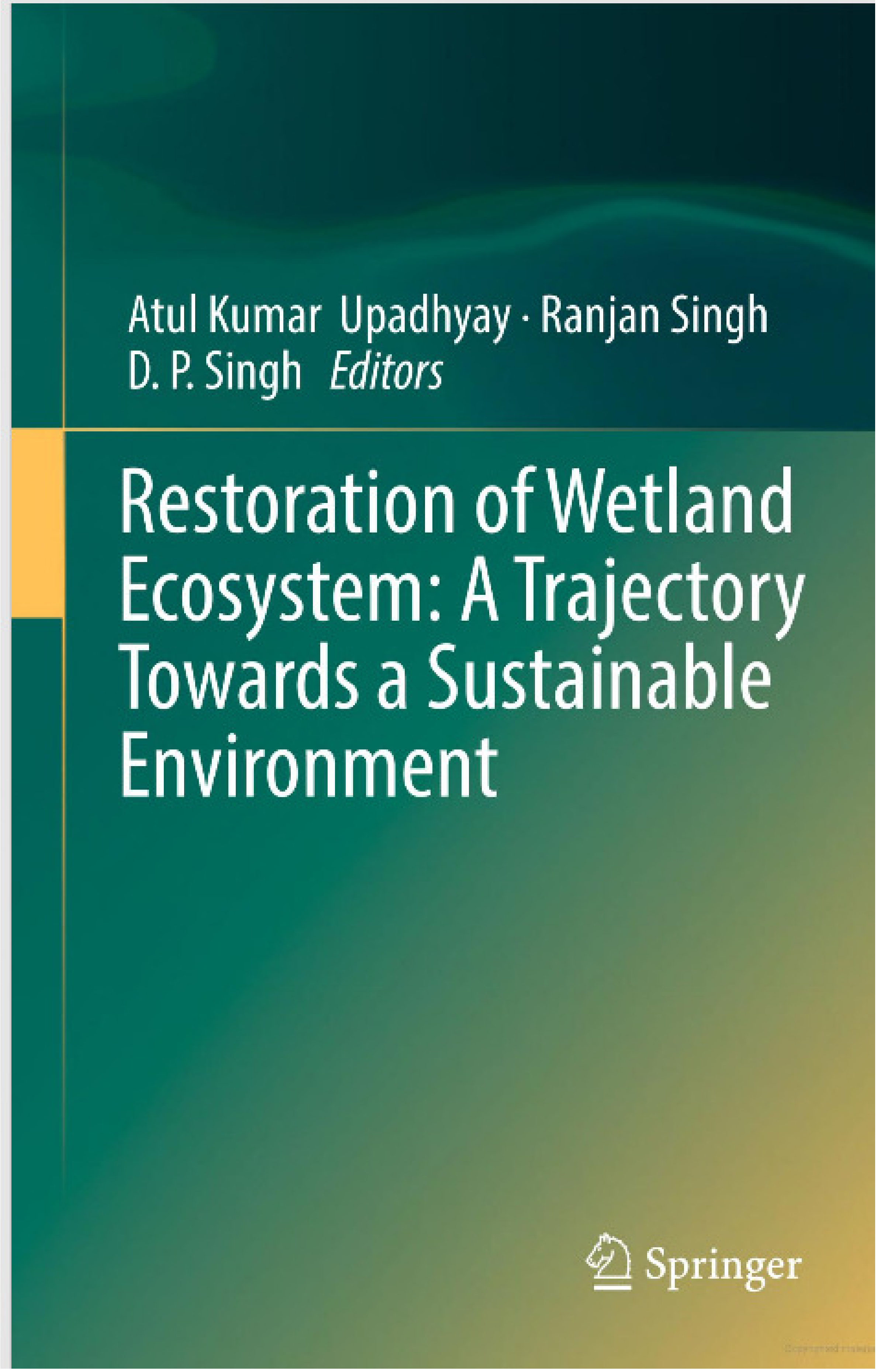

Atul Kumar Upadhyay · Ranjan Singh
D. P. Singh *Editors*

Restoration of Wetland Ecosystem: A Trajectory Towards a Sustainable Environment

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Microbes for Sustainable Development: Scope & Applications (MSDSA-2017)

CERTIFICATE

This is to certify that [✓ Mr.]/~~Ms.~~/~~Mrs.~~/~~Dr.~~/~~Prof.~~ Ranjan Singh

participated/[✓ presented] a Poster/Oral/Invited/Lead Lecture titled UV-B toxicity in Microalgae and Role of Antioxidant for management in this International Conference.

Vice Chancellor	**President**	**General Secretary**	**Organising Secretary**
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ICPEP-6

Sixth International Conference on Plants & Environmental Pollution

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CSIR-National Botanical Research Institute (CSIR-NBRI)

Lucknow -226001, India

November 27-30, 2018

Certificate of Participation

Prof./Dr./[✓ Mr.]/Ms. ....RANJAN SINGH..

attended the Sixth International Conference on Plants & Environmental Pollution, held at CSIR-National Botanical Research Institute (CSIR-NBRI), Lucknow, India during November 27-30, 2018. He/~~She~~ made ~~Oral~~/[✓ Poster] presentation at the Conference.

Nandita Singh

(K.J. Ahmad)	(R.D. Tripathi)	(Nandita Singh)	(Vivek Pandey)

NATIONAL SYMPOSIUM ON

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37th Annual Session of Academy of Environmental Biology, Lucknow

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(24-26 November, 2017)

Department of Zoology, Chaudhary Charan Singh University, Meerut

Certified that Prof. / Dr. / Mr. / Ms. Ranjan Singh

of Babasaheb Bhimrao Ambedkar University, Lucknow

delivered ~~Invited Lecture~~ / Participated / Presented Research paper entitled "Physiological and Biochemical

Response of Microalgae Under UV-B stress"

during the Symposium.

H. S. Singh
Prof. H. S. Singh
Pro Vice Chancellor

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Prof. A. K. Chaubey
Organizing Secretary

4th Lucknow Science Congress
LUSCON-2017

3rd & 4th March 2017

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This is to certify that Prof./Dr./Mr./Ms. RANJAN SINGH

from DES, SES, BBAU LUCKNOW has participated as Chairperson/delivered

Plenary Lecture/Inivited Talk/Oral Presentation/[✓ Poster] in 4th Lucknow Science Congress (LUSCON-2017) held on

3rd & 4th March, 2017 on the title of EFFECT OF SALINITY STRESS ON BIOMASS AND BIOFUEL PRODUCTION POTENTIAL ON MICROALGAE AND ROLE OF STRESS INDUCED MOLECULAR SIGNALING.

Dr. Naveen Kumar Arora
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actively participated in the scientific deliberations and also chaired technical session/delivered invited lecture/presented paper ([✓ oral]/poster) on topic EFFECT OF UVB STRESS ON MICROALGAE AND ITS BIOFUEL PRODUCTION POTENTIAL *at the Department of Zoology.*

Dr. Jamil Ahmad
Organizing Secretary

Gandhi Faiz-e-Aam College, Shahjahanpur

Prof. Aquil Ahmad
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