THE EFFECT OF DIFFERENT LIGHT INTENSITIES ON THE GROWTH AND STRESS RESPONSES OF CYANOBACTERIA

（シアノバクテリアの生長とストレスに係る光応答に関する研究）

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A dissertation submitted in partial fulfillment of the requirements for the degree of DOCTOR OF ENGINEERING in Environmental Science and Infrastructure Engineering

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Abstract

The continuous supply of nutrients like phosphorus to water bodies and global warming have promoted the presence of cyanobacteria globally. Due to the continuous spreading of harmful Cyanobacteria, the water environment is facing many challenges such as bad odor, releasing of cyanotoxins; and under the bloom, conditions losing clogging of water systems and reduction of aesthetic values. The biological and physical control of Cyanobacteria is getting increased attention as it is relatively environmentally sound and low cost in application. To understand the favorable and unfavorable light conditions and exposure durations of cyanobacteria, morphological and physiological responses of cyanobacteria to different light conditions and light exposure durations, and interaction of cyanobacteria with macrophyte species under different light conditions, a series of experiments were conducted. Experiments were conducted under controlled environmental conditions in incubators.

There is an information gap exists regarding light-induced oxidative stress and the species-specific behavior of cyanobacteria under various light levels. A study was conducted to evaluate the comparative effects of different light intensities on the growth and stress responses of two cyanobacteria species, *Pseudanabaena galeata* (strain NIES 512) and *Microcystis aeruginosa* (strain NIES 111), after periods of two and eight days. The cyanobacterial cultures were grown under the following different light intensities: 0, 10, 30, 50, 100, 300, and 600 $\mu mol \bullet m^{-2} \bullet s^{-1}$. The optical density (OD730), chlorophyll a (Chl-a) content, protein content, H_2O_2 content, and the antioxidative enzyme activities of catalase (CAT) and peroxidase (POD) were measured separately in each cyanobacteria species. *P. galeata* was negatively affected by light intensities lower than 30 $\mu mol \bullet m^{-2} \bullet s^{-1}$ and higher than 50 $\mu mol \bullet m^{-2} \bullet s^{-1}$. A range of 30 to 50 $\mu mol \bullet m^{-2} \bullet s^{-1}$ light was favorable for the growth of *P. galeata*, whereas *M. aeruginosa* had a higher tolerance for extreme light conditions. The favorable range for M. aeruginosa was 10 to 100 $\mu mol \bullet m^{-2} \bullet s^{-1}$

The diurnal variation oxidative stress and the antioxidant activities of cyanobacteria were tested to understand the oxidative stress and antioxidant activities under varying light conditions and their favorable and stressed phases during a single day. Two harmful cyanobacteria species, *Phormidium ambiguum* and *M. aeruginosa* were investigated under controlled environmental conditions. The photosynthetically active radiation (PAR) started at 0 μmol•m^{-2}•s^{-1} 06:00 h), increased by ~25 or ~50 μmol•m^{-2}•s^{-1}every 30 min, peaking at 300 or 600 μmol•m^{-2}•s^{-1} (12:00 h), and then decreased to 0 μmol•m^{-2}•s^{-1} by 18:00 h. The H_2O_2 and antioxidant activities were paralleled to light intensity. Higher H_2O_2 and antioxidant levels (guaiacol peroxidase, catalase (CAT), and superoxidase dismutase) were observed at 600 μmol•m^{-2}•s^{-1} than at 300 μmol•m^{-2}•s^{-1}. Changes in antioxidant levels under each light condition differed between the species. Significant correlations were observed between antioxidant activities and H_2O_2 contents for both species, except for the CAT activity of *P. ambiguum* at 300 μmol•m^{-2}•s^{-1}. Under each of the conditions, both species responded proportionately to oxidative stress; even under maximum light intensities (300 or 600 μmol•m^{-2}•s^{-1} PAR intensity), neither species was stressed. Studies using extended exposure durations are warranted to better understand the growth performance and long-term physiological responses of both species.

The influence of cyanobacteria on macrophytes was investigated by exposing *E. densa* plants to a low concentration of (OD_{730} = 0.04) *M. aeruginosa* for seven days, under different photosynthetically active radiation (PAR) intensities (0, 50, 100, 200, and 300 μmol•m^{-2}•s^{-1}). Experiments were conducted under controlled conditions inside incubators. The H_2O_2 content, antioxidant responses, pigmentation, and chlorophyll fluorescence parameters were measured. The biochemical parameters of *E. densa* varied with PAR intensity; under the 50 and 100 μmol•m^{-2}•s^{-1} PAR intensities, *E. densa* performed relatively better than under other intensities. When exposed to *M. aeruginosa,* the levels of the measured biochemical parameters reduced in *E. densa*.

Under unfavorable light conditions, the optical density of *M. aeruginosa* was also reduced when *E. densa* was also present. Even at low concentrations, the presence of *M. aeruginosa* in the water can have a negative effect on *E. densa* pigmentation and physiological parameters, though *E. densa* also has negative effects on *M. aeruginosa* growth. There were reduced OD_{730} and increased H_2O_2 formation in the *M. aeruginosa* was observed due to the co-existence with the *E. densa*.

The combination of light stress, together with existing measures (bubbling or mixing) can be used to suppress the growth of cyanobacteria further. Further research is recommended including investigating, recovery of cyanobacteria after high light exposure, macrophyte allopathy on cyanobacteria and influence of cyanobacterial circadian rhythm on light exposure. The physiological and growth responses of cyanobacteria under these conditions should be studied. Information such as diurnal stress phases of cyanobacteria, allopathic macrophyte species and circadian responses of cyanobacterial physiology are essential to formulate effective cyanobacteria control approaches.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Freshwater ecosystems

Freshwater ecosystems are mainly included lakes and ponds, rivers, streams, springs, bogs, and wetlands. Fresh water important for the survival of all most all the terrestrial organisms. The definition for the fresh water is, water resource with less than 0.5 parts per thousand dissolved salts (Dodds 2002). The ultimate source of freshwater is the precipitation of atmosphere in the form of rain and snow. It is estimated that the total volume of water on earth is about 1400 million km^3 of which only 2.5 %, or about 35 million km^3. The freshwater in lakes, rivers, soil moisture and relatively shallow groundwater basins are available for most organisms including humans. Among the total fresh water resources, the usable portion is only about 200 000 km^3 which is less than 1 % of freshwater and 0.01 % of all water on earth (UNEP 2002).

Fresh-water ecosystems are often categorized by two basic criteria based on water movement and the size of the water body. Lentic, lotic and wetlands are the three basic types of freshwater ecosystems in relation to water movement. Lentic ecosystems are slow moving water bodies such as pools, ponds and lakes where is lotic ecosystems with a unidirectional water movement along a slope in response to gravity like rivers and streams. Wetlands are the areas where the soil is saturated or inundated with water for at least part of the time (Boavida 1999). The movement of the waters is coupled with the physical, chemical and biological (biota of an area) and forming characteristic ecosystems (Figure 1-1).

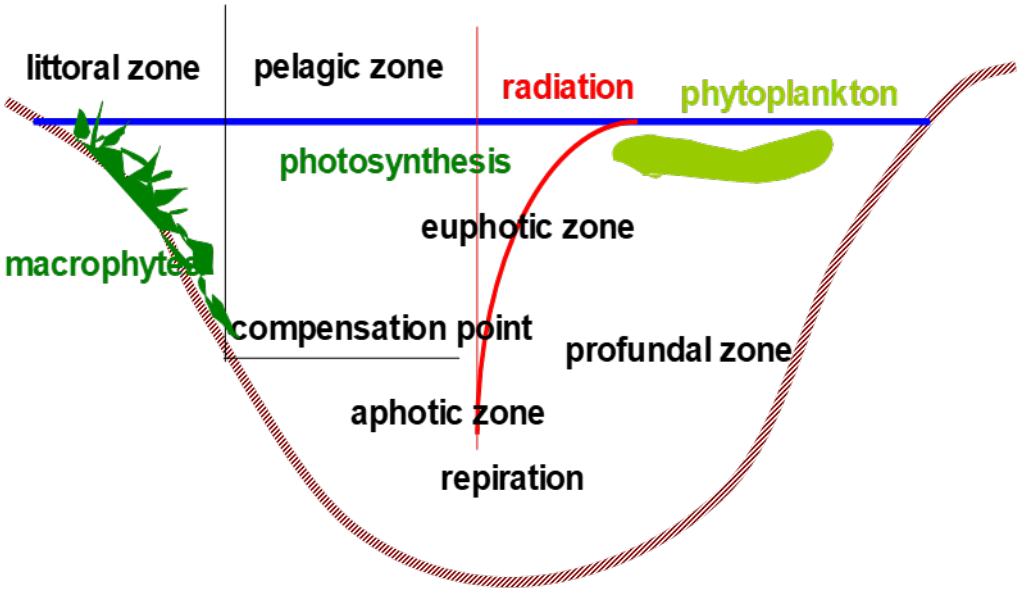


Figure 1-1: Natural environment and ecosystem

1.1.1 Physical environment in fresh waters

The light intensity and the quality of light (spectral quality) of light plays major on determine of the biota in an ecosystem. Depending on the geographical location, season cometic and weather conditions determining the amount of light received by a water body. Further, light penetration (attenuation) is controlled by dissolved and particulate material in the water column (Boavida 1999).

The temperature is also another important factor which is also associated with the light intensity or the season. In deeper freshwater ecosystems water column can be subjected to stratification, as water is warmed at the surface of a lake and having warmest water (epilimnion) and the deeper layers maintaining cooler stratum (hypolimnion). The region of sharp temperature changes (thermocline) between epilimnion and hypolimnion is called the metalimnion. These two layers are importance in the chemical cycling within lakes and consequently for the biota (Teneva et al. 2005). In colder seasons epilimnion become colder and the density of the water is reaching the level of density hypolimnion hen the cooling continues. Such situation is leading to mixing the

entire water column, known as turnover. Many temperate zones lakes undergo mixing in the spring and the fall, and stratification in the summer and winter.

1.1.2 Chemical environment in fresh waters

Oxygen occurs as dissolved oxygen (DO) and in combination with chemical or biological reactions. Oxygen is mixing with the water primarily from the atmosphere by diffusion and mixing. When biological demands for oxygen exceed oxygen supply, it can be depleted from and anoxic conditions can be accrued (Tanabe et al. 2018). This can be happened in hypolimnion during summer and in the winter when stratificated lakes strata's isolated from the atmospheric contact. On the other hand, Oxygen depletion may also occur in rivers due to excessive organic loading. Only a few of specialized bacteria and macro organisms can survived under anoxic conditions (Sinetova and Los 2016).

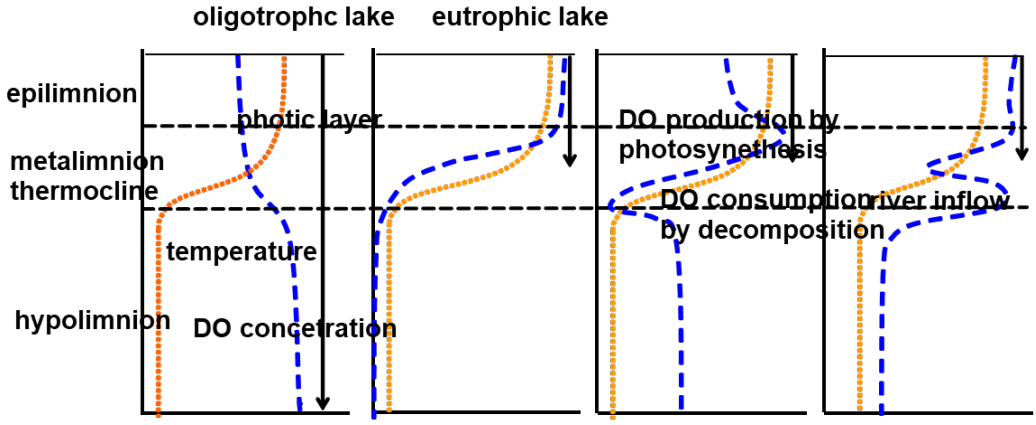


Figure 1-2: Vertical distribution of temperature and O2 concentration

Source: (Mikhail Gorbunov et al. 2007)

Carbon dioxide is also mixed with freshwater from the atmosphere, the respiration of plants and animal and carbonate minerals. Carbon dioxide is controlling the hydrogen ion concentration of water (pH) (Granelli-Piperno et al. 1998). Phosphorus is the shortest supply element in freshwater systems related to the biological demand. Phosphorus is ta limiting nutrient, and extra addition of it to fresh-water ecosystems

making numerous environmental problems. Due to human activities phosphorus can be added to water can increased growth of aquatic plants, algae and cyanobacteria at problematic rates (Figure 1-2). At extremely high rated it can limit growth of those aquatic organisms (Whiles Dodds 2002).

Nitrogen is presence as N_2, NO_2, NO_3 and NH_4 in water resources. Nitrogen may be supplied to aquatic system form precipitation and from soils, but its availability is regulated through bacterial processes. The extra supply of Nitrogen to water systems due to human activities also a present-day problem. Nitrogen shortage can be also accrued due to unavailability of source of supply satisfying the biological demand (Figure 1-3).

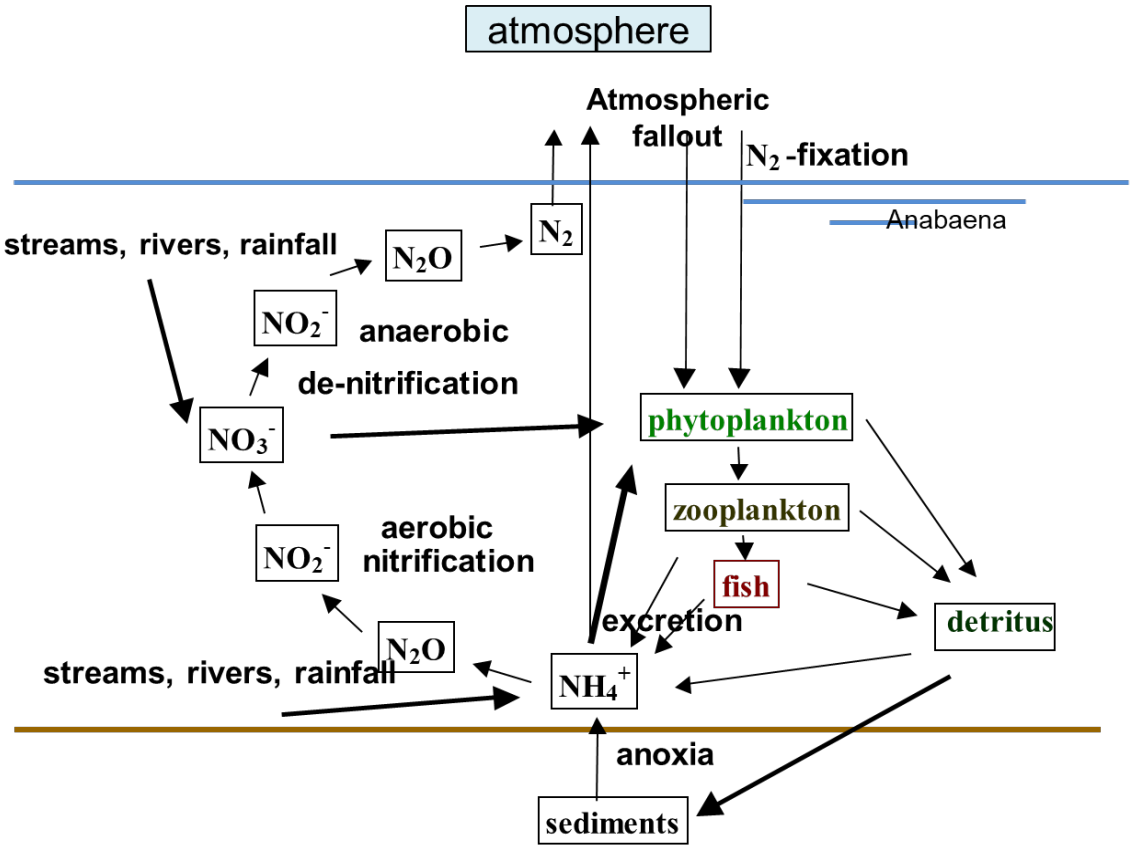


Figure 1-3 : Nitrogen cycle in water

1.1.3 Biodiversity in fresh waters

There are at least 12 percent of the animal species inhabit freshwater environments. Freshwater habitats support the same kinds of food webs readily identified in terrestrial communities. There are the autotrophic producers and heterotrophs, phagotrophic macroconsumers and saprotrophic decomposers, and microconsumers. Depending the availability in the different layers, bottom areas and water column (limnetic zone), major distinctions are made between organisms (Nakata and Ohme-Takagi 2014). Near the surface are communities of phytoplankton and the zooplankton occupied. Fishes and swimming insects can control their position in the water column independently of water movement. Fishes may also affect zooplankton, benthic invertebrates, vegetation, and lake sediments. Bacteria occur throughout fresh-water ecosystems in planktonic and benthic areas and play a major role in biogeochemical cycling. Most bacteria are heterotrophic, using reduced carbon as an energy source; others are photosynthetic or derive energy from reduced compounds other than carbon (Walter Dodds 2002). Plants including floating or submerged macrophytes forming a diverse community specially in shallow freshwater bodies determined by the composite of abiotic features such as light, temperature dissolved oxygen, carbon dioxide and other macronutrients (Robert G.wetzel 2001).

1.2 Cyanobacteria Bloom

1.2.1 Group and plankton

Cyanobacteria consist of a single cell of about 3 to 7 μm. Because it contains blue pigment, it belongs to the cyanobacteria. Because the shape of the cell's nucleus and chloroplast is less pronounced, it is considered a near-bacterial organism and is called a blue bacterium. Cyanobacteria are single-cell aquatic organisms, usually hundreds of cyanobacterial cells cluster together. Since the cells contain bubble nuclei, cyanobacteria can float on the surface of the water. When the cyanobacteria floating on

the surface of the water proliferates to a certain extent, it forms a bloom.(Brian A. Whitton and Malcolm Potts 2000)

1.2.2 Reproductive characteristics

Abnormal proliferation of cyanobacteria usually occurs in the strong solar radiation from May to October. Since cyanobacteria are single-celled aquatic organisms, their reproduction is accomplished by the division of individual cells. Its splitting and breeding speed is usually more than 1 times per day, and the highest can reach 3 to 5 times a day. When the content of cells in water is high, it can reach more than 1 million cells per liter of water.

1.2.3 Movement of cyanobacteria

According to the study on the living rules of cyanobacteria, it was found that cyanobacteria floated on the surface in the morning and at noon, gathered in large quantities, began to disperse in the afternoon, and then sunk to the water at night, and then repeated the above process on the second day. Studies have shown that cyanobacteria rely on regulating the size of the gas nucleus inside the cell to reach the floating and sinking. In the morning, the photosynthetic reaction of cyanobacteria is strengthened, and the gas nucleus becomes larger, so it floats up to the surface of the water. When the photosynthetic reaction is further strengthened, the pressure in the nucleus rises, and finally the weaker gas nucleus ruptures. The rupture of the bubble causes part of the cyanobacteria to buoyancy. Drop and settle below the water surface. Since the 10cm sunlight intensity is only a few percent of the incident sunlight, the cyanobacteria can rest underwater, waiting for the opportunity to rise again (Garcia-Pichel et al. 2020)

1.2.4 Status in the biological chain

In the bio-chain of the lake, usually plant algae are swallowed as bait for animal algae and fish and shrimp, and as long as this balance is not destroyed, a large amount of plant algae does not appear. However, since cyanobacteria survives in groups and float on the surface of the water, it is not easy to be preyed by animal algae. In a lake where cyanobacteria occur in large numbers, to maintain a sound biological chain, it is necessary to destroy the group life of cyanobacteria and its floating function (Visser et al. 2016)

1.2.5 toxicity of cyanobacteria

The massive accumulation of cyanobacteria not only destroys the landscape, makes the water smelly, but also emits the toxic substance Microcystin (MC-LR), which threatens the safety of drinking water. The toxicity of MC-LR is about 200 times that of green acid, which may cause liver dysfunction and even liver cancer. There are many foreign records of adverse reactions caused by contact or drinking cyanobacteria. In Hawaii and Japan, there are reports of dermatitis caused by exposure to cyanobacteria. In the United States, Australia, the United Kingdom, etc., there are reports of gastroenteritis, hepatitis, headache, dizziness, dysentery and other symptoms caused by drinking cyanobacteria. In addition, fish in areas where cyanobacteria occur in large numbers may also be toxic due to the predation of cyanobacteria. Vegetables washed with water may also be poisonous. According to research by Paerl (Paerl et al. 2001). The toxic content per 100 mg of cyanobacteria in the water body of the bloom is between 40 and 210 ug, which shows that the toxicity of cyanobacteria is very large.

1.2.6 Bloom

Cyanobacteria bloom is a phenomenon in which the cyanobacteria in the water body rapidly proliferate to form a macroscopic algae population or cause a change in the color of the water body. In severe cases, it can float on the surface of the water body to

form a layer of green algae mat, even algae pulp, cyanobacteria water. The root cause of China is that the water is enriched with nutrients such as nitrogen and phosphorus, which is another manifestation of eutrophication of water bodies. In recent years, cyanobacteria blooms have a high incidence, frequent occurrence and outbreak in aquaculture water (Figure 1-4).

In essence, cyanobacteria blooms are the result of material and energy conversion using cyanobacteria as a carrier. Among the waters rich in nutrients, some cyanobacteria often multiply in the summer, and form a layer of blue-green or scented foam on the surface of the water, known as "water bloom. “Large-scale cyanobacteria outbreaks are known as "green tides." The formation mechanism of cyanobacteria blooms and proposed a four-stage theoretical hypothesis: the growth of cyanobacteria and the formation of blooms can be divided into dormancy, resuscitation, and growth. ups and downs and four stages.

In each stage, the physiological characteristics and dominant environmental impact factors of cyanobacteria are different. In winter, the dormancy of cyanobacteria is mainly affected by low temperature and dark environment. The spring recovery process is mainly controlled by the temperature and dissolved oxygen of the lake sediment surface, while the materials and energy required for photosynthesis and cell division determine the growth of blooms in spring and summer. Once there are suitable meteorological and hydrological conditions, a large number of cyanobacteria that have accumulated in the water will float up to the surface of the water to form visible blooms. The most fundamental reason for the emergence of water blooms is that the pollutants discharged into the water body are far greater than the self-capacity of the water environment (Jenkins 1991).

When cyanobacteria such as *Microcystis* have excessive proliferation of cyanobacteria, the transparency of water is extremely low. The cyanobacteria are under low light for a

long time, and the formation of false vacuoles quickly causes the cells to rise rapidly. The increase of internal pressure is too late to rupture the pseudo-cavitation. The algae have risen to the surface layer with excessive light, forming spotted scum. When the scum is decomposed, it emits odor and dissolves oxygen in the water at night, which easily causes the fish to die of oxygen deficiency Moreover, cyanobacteria produce hydroxylamine or hydrogen sulfide after death, which is toxic to aquatic animals, destroys water bodies, and reduces the utilization value of water bodies.

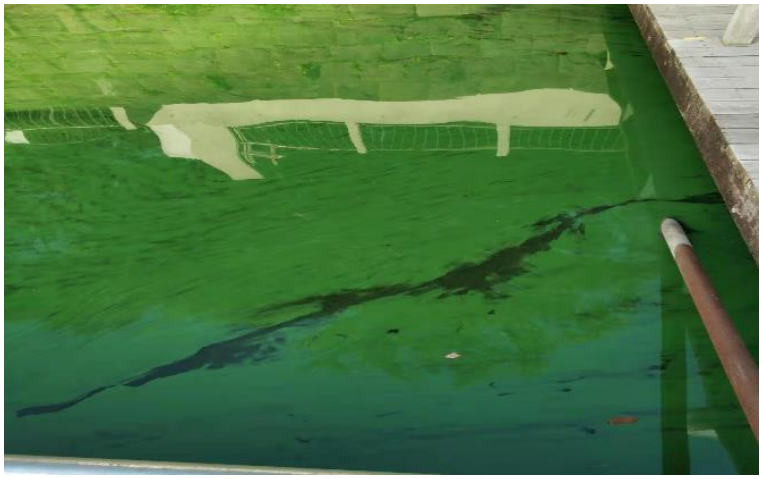


Figure 1-4 : Cyanobacteria bloom phenomenon
(Tokyo Imperial Palace, 2019.9.22)

1.3 General cause of cyanobacteria blooms

1.3.1 Internal factors - biological characteristics of cyanobacteria

The adaptation of cyanobacteria to high temperature, low light intensity and ultraviolet light can excessively ingest inorganic carbon and nutrients, and low nitrogen-phosphorus ratio is beneficial to the growth of cyanobacteria. The specific description is as follows:

After more than two months of cultivation in the pond waters, the large-scale production and energy conversion of plankton in the water, in addition to the rich nitrate, the other two nutrients such as phosphates and silicates have been consumed.

The cyanobacteria and a few bacteria have the ability to utilize free nitrogen in the air, causing the blue-green algae to accumulate a higher amount of protein nitrogen. This process is called biological nitrogen fixation, which increases the accumulation of nitrate and ammonia nitrogen. The more cyanobacteria like high nitrogen and low phosphorus have unique growth advantages.

Cyanobacteria blooms often spread in less than three or five days to cover the entire pond surface. The algae community has the ability to be unmatched and inhibited by other algae, and tends to be in an uncontrolled breeding state when the environment is suitable.

1.3.2 External factors- environmental conditions

Water bloom formation: a large number of cyanobacteria + hydrometeorological conditions.

Cyanobacteria growth: cyanobacteria + light + temperature + suitable environment.

The specific description is as follows:

1) In the continuous high temperature and high temperature weather, temperature is the main influencing factor from the algal phase ecosystem. Most algae can live in a wide temperature range, but the most suitable growth temperature is narrow (Cedhagen 1969). In general, diatom, algae, and yellow algae have a moderate temperature of 14 to 18 ° C, and green algae have a higher temperature of 20 to 23 ° C, while cyanobacteria are higher, and most prefer to grow at a temperature of 20 to 32 ° C. Among them, the most harmful Microcystis can grow at 10 to 40 °C, and the optimum temperature is 28 to 32 °C. The growth rate of cyanobacteria increases with the increase of water temperature. Under normal temperature conditions, some beneficial single-cell algae growth rate is not slower than cyanobacteria. Only when the temperature reaches 20 °C or above, and the water temperature is 25 to 35 °C, the growth rate of cyanobacteria is

faster than other algae. Therefore, due to the growth of other algae species, cyanobacteria are not likely to explode on a large scale under normal temperature conditions. Only when entering the hot season, the growth rate advantage of cyanobacteria will be reflected. Therefore, temperature is one of the main factors in the outbreak of cyanobacteria (Wells et al. 2015).

2) The water high nitrogen and low phosphorus organic matter content is abundant. There are two types of sources of nutrients such as nitrogen and phosphorus in aquaculture water:

Exogenous sources: mainly wastes imported into water bodies during the breeding process (uneat bait, manure and excrement of cultured objects, etc.) and agricultural fertilizers and poultry manure that enter the aquaculture water body with water flow.

Endogenous source: refers to the content of nitrogen and phosphorus in the water body of the sediment of aquaculture water that is released to the water body under certain conditions. During the breeding process, the residual bait, feces and excrement continuously enter the water body, part of the organic matter is directly dissolved in the water, and part of the sediment is deposited by the action of gravity, which not only increases the nutrient salt in the aquaculture water body, but also increases the nutrient salt in the sediment. This is a continuous gradual process (Cedhagen 1969).

In addition, in summer, there is a lot of drought and water shortage, and the amount of water supplement is often insufficient. The state of water with strong light, high temperature and high alkalinity inhibits the reproduction of other algae. When microcystis is in the right place, it will be born out of the wild.

3) Eutrophication, after entering the peak period of breeding, the eutrophication of the aquaculture water body, the excrement of the culture organism itself is also a kind of

pollution to the aquaculture water body. In the past we have often overlooked the self-contamination of farmed organisms. Therefore, it is often easier to explode cyanobacteria in ponds that do not change water frequently. If cyanobacteria are not adequately nutritious, it is difficult to grow.

4) The stocking structure is unreasonable. The excrement, corpse and other humus of aquatic plants in the aquaculture water can be indirectly provided for the breeding of algae through the decomposition of aerobic bacteria. If the stocking structure is unreasonable, the ratio of eating fish to filter-feeding fish (such as white peony) is out of balance, and the number of water quality updates in the hot season is small, which will facilitate the mass reproduction of plankton. Although these factors can not directly cause eutrophication of aquaculture water bodies, they can fundamentally change the water quality of aquaculture water bodies and facilitate the formation of eutrophication.

1.4 The harm of cyanobacteria

1.4.1 Consumption of dissolved oxygen in water

When the cyanobacteria in the aquaculture water forms water blooms, on the one hand, the oxygen produced by photosynthesis of phytoplankton is severely inhibited, and on the other hand, the atmosphere in the air is blocked from entering the aquaculture water body, thus causing serious shortage of dissolved oxygen in the aquaculture water body. The prolonged occurrence of hypoxia or sub-anoxic conditions causes the aquaculture water to continue to deteriorate, thereby directly or indirectly aggravating the accident (Huntington et al. 2006).

1.4.2 Reduce biodiversity

When the cyanobacteria in the aquaculture water forms an absolutely dominant population, the excessive proliferation of cyanobacteria exacerbates the continuous deterioration of the ventilation and illumination conditions of the aquaculture water,

inhibits the growth and reproduction of beneficial plankton in the aquaculture water, hinders the photosynthesis of other algae, and makes the culture Filamentous algae and planktonic algae in water cannot die by synthesizing the nutrients they need (Paerl 2014).

1.4.3 Producing toxic and hazardous substances

When a large number of cyanobacteria die, cyanobacterial toxins, a large amount of hydroxylamine and hydrogen sulfide and other toxic and harmful substances will seriously damage the aquaculture water and directly harm the cultured animals. In addition, the dead cyanobacteria will release a large amount of organic matter, which will emit odor and stimulate the growth of heterotrophic bacteria. Most of these heterotrophic bacteria are not beneficial bacteria for aquaculture animals, but are pathogenic bacteria, which further leads to the occurrence of secondary bacterial diseases (Pham and Utsumi 2018).

1.5 Features of cyanobacteria

1.5.1 Overview of cyanobacteria photosynthesis

Photosynthesis is the reaction process in which photosynthetic organisms (including green plants, algae, and photosynthetic bacteria) convert sunlight into chemical energy and then use this chemical energy to convert CO_2 and water into carbohydrates. Although the research on photosynthesis has been more than two hundred years old, it is still one of the major scientific problems that need to be solved urgently in the world (Pietsch et al. 2001). The photosynthesis process can be divided into photoreaction (including primary reaction, water cleavage, electron transfer and its coupled phosphorylation reaction) and dark reaction (carbonation assimilation), photoreaction on the thylakoid membrane, and dark reaction Performed in the cytoplasm. The results show that there are four main complexes on the thylakoid membrane of higher plants:

photosystem II (including core complex and light-harvesting complex-LHCII), photosystem I (including core complex and light-harvesting complex-LHCI), cytochrome b6f complex and ATP synthase. Two light systems are places where light energy is absorbed, transmitted, and transformed. All chlorophyll on the thylakoid membrane is present in these two photosystems, where chlorophyll b is only bound in the light-harvesting complex (Figure 1-5).

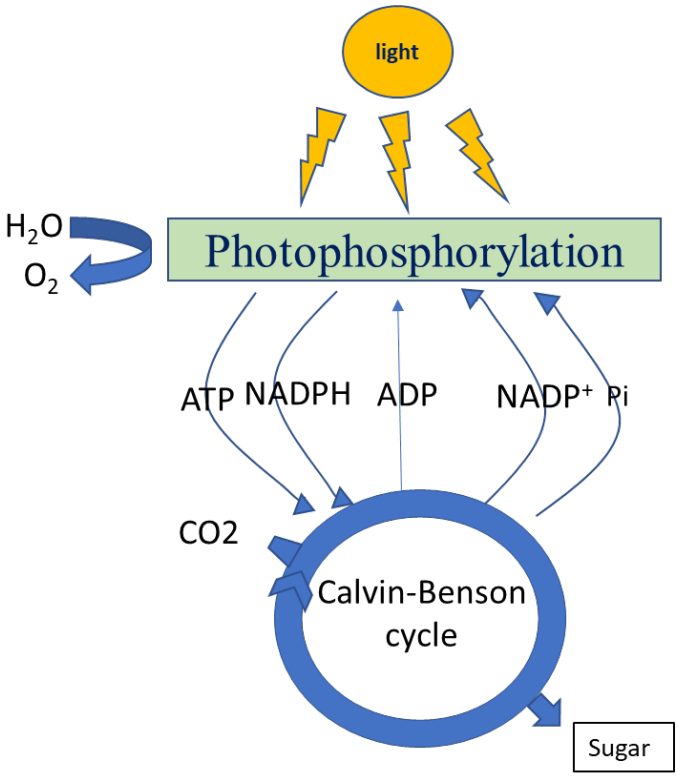


Figure 1-5: Overview of the two steps in the photosynthesis process

Source: (Purves et al. 2003)

Cyanobacteria include three membrane components: the outer membrane, the inner membrane (plasma membrane), and the thylakoid membrane (Stanier et al. 1979). The main component of the outer membrane is lipopolysaccharide (Zevenboom and Mur 1984), and the outer membrane and the inner membrane are peptidoglycan layers. The cyanobacterial cells are coated with organelles (such as chloroplasts, mitochondria, etc.), and their thylakoid membranes are directly dispersed in the cytoplasm to form a

lamellar structure, and there are no overlapping regions and non-folding regions (Virtanen et al. 2019). Electron transport on thylakoid membranes includes PSII, Cytb6f, PSI and ATP synthase enzymes, which are structurally and functionally similar to plants and eukaryotic algae. Cyanobacteria differ from higher plants and other algae in the composition of light-harvesting antennas and thylakoid membranes. Cyanobacteria contain only chlorophyll a, no chlorophyll a/b light-harvesting pigment complex (LHC), but phycobilin, which covalently binds to proteins to form phycobiliprotein. Various phycobiliproteins are organically assembled into phycobilisome, anchored to the surface of the thylakoids, as a light-harvesting pigment protein complex, functionally similar to the a/b light-harvesting antenna complex distributed across the membrane in higher plants (Murray and John K 1990). The cyan cystic membrane of the cyanobacteria not only distributes the photosynthetic electron transport chain, but also contains components of the respiratory electron transport chain, such as NAD(P)H dehydrogenase and Cytocides. The photosynthesis and respiration electron transport chains share the PQ library as an electron transporter (Robarts and Zohary 1987). The intersection of this photosynthetic electron transport chain and the respiratory electron transport chain also reflects the originality of cyanobacteria (Figure 1-6).

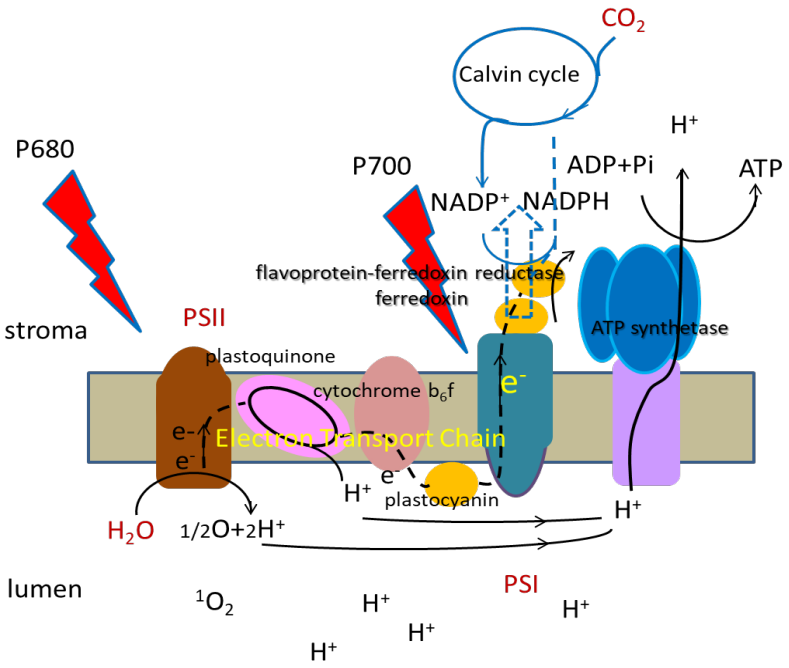


Figure 1-6: Plastoquinone reduction reactions

PSII is the center for (light-induced) water cleavage and plastoquinone reduction reactions (Loll et al. 2005). It is a multi-subunit pigment protein complex found in the thylakoid membranes of higher plants, eukaryotic algae, and cyanobacteria, including approximately 26 protein subunits encoded by nuclear and chloroplast genes (Murray glanzer and John K 1990). The core antenna of PSII consists of the pigment proteins CP43 and CP47, which bind most of the chlorophyll in PSII, with approximately 12-20 chlorophyll a molecules per protein (Qu et al. 2018). CP43 and CP47 are distributed around the P680, and the phycobilisomes are transmitted to the P680 along with the light energy captured by themselves. The CP43 protein is encoded by the psb C gene, and the psb C gene in the cyanobacteria Synechocystis 6803 is deleted. The contents of other peptide components D1, D2, and CP47 of PSII are significantly decreased, indicating the importance of CP43 for maintaining PSII structure and function. The CP47 protein is encoded by the gene psb B. Deletion of this gene results in a sharp

decrease in D1 and D2 protein content, which also demonstrates the importance of CP47 for maintaining PSII structural stability (Shen and Vermaas 1994).

The D1 and D2 proteins are the core proteins of the PSII reaction center, and their molecular weights are similar (32kD and 34k D). They are combined with the original electron donor (P680), the original electron acceptor (pheophyllin, Pheo a), and β-carotene. 4 auxiliary Chla molecules and non-heme iron (Barry and Alberts 1994). The D1 protein binds to QB via hydrogen bonding, while the D2 protein binds to QA via hydrogen bonding. The PSII reaction center also contains cytochrome b559 (Cytb559), which consists of two subunits that are distributed across the membrane and may play a role in electron transport (Barry and Alberts 1994).

1.5.2 Reactive oxygen species (ROS)

The triplet oxygen molecule ($3O_2$) is an excited state oxygen, and its outermost Valence electrons occupy their orbits through parallel spins. In order to oxidize atoms or molecules in a non-excited state, triplet oxygen needs to react with a partner phase that provides a pair of parallel spintronics and is suitable for its free electron orbitals. However, a pair of electrons usually spin in opposite directions, thus enhancing the strength of the reaction of triplet oxygen molecules with most organic molecules (Cadenas,E 1989). However, ground state oxygen may be converted to a more reactive form of ROS by energy transfer or electron transfer reaction. The former (energy transfer) generates singlet oxygen, while the latter (electron transport) produces superoxide, hydrogen peroxide, and hydroxyl groups through a series of reductions. In plants, ROS are constantly produced as a by-product of various metabolic pathways and remain in different parts of the cell (Saed-Moucheshi and Pessarakli el at. 2014). In the case of a stable physiological state, these molecules can be eliminated by antioxidant substances present throughout the cell. Under various adverse conditions, the balance between free radical production and clearance in cells may be disrupted, leading to a rapid increase in intracellular ROS levels (Foyer and Shigeoka 2011; Prasad

et al. 2013). In response to environmental changes, plants also produce ROS by activating various oxidases and peroxidases (Allan and Fluhr 1997; Satterfield and Bonnell 1955; Schopfer and Frahry 2001). This rapid increase in ROS concentration is known as the "oxidative burst" (Apostol and Low 1989). The external conditions that adversely affect plants can be other organisms, or non-biological, such as too much or too few physicochemical factors. Although plants may exhibit certain commonalities in the face of various stressful environments, the mechanisms of ROS caused by biotic or abiotic stress are different.

Environmental stress can be divided into damage-inducing stress and repair-inhibitory stress (Allakhverdiev and Murata 2006) according to its influence on PSII. Injury-induced stress includes strong visible light and ultraviolet light. Stresses that inhibit the repair activity include salt stress, low temperature stress, CO_2 restriction, and moderate-intensity heat stress. ROS is often an intermediate product of various stresses. In the past, classical theory believed that ROS directly led to photoinhibition, while (Allakhverdiev and Murata 2006) believed that ROS mainly inhibited the repair process of PSII. The main target of photodamage is the oxygen-evolving complex, especially the manganese clusters in the complex; followed by the photochemical reaction center. Electron transfer rates affect photodamage (Allakhverdiev et al. 2006), and repair of PSII is also associated with intracellular ATP levels (Kang et al. 2015). It seems that different environmental factors induce photoinhibition with a general rule, that is, environmental factors - limiting CO_2 fixation and reducing intracellular 3-PGA (3-phosphoglycerate) levels - reducing redox power (NADP +) - increasing ROS Yield - restricted protein synthesis - limits the repair of PSII (Figure 1-7).

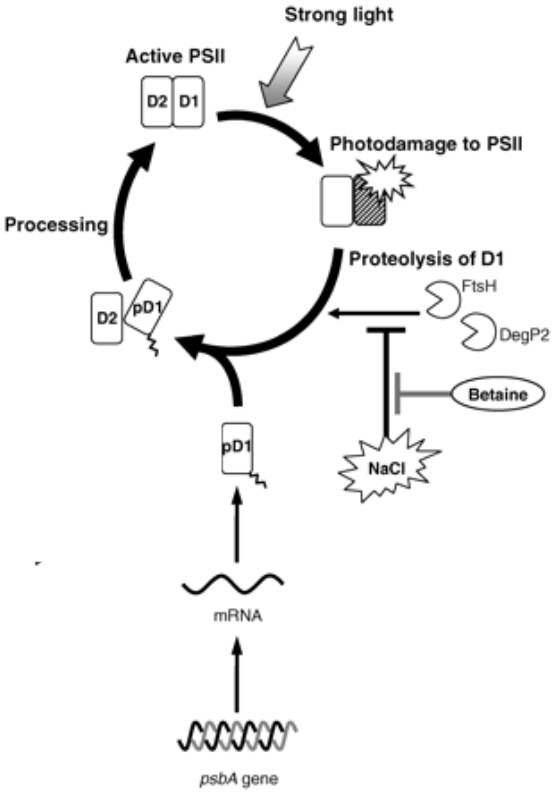


Figure 1-7: A hypothetical scheme for the stress-enhanced photoinhibition of PSII

Source: (Norikazu Ohnishi et al. 2006)

1.5.3 Inorganic carbon (CO_2)

When the concentration of inorganic carbon (HCO_3- and CO_2) in the environment or culture solution is very low, the photosynthesis and growth of cyanobacteria are restricted. The decrease in intracellular CO_2 fixation rate, in turn, exacerbates the degree of PSII photoinhibition (Kinoshita, Jin H. 1955; Sültemeyer et al. 1989). The view that has been accepted for many years is that when CO_2 supply is suppressed, light damage to PSII is exacerbated by the transitional reduction of QA (Yokthongwattana et al. 2001) However, some recent studies have found that the addition of glycolaldehyde (an inhibitor of ribulose phosphokinase) can reduce the rate of CO_2 fixation or by making a missense mutation in the gene encoding the large subunit of the Rubisco enzyme. Algae (*Chlamydomonas reinhardtii*) damages PSII, but inhibits repair of damaged PSII. The repair of D1 protein in spinach chloroplasts is also limited by insufficient CO_2 supply (Allakhverdiev and Murata 2006). Decreased intracellular 3-PGA reduces the

use of NADPH in the Calvin cycle and reduces the level of NADP+, which is the major electron acceptor of photosystem I (PSI), so the lack of NADP+ converts O_2 Accelerate the speed of ROS (Nakano and Asada 1981). In fact, blocking CO_2 fixation does increase H_2O_2 production (Asada and Badger 1984; Laboratories 1976; Radmer and Ollinger 1980). The inhibition of protein synthesis by insufficient CO_2 fixation may be due to the production of H_2O_2, and this hypothesis quickly gained strong evidence (Bolger et al. 1998).

1.5.4 Adaptation mechanism of cyanobacteria to environmental changes

Three billion years ago, life on earth began. As a relatively primitive life, cyanobacteria experienced a harsh environment in such a long period of time and still flourished. There are bound to be many ways to Adapt to various environmental stresses.

1) Free radical scavenging mechanism

In the presence of transition metal ions, hydrogen peroxide can react with superoxide to form hydroxyl (OH), and both superoxide and hydrogen peroxide have lower activity than ·OH. One risk faced by cells that produce these two active intermediates is that the two intermediates react to form a more active hydroxyl group. Since there is no known scavenger specific for hydroxyl groups, the only way to avoid oxidative damage is to control the formation reaction. Therefore, cells develop complex strategies to control the concentration of superoxide and hydrogen peroxide, and strictly control the concentration of transition metal ions such as Fe and Cu.

2) Non-enzymatic removal system of ROS

The main components of non-enzymatic antioxidants in the cell are ascorbate and glutathione (GHH). Secondary components include tocopherol, flavonoids, alkaloids and alkaloids. Carotenoids. Mutant strains with decreased ascorbic acid or glutathione levels are very sensitive to environmental stress. GSH is oxidized by ROS to oxidized glutathione (GSSG), and ascorbic acid is oxidized to monodehydroascorbate (MDA)

and dehydroascorbate (DHA). Through the ascorbate-glutathione cycle, GSSG, MDA, DHA are reduced to GSH and ascorbic acid. Plants increase GSH biosynthetic enzyme activity(Vanacker, Carver, and Foyer 2000) and GSH levels (Noctor et al. 2002), when subjected to stress conditions such as cold, heat, pathogen invasion and drought. A higher ratio of intracellular reduced ascorbic acid and oxidized glutathione is necessary for clearance of ROS. The maintenance of the antioxidant state of reduction requires the catalysis of glutathione reductase (GR), monodehydroascorbate reductase (MDAR) and dehydroascorbic reductase (DHAR), which provide reductive power by NADPH (Nakano and Asada 1981). At present, little is known about the role of flavonoids and carotenoids in the elimination of ROS, but the transient expression of β-carrotyl hydroxylase in Arabidopsis leads to an increase in the content of lutein in the chloroplast, thus making it exposed to intense light.

3) Enzyme clearance mechanism of ROS

Enzymes capable of clearing ROS in cells include: superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT). Superoxide dismutase first turns superoxide into hydrogen peroxide, and then APX, GPX and CAT convert H_2O_2 to water (Das and Roychoudhury 2014). Unlike CAT, APX requires a regeneration system for ascorbic acid and GSH, the ascorbate-glutathione cycle. APX catalyzes the reaction of H_2O_2 with ascorbic acid to form H_2O and MDA. MDA is reduced to ascorbic acid by its reductase, and MDA can also undergo disproportionation to form dehydroascorbic acid. The dehydroascorbic acid reacts with GSH under the catalysis of DHAR to form ascorbic acid and GSSG. Finally, GR uses NADPH as a reducing force to re-restore GSSG to GSH. Similar to APX, GPX directly uses GSH as a reducing agent to catalyze the production of water by H_2O_2. Different enzymes are often distributed in different organelles, such as chloroplasts, mitochondria, peroxisomes, and also in cytosol and apoplast. GPX is located in the cytosol, and CAT is mainly located in the peroxisome. The function of various antioxidant enzymes can be determined by genetic modification. Altering the balance

of intracellular ROS-clearing enzymes triggers compensatory mechanisms. For example, when CAT activity is reduced, other enzymes with H_2O_2 clearance, such as APX and GPX, undergo upregulation (Liu et al. 2018).

4) Measures for cyanobacteria to adapt to inorganic

The photosynthesis of cyanobacteria requires absorption of CO_2 from the surrounding environment. The enzyme that fixes CO_2 in cyanobacterial cells is Rubisco, which has about 200-300 μmol L^{-1}(Badger et al. 1980). If the concentration of CO_2 in the cell is only 10-12 μmol L^{-1} (the concentration of CO_2 in a typical water environment when the air is in equilibrium), it is almost impossible to carry out CO_2 assimilation. Fortunately, most cyanobacteria have developed an increase. The mechanism of intracellular CO_2 concentration—CO_2 concentration mechanism (CCM) (Asada and Badger 1984). After transporting across the plasma membrane, HCO_3-accumulates in cyanobacterial cells, and HCO_3- accumulated in the cytoplasm enters the carboxylate through diffusion, and is dehydrated by carbonic anhydrase (CA) to form CO_2, which causes CO_2 around Rubisco. The concentration is increased, a considerable part of the CO_2 is fixed by Rubisco, and the remaining CO_2 is partially recovered by the CO_2 transport pathway depending on the light energy during the outward diffusion process. There is an inducible Ci transmembrane transport mechanism (including HCO_3- and CO_2 transport) in cyanobacterial cells, which enables it to accumulate more than 500-1000 times more extracellular Ci in cells (Harel et al. 2004; Sültemeyer et al. 1989). Regardless of the form of Ci provided by cyanobacteria, it is always accumulated in the cytosol as HCO_3^-. The transport capacity of cyanobacterial inorganic carbon varies with changes in the external environment, especially the concentration of inorganic carbon (Badger et al. 1980).

The CCM of cyanobacteria grown at high CO_2 levels (2-5% CO_2) has a low affinity for Ci, with high apparent K0.5 (CO_2) and K0.5 (HCO_3^-), and intracellular accumulation of Ci the ability is weak. When adapted to the growth environment of restricted CO_2

levels (0.002-0.035% CO_2), its affinity for CO_2 and HCO_3^- increased, intracellular Ci accumulation capacity increased, and the number of carboxyl and Rubisco increased. It is for these reasons that cyanobacterial cells grown in a low CO_2 environment are capable of taking up Ci from a substrate that is almost carbon depleted for photosynthesis.

1.6 Existing research

1.6.1 Research on algae removal methods in countries around the world

The methods of removing algae can be generally divided into two major categories: direct methods and indirect methods.

1) Direct method

Direct methods can be divided into three major categories: chemical methods, physical methods and biological methods. The chemical method is to put in an algicide or to carry out salt treatment. This method is fast and effective, but the possibility of secondary pollution is great. Physical methods include ozone treatment, activated carbon filtration, and artificial physical salvage. This method is effective and costly, and the cost is low. Biological methods are a good method, but they can also cause damage to the biological chain, causing a certain organism to multiply and bring new problems.

2) Indirect method

Mainly refers to the method of removing or reducing the concentration of nutrient salt to control the growth of algae, which can be roughly divided into watershed countermeasures, lake countermeasures and emergency measures.

a. Watershed countermeasures. Mainly to reduce the input of nutrient load in the lake, to prevent the occurrence of eutrophication by controlling the amount of lake pollution sources. In the long run, in order to fundamentally control the eutrophication of water

bodies, it is necessary to reduce the input of nutrients to water bodies. The main measures include sewage interception engineering, wastewater resource utilization, and wastewater phosphorus and nitrogen removal technologies. China's current water pollution is very serious. Reducing the discharge of some wastewater will not change the eutrophication status of the water body, and it is necessary to comprehensively control the source pollution. The investment is very large and will affect the economic development. It is difficult to see the effect in the short term.

b. Countermeasures in the lake. The countermeasures in the lake include two aspects: dredging, removal of organic matter accumulated in the river or in the lake for a long time, and removal of internal pollution sources. After the implementation of the watershed countermeasures to a certain extent, this measure is feasible, but if it is implemented too early, it will definitely bring about a new siltation. The nutrient salt in the water is consumed by various means to reduce the nutrient concentration in the lake water to control the growth of the algae. This method includes purification of aquatic plants, reproduction of benign algae, and the like. The use of this method requires consideration of the recovery of aquatic plants and benign algae in order to prevent decay in water and become a new source of pollution. It should be noted that after the algae in the lake are propagated and recovered, the nutrient salt in the water body cannot be greatly reduced. Taking Taihu Lake in China as an example, the total phosphorus content in Taihu Lake is about 660t, the total amount of phosphorus consumed is about 66t when algae occur once, and the largest area of cyanobacteria in Taihu Lake is about 20% of the total lake area. The best cyanobacteria separation technology Only about 50% can be separated, so that when the algae occurs once, 6.6 tons of phosphorus can be consumed by separation, which is only 1% of the total amount of phosphorus. It can be seen that the total amount of phosphorus excluded by algae separation technology is a drop in the amount of phosphorus in Taihu Lake.

c. Emergency measures in the lake. It mainly includes rectifying membrane, aeration, etc., which is a method of controlling the growth of algae by changing the distribution of nutrient salts by flow. Japan has done a lot of research in this area and found that the method is practical to some extent.

CHAPTER 2

RESEARCH PURPOSES AND OBJECTIVES

2.1 Research purposes

Eutrophication of freshwater system rapidly increasing worldwide during the last few decades. Cyanobacterial blooms are one of the oldest threats in fresh, brackish and marine water which decrease light penetration through the water, cause the depletion of dissolved oxygen that causes mortality of aquatic life. Cyanobacteria are known as oxygenic autotrophs constituting the largest and most diverse communities of photosynthetic prokaryotes which plays an imperative role in carbon and nutrient cycling in aquatic systems as well as it degrades water quality, safety and ecosystem management from last few decades. From the environmental aspect, cyanobacterial blooms and its effects have been reported in the scientific literatures for more than a century, and the probability and severity of the concern have escalated in recent decades. Regardless the toxicity, mass development of cyanobacteria gives rise to negative consequences of eutrophication, ecosystem imbalances and scenic impairments. In addition, with regards to the interests of human and animal health, the ability of producing toxic secondary metabolites becomes an environmental issue and big challenge in environmental management.

Microcystis aeruginosa and green algae are the planktonic cyanobacteria that form water blooms, and also is a bloom-forming species, posing serious social and ecological hazards and these two algae often appear in the same water bloom. Responsible for the synthesis of secondary toxic metabolites, known as microcystins, which affect water safety and thereby human health. *P. galeata* is a non-bloom-forming species yet forms odorous 2-methylisoborneol (2-MIB), causing operational issues with water supply. *P. ambiguum* is a non-heterocystous filamentous cyanobacterial species categorized as a benthic cyanobacterium. In addition to these concerns that arise with these species, the worldwide occurrence has increased as eutrophication and global warming together provide suitable conditions for vigorous growth, so in-depth study of the environmental

conditions of the growth of is important to reveal the mechanism and prevention of bloom.

With the increasing frequency of cyanobacteria blooms, various control measures are being applied to control their growth. chemical methods, is fast and effective, but the possibility of secondary pollution is great, physical methods is effective and costly, Biological methods sometimes they can also cause damage to the biological chain, causing a certain organism to multiply and bring new problems. Also mixing of water in lakes or reservoirs mainly focus on exposing cyanobacteria to low light. However, the photosynthetic organisms of cyanobacteria can be stressed by high light exposure and the excess energy produces reactive oxygen species (ROS), leading to severe photodamage of cellular components. leading to severe photodamage of cellular components. These studies provide information about adaptation capacities of cyanobacteria blooms for helping to predict their occurrence under various environmental conditions. The high-light intensity at which growth is lowest could be used to develop a mechanism or improve the present methods, which are based on low light exposure, to control cyanobacteria effectively in water bodies.

2.2 Research objective

The objectives of the present research are to,

1. Elucidate the cyanobacteria growth, stress response and their relationships with different light intensities.

2. Investigate the critical light conditions which cyanobacteria antioxidant capacity reduced, and adoptability of highlight induced oxidative stress to suppress the cyanobacterial growth.

To achieve the objectives three main studies were conducted.

STUDY 1 Oxidative stress and antioxidant responses of two cyanobacteria under diurnally varying light conditions.

STUDY 2 Effects of light intensity and exposure period on the growth and stress responses of two cyanobacteria.

STUDY 3 Interaction of plants and cyanobacteria under different light intensities.

CHAPTER 3

OXIDATIVE STRESS AND ANTIOXIDANT RESPONSES OF TWO CYANOBACTERIA SPECIES *PHORMIDIUM AMBIGUUM* AND *MICROCYSTIS AERUGINOSA* UNDER DIURNALLY VARYING LIGHT CONDITION

3.1 Introduction

The growth and spread of cyanobacteria have increased, thus threatening today's water bodies and supplies worldwide (Pham and Utsumi 2018; Trolle et al. 2019). Global warming and abundant nutrition supply have promoted the spread of cyanobacteria, which, among others, generate bad odors by producing substances such as 2-methylisoborneol, releasing cyanotoxins, and forming blooms, thus making many water bodies unusable (Butakova 2013; Izaguirre et al. 1982; Kakimoto et al. 2014). In addition, some cyanobacterial species can produce allelochemicals that are harmful to other aquatic species (Ghadouani, Pinel-Alloul, and Prepas 2003; Monserrat, Yunes, and Bianchini 2001; Pflugmacher 2004). Therefore, numerous studies have focused on suppressing or preventing their growth, globally (Paerl et al. 2011; Rajasekhar et al. 2012; Rodriguez-Molares et al. 2014).

During cyanobacteria-control efforts, chemical-control measures are discouraged due to their potentially harmful secondary effects on ecosystems (Grandgirard et al. 2002; Jančula and MarŠálek 2011; Lake et al. 2003), while non-chemical methods require knowledge of the interactions of cyanobacteria with the natural environment, their responses to changing environmental factors or stresses, and their interaction with other species (allopathy). Currently, this approach is being extensively studies by various research groups). In addition, many studies have focused on the physiology and morphology of cyanobacteria under natural and laboratory-derived conditions (Dillon et al. 2002; Dobretsov et al. 2011). Despite those findings, however, knowledge gaps remain to be filled.

With the increasing frequency of cyanobacteria blooms, various control measures are being applied to control their growth Biological, chemical, and physical factors collectively determine the occurrence and distribution of cyanobacteria in the

environment (Celeste el at. 2013; Paerl et al. 2001; Watson 2003). Physical factors, such as the temperature and light influence the growth and distribution of cyanobacteria (Paerl 2014; Rastogi, Madamwar, and Incharoensakdi 2015). To establish a presence in an ecosystem, cyanobacteria require temperature and light-intensity conditions within suitable ranges, which can vary between cyanobacterial species (Briand et al. 2004; Paerl 2014; Rucker et al. 2009). Under preferable environmental conditions for most cyanobacteria, such as tropical and subtropical conditions, temperature factors do not show significant diurnal variation (Qi et al. 2018). However, the light conditions certainly change, regardless of the geological location. In natural ecosystems, the sunlight typically peaks at approximately noon-hour and then gradually decreases following that point into the evening. This light cycle also affect the physiological conditions of cyanobacteria in a diurnal manner (Saha et al. 2016), and extreme light conditions (high or low) can be disadvantageous for cyanobacteria.

The photosynthetic species produce reactive oxygen species (ROS) as a byproduct of photosynthesis process which is harmful when accumulated in cells (Anthony, Warczak, and Donohue 2005). Therefore a balance between ROS generation and antioxidant activities is required to maintain cell homeostasis (Choudhury et al. 2013). Unfavorable conditions, including excess light, can disturb this balance, leading to oxidative stress (Latifi, Ruiz, and Zhang 2009; Szymańska et al. 2017). When the solar eradiation is varying diurnally, the production of ROS in cyanobacteria then varies, and the antioxidants balance should therefore be adjusted accordingly. Both ROS production and antioxidant activities of cyanobacteria under diurnally varying light conditions have yet to be fully elucidated. This will deepen our understanding of the diurnal variation of cyanobacteria physiology and helps to determine the effective time scale to apply cyanobacteria control measures such as bubbling and mixing which light is the primary factor (Visser et al. 2016).

In this study, the oxidative stress (H_2O_2) and antioxidants (guaiacol peroxidase [GPX], catalase [CAT], ascorbic peroxidase [APX], and superoxidase dismutase [SOD]) responses of cyanobacteria to diurnal changes in the light intensity were studied. Two photosynthetically active radiation (PAR) levels, 300 and 600 $\mu mol \bullet m^{-2} \bullet s^{-1}$, were selected as maximum light intensities, and the responses of two cyanobacterial species, *Phormidium ambiguum* and *Microcystis aeruginosa*, were tested for gradually varying light conditions. *P. ambiguum* is a non-heterocystous filamentous cyanobacterial species categorized as a benthic cyanobacterium (Berrendero et al. 2016) while *M. aeruginosa* is a floating (buoyant) and colony foaming type cyanobacterium (Xiao, Li, and Reynolds 2018). Both cyanobacterial species used in this study are known for their harmful environmental effects, due to their cytotoxin release, as well as their increasing growth in tropical and subtropical water bodies (Princiotta, Hendricks, and White 2019; Teneva et al. 2005).

3.2 Materials and Methods

3.2.1 Cyanobacteria cultures and incubation

P. ambiguum (strain NIES 2119) and *M. aeruginosa* (strain NIES 111) were obtained from the National Institute for Environmental Studies (Japan). Both species were cultured for 14 days at 20°C under a 12 h:12 h light–dark cycle inside an incubator (MIR-254, Sanyo, Tokyo, Japan). Light was provided with cool white fluorescent lamps, and the light intensity was maintained at 20–30 $\mu mol \bullet m^{-2} \bullet s^{-1}$ PAR intensity. The nutrient medium was 100% BG-11 (Rippka, Deruelles, and Waterbury 1979). During the incubation period, each culture was manually shaken three times per day during the light phase.

Table 3-1: BG11 nutrient composition

Macronutrients	Mg/L
$NaNO_3$	1500.0
K_2hpo4	40.00
$MgSO_4.7H_2O$	75.00
$CaCL_{2.2}H_2O$	36.00
Citric acid	6.00
Na_2CO_3	20.00
Na_2EDTA	1.00
Ferric ammonium citrate	6.00

Micronutrients	Mg/L
H_3BO_3	2.86
$MnCL_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.222
$Na_2MoO_4.2H_2O$	0.39
$CuSO_4.5H_2O$	0.079
$Co(NO_3)_2.6H_2O$	0.0494

3.2.2 Experimental setup and procedure

Following the 14-day incubation, 3 replicate conical flasks (500 ml Pyrex clear glass conical flasks) from each of the *P. ambiguum* and *M. aeruginosa* cyanobacteria cultures were made maintaining the 0.6 ± 0.02 optical density measured at 730 nm (OD_{730}) using a spectrophotometer (UVmini-1240, Shimadzu, Kyoto, Japan). The dilution of the cyanobacteria culture was accomplished with BG11 nutrient medium. In all experiments, the temperature was maintained at 20 °C in an incubator, whereas the lighting conditions changed from 0 µmol•m^{-2}•s^{-1} (at 06:00 h) to 300 or 600 µmol•m^{-2}•s^{-1} (at 12:00 h) through changing the lighting intensity by ~25 or ~50 µmol•m^{-2}•s^{-1} at every 30 min with a VBP-L24-C2 light (Insight, Valore, Japan). Then, the light intensity was decreased at the same rate until 18:00 h. The lighting condition was controlled with warm light-emitting diode panel lights, and the light intensity was measured using a quantum flux meter (Apogee, MQ-200, USA). Cyanobacteria samples from each flask were collected for analysis every 3h, at 6:00, 9:00, 12:00, 15:00, 18:00, and 21:00 h. To facilitate mixing, each flask was manually shaken at the time of sampling.

3.2.3 H_2O_2 concentration

Cellular H_2O_2 contents were estimated according to standard methods (Jana and Choudhuri 1984). Briefly, 1 ml was collected from each flask, and the supernatants were removed by centrifugation at 10,000 × *g* for 10 min at 4 °C. The cell pellets were

washed once with ultrapure water (Milli-Q direct 5). To extract cellular H_2O_2, cells pellets were homogenized in 1 ml of 0.1 M pH 6.5 phosphate buffer and centrifuged at 10,000 × *g* for 10 min at 4 °C. Then, 750 µl of 1% titanium chloride in 20% H_2SO_4 (v/v) was added to initiate the reaction. The optical absorption was measured at 410 nm using a spectrophotometer (UVmini-1240) following centrifugation (10,000 × *g* for 5 min) at room temperature (25 ± 2°C). The H_2O_2 concentration was determined using a standard curve prepared using a series of samples with known H_2O_2 concentration.

3.2.4 GPX-activity assay

The GPX activity was assayed as described by Hoda et al. (Senousy et al. 2020) and MacAdam et al. (MacAdam et al. 1992) with modifications. Cyanobacteria cells were harvested by centrifuging 1 ml samples at 10,000 × *g* at 4 °C for 10 min and removing the supernatant and cell pellets were homogenized in 1 ml potassium phosphate buffer (100 mM, pH 7.0). Then 65 µl of enzyme extract was mixed with 920µl of potassium phosphate buffer (100 mM, pH 7) containing 20 mM guaiacol. Then with the addition of 15µl of 0.6% H_2O_2, the reaction was started, and the absorbance change was recorded at 470 nm every 10 s for 3 min using UV mini-1240. GPX activity was calculated using an extinction coefficient of 26.6 mM/cm.

3.2.5 CAT-activity assay

CAT activity was measured using the method described by Aebi (Aebi 1984). One milliliter of each culture was centrifuged at 10,000 × *g* at 4 °C for 10 min, the supernatant was removed, and the cell pellets were homogenized in 1 ml potassium phosphate buffer (50 mM, pH 7.0) containing 0.1 mM EDTA. After centrifuging again (10,000 × *g* at 4 °C for 10 min), the supernatant was collected as the enzyme extract. The CAT activity was measured by reacting 15 µl of 750 mM H_2O_2, 920 µl of potassium phosphate buffer, and 65 µl of extract supernatant. Optical absorption was measured at 240 nm using UV mini-1240. The measurements were recorded every 10

s for 3 min, and the CAT activity was calculated using an extinction coefficient of 39.4 mM/cm.

3.2.6 APX-activity assay

APX activity was assayed as described by Nakano and Asada (Nakano and Asada 1981). The decrease in absorbance at 290 nm was recorded every 10 s for 3 min using UV mini-1240. Each reaction mixture was performed in a 1-ml volume. Initially, 920 µl of 50 mM phosphate buffer (pH 7.0) containing 5 mM EDTA was mixed with 15 µl of 0.5 mM ascorbic acid. Then, each reaction was started routinely by adding 15 µl of 1 mM H_2O_2. Calculations were performed using a molar extinction coefficient for ascorbate of 2.8 mM/cm.

3.2.7 SOD-activity assay

SOD activities were determined by performing nitro blue tetrazolium (NBT) assays, as described previously (Ewing and Janero 1995). Each sample was mixed with 10 µl of 750 µM NBT, 10 µl of 130 mM methionine, 70 µl of 50 mM phosphate buffer with 100 µM EDTA (pH 7.8), and 10 µl of 20 µM riboflavin solution. The reactions were carried out for 5 min, and the absorbances were recorded at 560 nm using UV mini-1240. Blank reactions were prepared by substituting the sample with an equal volume of 50 mM phosphate buffer (pH 7.8). One unit of SOD activity was defined as the amount of SOD that inhibited the rate of formazan production by 50% at 25°C.

3.2.8 Data Analysis

One-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests were performed to test statistical significance of variations among the means of sample groups. Data was normalized relative to the starting group (06:00h), by dividing the results of each group by the corresponding 06:00 h group for each replicate. Significant differences between experimental groups of *P. ambiguum* and *M. aeruginosa* were

evaluated using Student's t-test assuming equality of variance. Pearson's correlation analysis was used to evaluate correlations between parameters. Statistical analyses were performed by using IBM SPSS Statistics for Windows, Version 25.0. (Armonk, NY, USA: IBM Corp).

3.3 Results

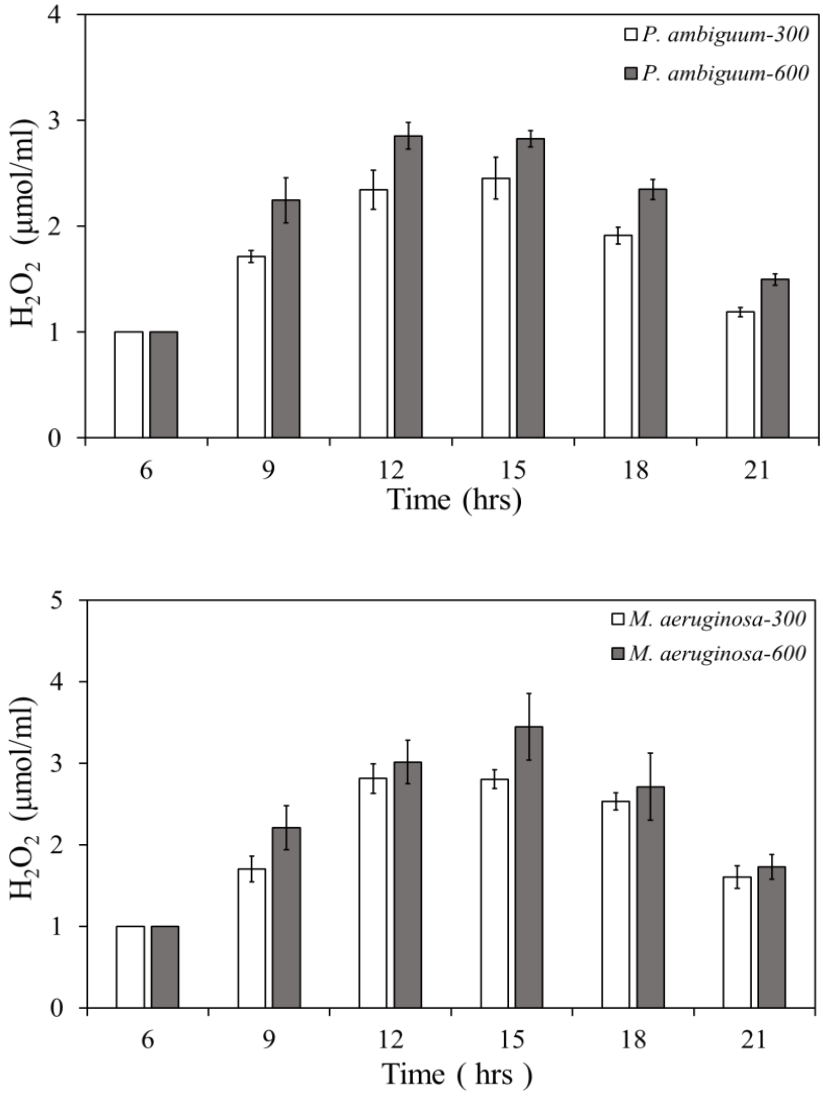


Figure 3-1: Diurnal variations in the H_2O_2 contents of *P. ambiguum (*strain NIES 2119*) and M. aeruginosa (*strain NIES 111*).* The numbers 300 and 600 represent the maximum photosynthetically active radiation (PAR) intensities (in $\mu mol \bullet m^{-2} \bullet s^{-1}$), where the maximum PAR intensity was reached at 12:00 h. The error bars represent the standard deviations.

The cellular H_2O_2 contents of *P. ambiguum* and *M. aeruginosa*, increased with increasing light intensity and peaked between 12:00 h and 15:00 h (Figure 3-1). The H_2O_2 content decreased thereafter in parallel with decreasing light intensity. The cyanobacteria were exposed to dark at 18:00 h; however, even at 21:00 h, the H_2O_2 content had not reached the initial level measured at 06:00 h. ANOVA testing of *P. ambiguum* exposed to a PAR intensity of 300 $\mu mol \bullet m^{-2} \bullet s^{-1}$ groups 12:00 h and 15:00 h, and the rest of the time points were differ individual y ($P < 0.01$, $F = 97.839$). ANOVA testing of *P. ambiguum* exposure to a PAR intensity of 600 $\mu mol \bullet m^{-2} \bullet s^{-1}$ grouped 06:00 h, 09:00, and 18:00 h, and 12:00, 15:00 h, and 21:00 h ($P < 0.01$, $F = 167.265$). ANOVA testing of *M. aeruginosa* revealed grouping at 06:00 h, 09:00 and 21:00 h, 12:00 h and 15:00 h, and 18:00 h ($P < 0.01$, $F = 182. 714$) after exposure to a PAR intensity of 300 $\mu mol \bullet m^{-2} \bullet s^{-1}$. For *M. aeruginosa* exposure to a PAR intensity of 600 $\mu mol \bullet m^{-2} \bullet s^{-1}$, ANOVA testing distinguished significant difference between each time point ($P < 0.01$, $F = 106.817$). Comparing the results obtained after exposure to PAR intensities of 300 and 600 $\mu mol \bullet m^{-2} \bullet s^{-1}$ showed that the H_2O_2 contents of *P. ambiguum* and *M. aeruginosa* differed significantly at each time point ($P < 0.01$ for each time point).

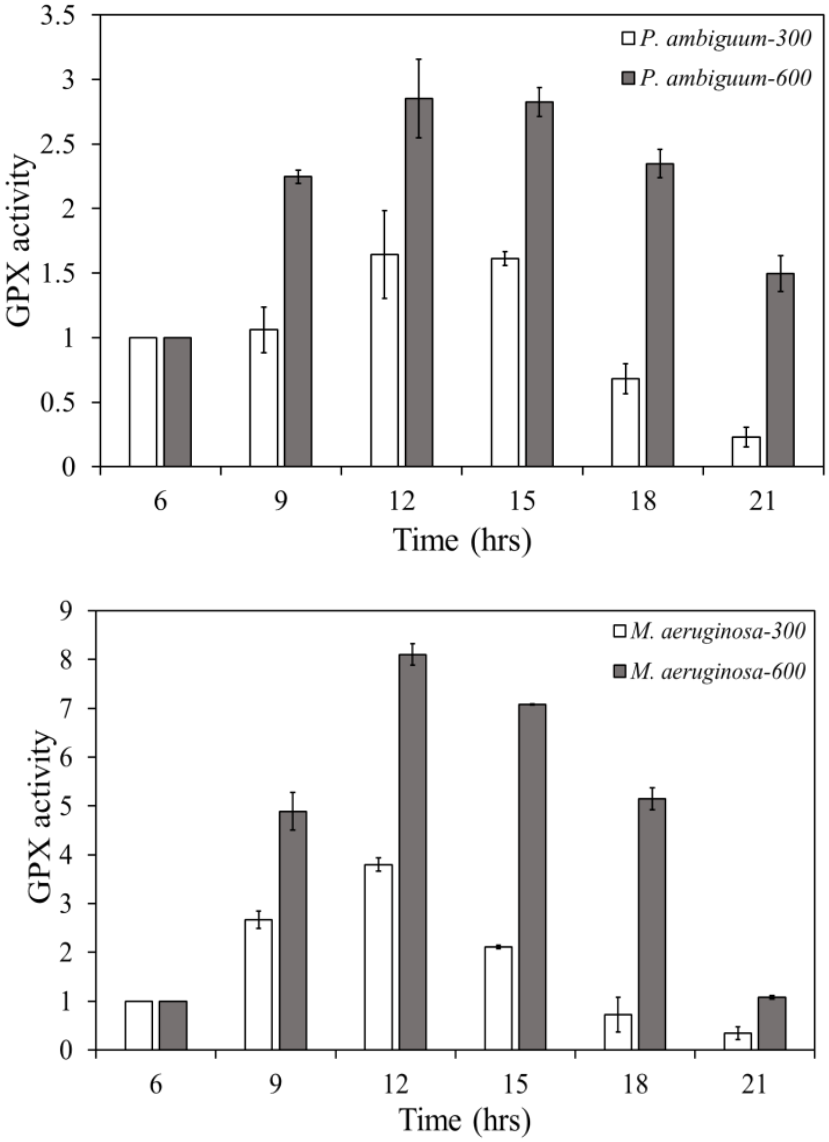


Figure 3-2: Diurnal variations in the guaiacol peroxidase (GPX) activities of *P. ambiguum* (strain NIES 2119) and *M. aeruginosa* (strain NIES 111). The numerals 300 and 600 represent the maximum PAR intensities (in $\mu mol \bullet m^{-2} \bullet s^{-1}$), where the maximum PAR intensity was reached at 12:00 hours. The error bars represent the standard deviations.

The GPX activities of both *P. ambiguum* and *M. aeruginosa* increased over time and reached a maximum after 12 h when the maximum light intensity reached a PAR intensity of 300 or 600 $\mu mol \bullet m^{-2} \bullet s^{-1}$ (Figure 3-2). With decreasing light intensity, the GPX activities of both species decreased for both light intensities. However, with the 300 $\mu mol \bullet m^{-2} \bullet s^{-1}$ PAR-intensity group, under minimum light intensity at 18:00 h and

continued darkness at 21:00 h, the GPX activity decreased even further than the starting GPX activity at 06:00 h. With the 600 μmol•m^{-2}•s^{-1} PAR-intensity group, the GPX activity of both species decreased, but the GPX activity of *P. ambiguum* remained higher than the starting GPX activity. For *M. aeruginosa*, the GPX activity at 21:00 h was the same as the starting GPX activity. For *P. ambiguum*, ANOVA testing of the 300 μmol•m^{-2}•s^{-1} PAR-intensity group revealed statistical differences at 06:00, 09:00 and 15:00 h, 12:00 h, 15:00 and 18:00 h, and 21:00 h ($P < 0.01$, $F = 16.945$). For *P. ambiguum*, ANOVA testing of the 600 μmol•m^{-2}•s^{-1} PAR-intensity group revealed significant differences at 06:00 and 09:00 h, 12:00 and 15:00 h, 18:00 h, and 21:00 h ($P < 0.01$, $F = 35.562$). ANOVA testing of *M. aeruginosa* revealed that the GPX activities following exposure to a PAR intensity of 300 μmol•m^{-2}•s^{-1} differed significantly at 06:00, 18:00 and 21:00 h, 12:00 h, and 09:00 and 15:00 h ($P < 0.01$, $F = 18.050$). For *P. ambiguum*, ANOVA testing of the 600 μmol•m^{-2}•s^{-1} PAR-intensity group revealed significant differences at 06:00 and 21:00 h, 06:00, 09:00 and 18:00 h, 09:00, 15:00 and 18:00 h, and 12:00 h ($P < 0.01$, $F = 13.418$). Comparison between groups exposed to PAR intensities of 300 and 600 μmol•m^{-2}•s^{-1} showed that the GPX activities of *P. ambiguum* and *M. aeruginosa* differed significantly at each time point ($P < 0.01$ for each time point).

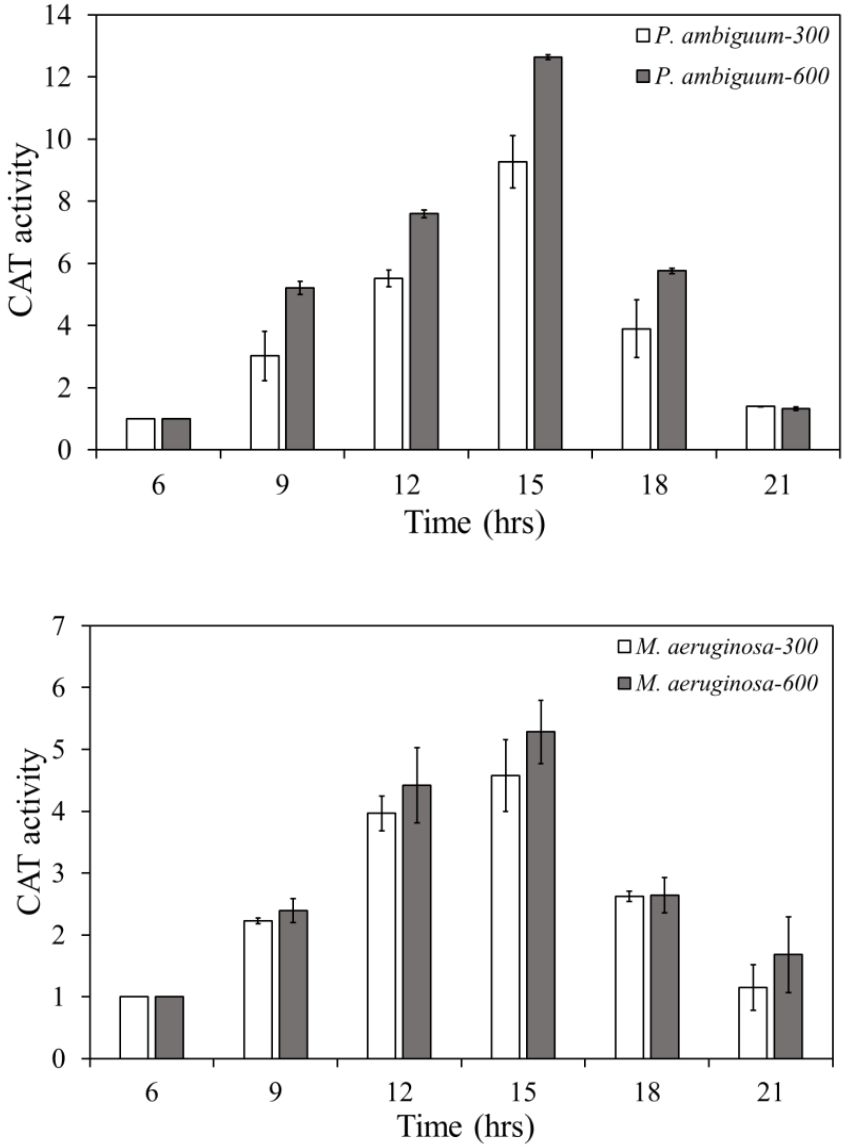


Figure 3-3: Diurnal variations in the catalase (CAT) activities of *P. ambiguum* (strain NIES 2119) and *M. aeruginosa* (strain NIES 111). The numerals 300 and 600 represent the maximum PAR intensities (in $\mu mol \bullet m^{-2} \bullet s^{-1}$) for two different treatment conditions, where the maximum PAR intensity was reached at 12:00 h. The error bars represent the standard deviations.

The CAT activities of both *P. ambiguum* and *M. aeruginosa* increased over time but showed a delayed response as the maximum CAT activities were reached at 15 h (which is 3 h after the maximum light intensities of 300 or 600 $\mu mol \bullet m^{-2} \bullet s^{-1}$ PAR intensities were reached), as shown in Figure 3-3. Decreasing light intensities were paralleled by reduced CAT activities, although the CAT activity did not reach the initial CAT level,

even at 21:00 h. For *P. ambiguum*, ANOVA testing of the 300 µmol•m^{-2}•s^{-1} PAR-intensity grouped CAT activities at 06:00, 09:00, and 21:00 h, 09:00, 18:00 and 21:00 h, 12:00 and 15:00 h ($P < 0.01$, $F = 20.489$). For *P. ambiguum*, ANOVA testing of the 600 µmol•m^{-2}•s^{-1} PAR-intensity grouped at 06:00 and 21:00 h, 09:00, 12:00 and 18:00 h, and 15:00 h ($P < 0.01$, $F = 41.935$). ANOVA testing for *M. aeruginosa* showed that the CAT activities of 300 µmol•m^{-2}•s^{-1} PAR intensity were grouped at 06:00 and 21:00 h, 9:00, and 21:00 h, 09:00 and 18:00 h, and 12:00 and 15:00 h ($P < 0.01$, $F = 24.520$). The ANOVA testing for the 600 µmol•m^{-2}•s^{-1} PAR-intensity grouped CAT activities at 06:00 and 21:00 h, 09:00 and 18:00 h, 12:00 h, and 15:00 h ($P < 0.01$, $F = 35.619$). Comparisons between the 300 and 600 µmol•m^{-2}•s^{-1} PAR-intensity groups showed that the CAT activities of *P. ambiguum* differed significantly at 09:00, 12:00, 15:00, and 18:00 h ($P < 0.01$), although the CAT activities at 06:00 and 21:00 h were not different ($P > 0.05$). The CAT activities between the 300 and 600 µmol•m^{-2}•s^{-1} PAR-intensity groups of *M. aeruginosa* also differed at 09:00, 15:00, and 21:00 h ($P < 0.05$).

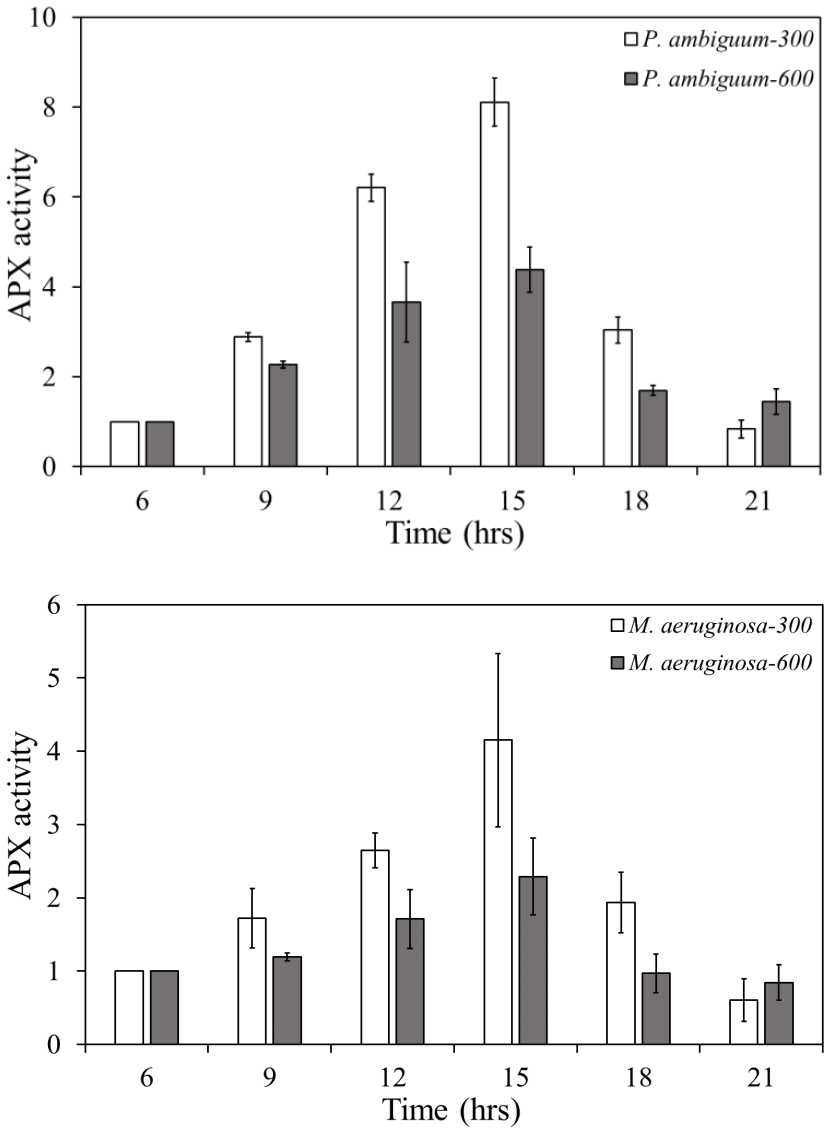


Figure 3-4: Diurnal variations in the ascorbic peroxidase (APX) activities of *of P. ambiguum* (strain NIES 2119) *and M. aeruginosa* (strain NIES 111). The numerals 300 and 600 represent the maximum PAR intensities (µmol•m^{-2}•s^{-1}), where the maximum PAR intensity was reached at 12:00 h. The error bars represent the standard deviations.

The APX activities of both *P. ambiguum* and *M. aeruginosa* increased with increasing light intensity, but delayed response time was observed (as found with the CAT activities), where the maximum APX activity was reached at 15:00 h (3 h after the maximum light intensities were reached, i.e., PAR intensities of 300 or 600 µmol•m^{-2}•s^{-1}). With subsequent decreasing light intensity, the APX activities of both species

decreased and reached the initial APX activity at 21:00 h. For *P. ambiguum* exposed to a PAR intensity of 300 μmol•m^{-2}•s^{-1}, ANOVA testing grouped APX activities at 06:00 and 21:00 h, 09:00 and 18:00 h, and 12:00 and 18:00 h ($P < 0.01$, $F = 35.599$). For *P. ambiguum* exposed to a PAR intensity of 600 μmol•m^{-2}•s^{-1}, ANOVA testing grouped APX activity at 06:00, 18:00 and 21:00 h, 09:00, 18:00 and 21:00 h, and 12:00 and 15:00 h ($P < 0.01$, $F = 15.069$). ANOVA testing for *M. aeruginosa* exposed to a PAR intensity of 300 μmol•m^{-2}•s^{-1} grouped APX activities at 06:00, 18:00 and 21:00 h, 06:00, 09:00 and 15:00 h, and 12:00 h ($P < 0.01$, $F = 18.050$). For *M. aeruginosa* exposed to a PAR intensity of 600 μmol•m^{-2}•s^{-1}, ANOVA testing groped APX activities at 06:00 and 21:00 h, 06:00, 09:00 and 18:00 h, 09:00, 15:00 and 18:00 h, and 12:00 h ($P < 0.01$, $F = 13.418$). Comparisons between the 300 and 600 μmol•m^{-2}•s^{-1} PAR-intensity groups showed that the differences in APX activities for both species were significantly higher in the 300 μmol•m^{-2}•s^{-1} PAR-intensity groups from 09:00 to 18:00 h ($P < 0.01$ for each light condition for both species). The 21:00 h APX activities of *P. ambiguum* were significantly lower in the 300 μmol•m^{-2}•s^{-1} PAR-intensity group than in the 600 μmol•m^{-2}•s^{-1} PAR-intensity group ($P < 0.01$), although no differences were observed for *M. aeruginosa* (Figure 3-4).

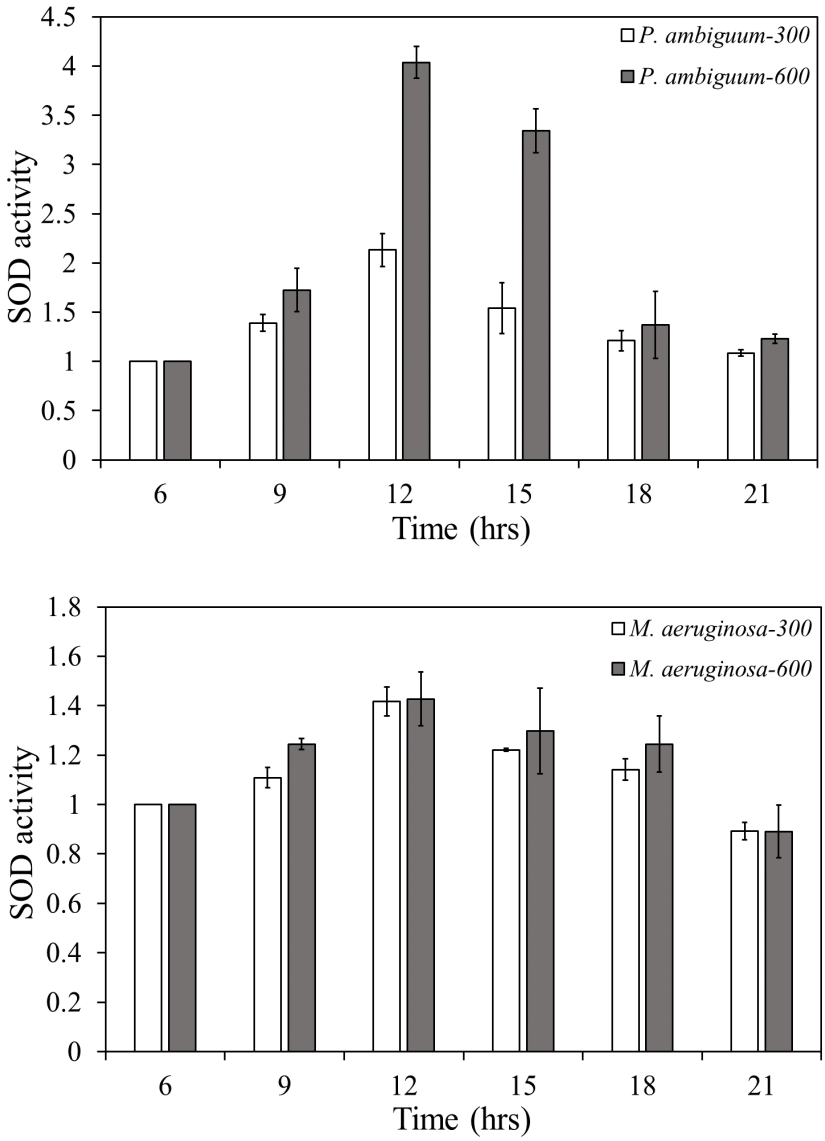


Figure 3-5： Diurnal variations in the super oxidase dismutase (SOD) activities of P. ambiguum (strain NIES 2119) and *M. aeruginosa* (strain NIES 111). The numerals 300 and 600 represent the maximum PAR intensities (in μmol•m^{-2}•s^{-1}) for two different treatment conditions, where the maximum PAR intensity was reached at 12:00 h. The error bars represent the standard deviations.

The SOD activities of both *P. ambiguum* and *M. aeruginosa* increased with the light intensity (Figure 3-5). With decreasing light, the SOD activity decreased for both species and approached the starting level at 21:00 h, for both 300 and 600 μmol•m^{-2}•s^{-1} PAR conditions. For P. ambiguum exposed to a PAR intensity of 300 μmol•m^{-2}•s^{-1}, ANOVA testing grouped SOD activities at 06:00, 18:00 and 21:00 h, 09:00 and 15:00 h, and 12:00 h ($P < 0.01$, $F = 30.725$). For P. ambiguum exposed to a PAR intensity of

600 µmol•m^{-2}•s^{-1}, ANOVA testing grouped SOD activities at 06:00, 18:00 and 21:00 h, 09:00, 18:00 and 21:00 h, 12:00 h, and 15:00 h ($P < 0.01$, $F = 65.914$). ANOVA testing for *M. aeruginosa* showed that the 300 µmol•m^{-2}•s^{-1} PAR-intensity grouped SOD activities at 06:00 and 21:00 h, 06:00 and 09:00 h, 09:00, 15:00 and 18:00 h, and 12:00, 15:00 and 18:00 h ($P < 0.01$, $F = 10.859$). For *M. aeruginosa* exposed to a PAR intensity of 600 µmol•m^{-2}•s^{-1}, ANOVA testing grouped SOD activities at 06:00 and 21:00 h, 06:00, 09:00, 15:00 and 18:00 h, and 09:00 and 12:00 h ($P < 0.01$, $F = 7.313$). Comparisons between the 300 and 600 µmol•m^{-2}•s^{-1} PAR-intensity groups indicated that the SOD activities of *M. aeruginosa* differed at 12:00 and 15:00 h ($P < 0.01$) and that *M. aeruginosa* showed no significant differences between 300 and 600 µmol•m^{-2}•s^{-1} PAR-intensity conditions.

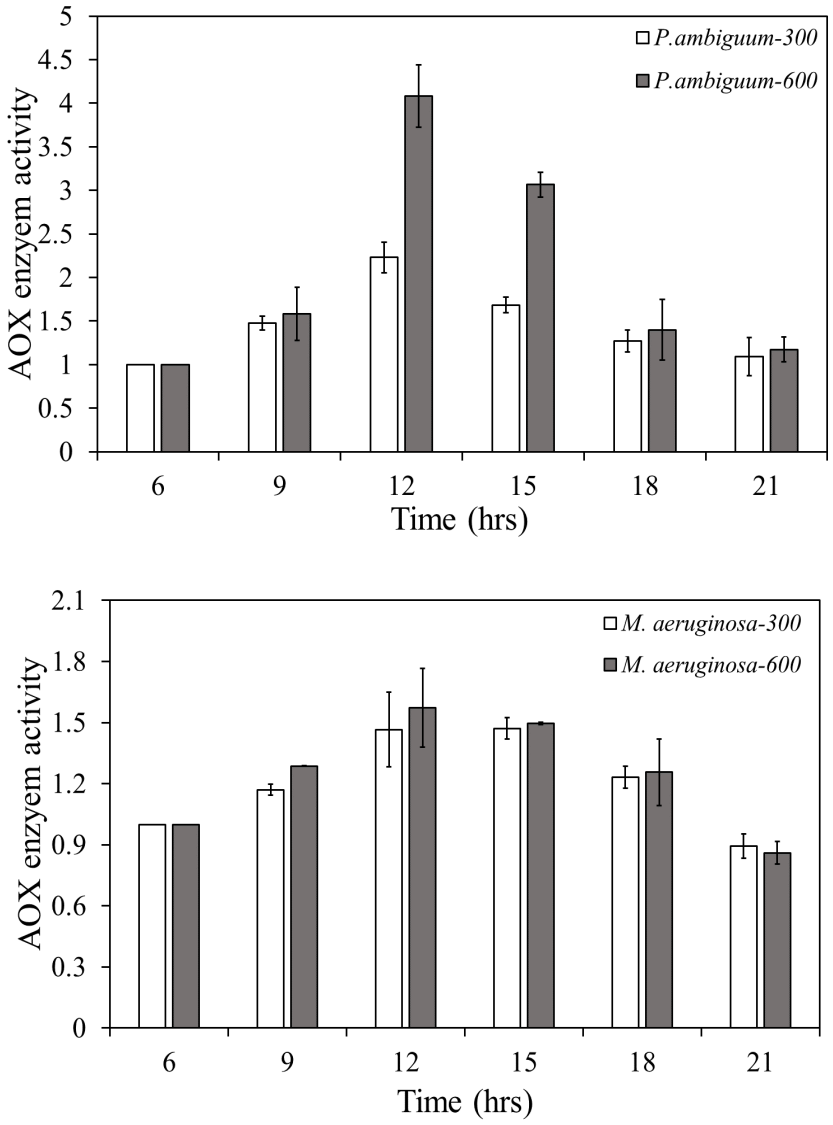


Figure 3-6: Diurnal variations in the total antioxidant (AOX) activity of *P. ambiguum* (strain NIES 2119) and *M. aeruginosa* (strain NIES 111). The numerals 300 and 600 represent the maximum PAR intensities (in $\mu mol \bullet m^{-2} \bullet s^{-1}$) for two different treatment conditions, where the maximum PAR intensity was reached at 12:00 h. The error bars represent the standard deviations.

The total antioxidant (AOX) activities of both *P. ambiguum* and *M. aeruginosa* increased with the light intensity (Figure 3-6). With decreasing light, the AOX activity decreased, and for both species, the AOX activity approached the starting level at 21:00 h, under both the 300 and 600 $\mu mol \bullet m^{-2} \bullet s^{-1}$ PAR conditions. Interestingly the AOX activities following exposure to a PAR of 300 or 600 $\mu mol \bullet m^{-2} \bullet s^{-1}$ differed significantly at 12:00 h and 15:00 h, with *P. ambiguum* while those for M. aeruginosa only exhibited

a significant difference at 09:00 h ($P < 0.01$). In other cases, no significant differences were observed. ANOVA testing of *P. ambiguum* exposed to a PAR intensity of 300 $\mu mol \bullet m^{-2} \bullet s^{-1}$ grouped AOX activities at 06:00 and 21:00 h, 18:00 and 21:00 h, 09:00 and 15:00 h, and 12:00 h ($P < 0.01$, $F = 41.711$). For *P. ambiguum*, ANOVA testing after exposure to PAR intensity of 600 $\mu mol \bullet m^{-2} \bullet s^{-1}$ grouped AOX activities at 06:00, 18:00 and 21:00 h, 09:00, 18:00 and 21:00 h, 15:00 h, and 12:00 h ($P < 0.01$, $F = 79.973$). ANOVA testing for *M. aeruginosa* after exposure to a PAR intensity of 300 $\mu mol \bullet m^{-2} \bullet s^{-1}$ grouped AOX activities at 06:00 and 21:00 h, 09:00 and 18:00 h, and 12:00 and 15:00 h ($P < 0.01$, $F = 26.143$). For M. aeruginosa exposed to 600 $\mu mol \bullet m^{-2} \bullet s^{-1}$ PAR intensity, ANOVA grouped at 06:00 and 21:00 h, 06:00 and 18:00 h, 09:00, 15:00 and 18:00 h, and 12:00 and 15:00 h ($P < 0.01$, $F = 12.466$).

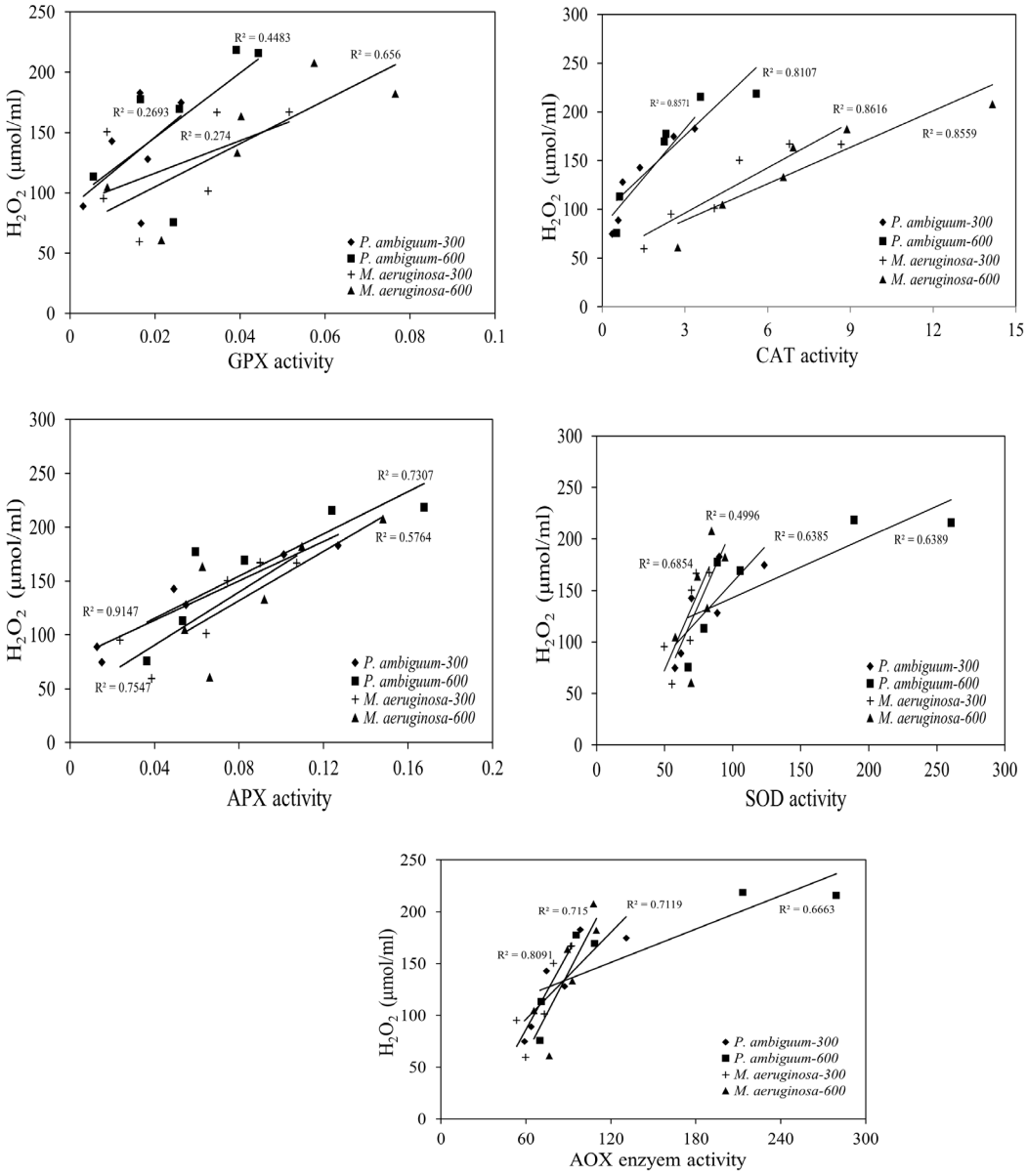


Figure 3-7: Linear-regression relationships between the H_2O_2 contents and the antioxidant activities (GPX, CAT, APX, SOD, and AOX) of *P. ambiguum* (strain NIES 2119) and *M. aeruginosa* (strain NIES 111). The numerals 300 and 600 represent the maximum PAR intensities (in $\mu mol \bullet m^{-2} \bullet s^{-1}$) for two different treatment conditions, where the maximum PAR intensity was reached at 12:00 h.

The relationships between the H_2O_2 levels and those of the antioxidants (CAT, APX, GPX, and SOD) and the AOX levels were significantly linearly correlated, with the exception of the GPX activity of *P. ambiguum* under the illumination of 300 $\mu mol \bullet m^{-2} \bullet s^{-1}$ (Figure 3-7). Pearson's correlation test results are presented in Table 3-2. Among the antioxidants tested, GPX consistently showed low R^2 values and exhibited higher

variance. Out of all antioxidants tested, the CAT activity showed the highest R^2 values (>0.75), confirming that less variance occurred. When the AOX values were considered, the R^2 showed relatively higher values (>over 0.72).

Table 3-2: Pearson's correlation test results of the correlation between H_2O_2 and antioxidant levels, i.e., guaiacol peroxidase (GPX), catalase (CAT), ascorbic peroxidase (APX), super oxidase dismutase (SOD), and total antioxidants (AOX)

Condition	Parameter	R^2	P value
M. aeruginosa – 300 [1]	SOD	0.780	P < 0.01
	APX	0.652	P < 0.01
	CAT	0.893	P < 0.01
	GPX	0.539	P < 0.05
	AOX	0.856	P < 0.01
M. aeruginosa - 600	SOD	0.526	P < 0.05
	APX	0.683	P < 0.01
	CAT	0.924	P < 0.01
	GPX	0.692	P < 0.01
	AOX	0.720	P < 0.01
P. ambiguum - 300	SOD	0.748	P < 0.01
	APX	0.962	P < 0.01
	CAT	0.824	P < 0.01
	GPX	0.383	P > 0.05
	AOX	0.803	P < 0.01
P. ambiguum - 600	SOD	0.784	P < 0.01
	APX	0.738	P < 0.01
	CAT	0.830	P < 0.01
	GPX	0.624	P < 0.01
	AOX	0.796	P < 0.01

3.4 Discussion

The H_2O_2 contents and the antioxidant activities of *P. ambiguum* and *M. aeruginosa* were highly responsive to the diurnal variations in light intensity. In this study, the only variable factor was the light intensity where H_2O_2 levels were high during times of higher light intensities and decreased at lower light intensities. When cellular H_2O_2 level increases, the antioxidant activities correspondingly increase to prevent damage induced by oxidative stress (Lin and Scott 2012; Pruchniak, Araźna, and Demkc 2016).

As observed with the H_2O_2 levels, the antioxidant activities also varied during the same time frame and followed the H_2O_2 levels, which increased at higher light intensities and decreased at lower light intensities. The antioxidant activities of both species were correlated linearly with the H_2O_2 contents. Although the H_2O_2–antioxidant relationships were varied from strong to weak (depending on the antioxidant species), overall, our findings suggest that the antioxidant levels of both species responded to the cellular H_2O_2 level accordingly.

The H_2O_2 and antioxidant responses followed the same trends for both maximum-light intensity conditions (PAR intensities of 300 or 600 $\mu mol \bullet m^{-2} \bullet s^{-1}$). Under the maximum PAR intensity of 600 $\mu mol \bullet m^{-2} \bullet s^{-1}$, the cyanobacteria received approximately twice the photon energy of the group with a maximum PAR of 300 $\mu mol \bullet m^{-2} \bullet s^{-1}$. Therefore, it can be anticipated that the experiment groups which receive higher photon energy undergo an enhanced rate of photosynthesis. This is evidenced by the increased H_2O_2 formed after exposure to a higher light intensity (Exposito-Rodriguez et al. 2017; Page et al. 2012). However, at higher light intensities where the photon energy exceeds tolerable levels for the photosystem, photoinhibition occurs to prevent photodamage (Allakhverdiev and Murata 2006; Virtanen et al. 2019), during which H_2O_2 production is reduced with higher light exposure (Collén, Pedersén, and Collén 1996; Estervig and Wang 1984; Vanderauwera et al. 2005). As the H_2O_2 contents correlated directly with light intensity, even at higher intensities, PAR intensities under 600 $\mu mol \bullet m^{-2} \bullet s^{-1}$ did not subject either cyanobacterial species to photo stress. However, this study only involved a single day diurnal variation, and the H_2O_2 levels of the cells did not reach the starting H_2O_2 conditions at 06:00 h (even at 21:00 h) for both species. The antioxidant activities almost decreased to the initial conditions by 21:00 h. Therefore, it is possible that cells may undergo oxidative stress during dark conditions due to the lack of antioxidant activities. If the H_2O_2 was continued to be presence in cells, the protein synthesis of photosystems will be inhibited (Latifi et al. 2009) and in long duration, cell function reduced and even cell deaths can be occurred (Brutemark et al.

2015). Therefore, an extended exposure period is required to better understand the fate of the remaining H_2O_2 and adaptation responses.

The antioxidant levels were differed between the two species, where the response level was lower for *M. aeruginosa* than *P. ambiguum*, except for GPX. Under high H_2O_2 contents, the AOX activity was highly elevated in *P. ambiguum*, but in the dark, both species reached the starting AOX activity level at 21:00 h. This finding suggests that, *M. aeruginosa* is less tolerant to oxidative stress than *P. ambiguum* (Foyer and Shigeoka 2011; Latifi et al. 2009). Concerning the correlation between antioxidant responses and H_2O_2 contents, both species demonstrated significant linear relationships (with the only exception being for GPX of *P. ambiguum* under a maximum PAR of 300 $\mu mol \bullet m^{-2} \bullet s^{-1}$). Therefore, despite the high AOX content of the *P. ambiguum* both species were able to maintain balanced antioxidant activity under every light conditions of the single day exposure.

The difference in antioxidant levels of the two species can be related to their behavioral characteristics. The *M. aeruginosa* is a buoyant species which floating in range of depths might have higher tolerance to oxidative stress (Muhetaer et al. 2020; Zevenboom and Mur 1984) than benthic *P. ambiguum*. However, in the present study, both species experienced same light intensity variance as the cultures were mixed periodically. The non-different H_2O_2 contents between the two species suggesting that both species experienced similar level of oxidative stress. Therefore, less oxidative stress tolerance of *P. ambiguum* triggered the antioxidants activity at relatively higher rate. Conversely, nonenzymatic antioxidants, primarily carotenoids, protect against ROS in phototrophs including cyanobacteria (Edge, McGarvey, and Truscott 1997; Latifi et al. 2009). The nonenzymatic antioxidants can neutralize ROS prior to triggering the antioxidant enzymes. However, the carotenoid content is reported to be high in *P. ambiguum* than *M. aeruginosa* (Borase, Dhar, and Singh 2013; Paerl 1984; Sheng et al. 2019); therefore, it is challenging to determine whether the low antioxidant

activity reported in *M. aeruginosa* is due to involvement of nonenzymic antioxidant over the *P. ambiguum*.

Our previous study on the effects of 8 days of exposure to non-varying, high-light intensities (300 and 600 μmol•m^{-2}•s^{-1}) confirmed that the OD_{730} and chlorophyll a contents of cyanobacteria (*Pseudanabaena galeata* and *M. aeruginosa*) were significantly reduced, which was associated with oxidative stress (Muhetaer et al. 2020). Although the present research confirmed the relationships between varying oxidative stress and antioxidant responses with the light intensity, further investigation into the longer-term effects on the growth and pigmentation of cyanobacteria is warranted. Longer exposure duration will help to better understand the growth performance and physiological responses of cyanobacteria to diurnally varying light conditions. Further, there can be a circadian rhythm in the physiology of cyanobacteria which the cellular conditions can be changed diurnally regardless of the prevailing conditions (Golden et al. 1997). In future studies, the circadian rhythm of the cyanobacterial species also should be considered.

CHAPTER 4

EFFECTS OF LIGHT INTENSITY AND EXPOSURE PERIOD ON THE GROWTH AND STRESS RESPONSES OF TWO CYANOBACTERIA SPECIES: *PSEUDANABAENA GALEATA* AND *MICROCYSTIS AERUGUNOSA*

4.1 Introduction

Eutrophication and global warming have promoted cyanobacteria growth in freshwater systems worldwide during the last few decades and it is expected to increase in the future (Trolle et al. 2015) . Cyanobacterial blooms are one of the oldest threats in fresh, brackish and marine water which decrease light penetration through the water, cause the depletion of dissolved oxygen that causes mortality of aquatic life (Wang et al. 2015). Cyanobacteria are known as oxygenic autotrophs constituting the largest and the most diverse communities of photosynthetic prokaryotes (Demoulin et al. 2019). They play an imperative role in carbon and nutrient cycling in aquatic systems as well as they degrade water quality and safety causing numerous difficulties in ecosystem management (Yan et al. 2019). From the environmental aspect, cyanobacterial blooms and its effects have been reported in the scientific literatures for more than a century, and the probability and severity of the concern have escalated in recent decades (Śliwińska-Wilczewska et al. 2019). Further the mass development of cyanobacteria gives rise to negative consequences of eutrophication, ecosystem imbalances and scenic impairments (Mazur-Marzec et al. 2013). In addition, the ability to produce toxic secondary metabolites becomes an increasing environmental issue that challenging not only human health but also animal and plant health leading to ecosystem destruction (Codd 2005;Pearson et al. 2010). Though the problems are such, there is still gaps to be filled in the knowledge on cyanobacteria.

In particular, the knowledge enrichment of the appraisal of environmental factors associated with cyanobacterial growth and metabolism becomes important in addressing real world problems. Different researchers have been documented divergences of cyanobacterial growth responses to environmental predictor variables in comparison to many other freshwater taxa (Paerl and Huisman 2009; O'neil et al. 2012). At the same time, this subject has been extensively mentioned in connection to climate change and anthropogenic activities associated with land use changes are also being experienced worldwide (Huisman et al. 2018; Barros et al. 2019). Among a number of

environmental drivers such as water temperature, water column irradiance, stratified water column coupled with long residence time, availability of N and P, turbidity and salinity of water etc., the light intensity is an important determinant of cyanobacterial blooms growth (Berg and Sutula 2015). The growth patterns of the cyanobacteria respond to different light intensities by morphological and physiological changes (Khatoon et al. 2018) and the detailed investigation of these changes is vital in formulation of both proactive and reactive planning for freshwater resources management.

P. galeata and *M. aeruginosa* are two resounding cyanobacteria species that are known to degrade the water quality and water safety of many parts of the. *P. galeata* is a non-bloom-forming species yet forming odorous 2-methylisoborneol (2-MIB) and it causes operational issues in the water supply (Kakimoto et al. 2014), on the other hand, *M. aeruginosa* is a bloom forming species and also responsible for the synthesis of secondary toxic metabolites as hepatotoxins known as microcystins that also affects the water safety and thereby human health (Dang et al. 2012; Princiotta et al. 2019) with serious social and ecological concerns. Beside these concerns arises with both species, the worldwide occurrence has increased as the eutrophication and global warming together providing suitable conditions for vigorous growth (Glibert 2019; Lürling at al. 2017).

With the increasing frequency of cyanobacteria blooms, various control measures are being applied to control their growth (Visser et al. 2016). Methods such as mixing of water in lakes or reservoirs mainly focus on exposing cyanobacteria to low light. However, the photosynthetic organisms of cyanobacteria can be stressed by high light exposure and the excess energy produces reactive oxygen species (ROS), leading to severe photodamage of cellular components (Beardall 2017; Muramatsu and Hihara 2012). The growth of *Microcystis* and *Anabaena* species under low light levels (25

μmol•m^{-2}•s^{-1}) is high, which decreases under high light levels (200 μmol•m^{-2}•s^{-1}) (Beardall 2017; Venugopal et al. 2006).

Although high light levels can negatively affect cyanobacteria species, its oxidative stress, antioxidant, pigmentation, and protein contents responses and their relationships are not currently well understood. Therefore, we aimed to explore the responses of *P. galeata* and *M. aeruginosa* to low-light and high-light stressors. H_2O_2, antioxidant enzymes (catalase (CAT) and peroxidase (POD), chlorophyll a (Chl-a), protein contents, and optical densities (OD_{730}) were measured under different light conditions. The growth performance of these two species was calculated and validated with cyanobacteria growth models proposed by Steele (Steele 1965), Platt and Jassby (Platt and Jassby 1976), and Peeters and Eilers (Peeters and Eilers 1978) to determine the applicability of findings for cyanobacteria control.

4.2 Materials and Methods

4.2.1 Cyanobacterial cultures and incubation

The cyanobacterial species *P. galeata* and *M. aeruginosa* strains were obtained from the National Institute for Environmental Studies (NIES), Ibaraki, Japan. The samples were cultured in BG-11 medium (Stanier et al. 1979) and acclimatized for 14 days inside an incubator (MIR-254, Sanyo, Japan) at 20 °C with shaking 3 to 5 times manual shaking per day. The samples were cultured under controlled light condition with photon flux level 20 – 30 μmol•m^{-2}•s^{-1} emitted from cool white fluorescent lamps (5000 K color temperature). The cycle of light condition to dark condition was maintained at 12 hours light and 12 hours darkness (Guan et al. 2004) with the use of automatic time set up device (REVEX PT7, Japan).

4.2.2 Growth experimental setup

All the experiments were conducted inside an incubator (MIR-254, Sanyo, Japan) at temperature 20°C constantly throughout the experiment period. To characterize the response of growth to different light condition, incubated *P. galeata* (NIES 512) and *M. aeruginosa* (NIES 111) cells were separately subjected to 7 different photon flux levels as 0, 10, 30, 50, 100, 300 and 600 $\mu mol \bullet m^{-2} \bullet s^{-1}$ and a constant temperature of 20°C were used to calculate saturation light intensities. Light was supplied from cold white fluorescent lamp sources. The cultures were kept in an illumination cycle of 12 hours of light and 12 hours of darkness (12L:12D) respectively. The value 0 $\mu mol \bullet m^{-2} \bullet s^{-1}$ light intensity means there is no light source and the cultures were maintained 24 hours darkness. The light intensities were measured by quantum sensor (ml-020P, EKO instruments Co., LTD, USA) read by voltage logger (LR5041, HIOKI, Japan) as a voltage output (mV). These light conditions were maintained inside the incubator with setting constant temperature 20°C during experiment period. The cells which were cultured under the different light were collected for Chl-a and enzyme analysis for every 2 days interval. To ensure homogenous exposure of cells to the light environment, the cyanobacteria cultured flasks were shaken gently 5 times a day. Each treatment were maintained triplicates.

4.2.2 Measuring Protein

The concentration of protein was measured using the Bradford method (Marion 1976). Crude protein extract was discolored with Coomassie (G-250). After incubation at room temperature (25 ± 2 °C) for 10 min, the absorbance was measured at 595 nm using a UV–vis spectrometer (UVmini-1240, Shimadzu, Kyoto, Japan). Protein was diluted with the same buffer and stained with Coomassie (G-250) dye used to prepare the standard curve and deionized water was used as the blank.

4.2.3 Measuring optical density (OD730)

To estimate the growth of cyanobacteria, optical density (OD_{730}) was measured by taking 1 ml sample from each flask. OD_{730} was measured with a UV-vis spectrophotometer (UVmini-1240, Shimadzu, Japan) at the optical absorption of 730 nm by using the methodology proposed by (Axler and Owen 1994; Association et al. 1915).

4.2.4 Measuring Chlorophyll-a concentration

The concentration of Chlorophyll-a (Chl-a) in cyanobacteria samples were measured according to Holm (Holm 1954; Romo 1994). The concentration of Chl-a in the cyanobacteria samples was measured according to methods followed by Holm and Romo. The 1 mL cell suspensions of the two species was centrifuged separately at 10,000 $\times g$ for 10 min at 4 ℃ and the supernatant was removed. The cell pellet was washed once with Milli-Q water and extracted in 1 mL of 80% acetone. The mixture was shaken vigorously and maintained in darkness overnight at room temperature (25 ± 2 °C). Then, the sample was again centrifuged at 5000 rpm and the supernatant was measured with a UV–vis spectrophotometer (UVmini-1240, Shimadzu, Kyoto, Japan) at absorptions of 660 and 645 nm. To correct the absorbance for pheophytin a, the samples were acidified with 0.1 N HCL and the absorbance was measured again. The chlorophyll a content is calculated by using equation (1).

$$Chl - a = (9.76 \times A_{660}) - (0.99 \times A_{645}) \quad (1)$$

where, $Chl - a$ is chlorophyll a content expressed in µg per mL, A_{660} and A_{645} are absorbance at 660 nm and 645 nm respectively.

4.2.5 Measuring H_2O_2 concentration

The method specified by Jana (Jana and Choudhuri 1984) was employed for measurement of H_2O_2 concentration in the cultured cyanobacteria samples. *P. galeata* (NIES 512) and *M. aeruginosa* (NIES 111) cell pellet were obtained by centrifuging at

10,000 g for 5 min and removing the supernatant. The cell pellets were washed once with Milli-Q water and homogenized in 1 ml of 0.1 M pH 6.5 phosphate buffer to extract internal H_2O_2. Hereafter, the homogenate was centrifuged at 10,000 g for 10 minutes at 4°C and the extract was used for H_2O_2 estimation. A reaction mixture of 0.1% titanium chloride in 20% H_2SO_4 (v/v) was added to the supernatant, and after 1 min incubation period, the mixture was centrifuged at the room temperature (25±2 °C) and the absorbance was measured at 410 nm with UV-vis spectrophotometer (UVmini-1240, Shimadzu, Japan). H_2O_2 concentration was determined by pre-prepared standard curve for known concentration series. The extinction coefficient of 0.28 $mmol^{-1}cm^{-1}$ was used to calculate the concentration of H_2O_2 in µmol ml^{-1}.

4.2.6 Measuring CAT activity

The CAT activity was measured by the method proposed by Aebi (Aebi 1984). The cyanobacteria cells were homogenized in phosphate buffer (pH 7.0), supernatant liquid was taken as enzyme extract after centrifuged at 12000 g at 4°C. The decrease in absorbance at 240 nm was recorded for 3.0 min. The CAT activity was calculated using the extinction coefficient of 39.4 $mM^{-1}cm^{-1}$.

4.2.7 Measuring POD activity

The POD activity was measured based on guaiacol oxidation proposed by MacAdam (MacAdam et al. 1992). The reaction mixture contained 920 µl of 100 mM Potassium phosphate buffer (pH 6.8), 15 µl of 0.6% of H_2O_2 and 65 µl of enzyme extract. The increase in absorbance was measured at 420 nm in every 10 s for 3 min.

4.2.8 Cell growth measurement and different model fitting

The cell growth of cyanobacteria was measured by using optical density (OD_{730}) measurement. The OD_{730} was measured by UV-vis spectrophotometer (UVmini-1240, Shimadzu, Kyoto, Japan).

The cell growth measurements of present study were compared with the three cell growth models proposed for cyanobacteria growth (Steele 1965; Platt and Jassby 1976; Peeters and Eilers 1978). The cell growth of cyanobacteria was measured by using optical density (OD_{730}), was applied to the growth rate equation (1) proposed by (Foy et al. 1976). Then the calculated growth rates were compared with the three model outputs obtained for the different light intensities of present experiment.

$$\mu = \frac{(logOD_t - logOD_0)}{t} \times 3.32 \quad (2)$$

Where, μ is cell growth rate, T is time in days, OD_t is optical density after t days and OD_t is optical density at beginning of the experiment zero time. Different models proposed by Steele, Platt and Jassby and Peeters and Eilers were fitted to our experimental observations (Sabour et al. 2009).

The model proposed by Steele (Steele 1965) named as Model I and is written as:

$$\mu_{T,I} = \mu_{maxT} \times I/I_{optT} \times \exp\left(I - I/I_{optT}\right) \quad (3)$$

Where $\mu_{T,I}$ is growth rate at light intensity *I* and μ_{maxT} and I_{optT} are the estimated maximal growth rates and optimal light intensity at temperature respectively.

The model proposed by Platt and Jassby (Platt and Jassby 1976) named as Model II and is written as:

$$\mu_{T,I} = \mu_{maxT} \times \tanh[\alpha \times (I - I_c)/\mu_{maxT}] \quad (4)$$

Where α represents the growth efficacy, Ic is estimated light intensity without growth (Ic≥0), and tanh is the hyperbolic tangential function.

The model proposed by Peeters and Eilers (Peeters and Eilers 1978) named as Model III and is written as:

$$\mu_{T,I} = 2 \times \mu_{maxT} \times (1 + \beta) \times \left(I/I_{optT}\right) / \left[\left(I/I_{optT}\right)^2 + 2 \times \left(I/I_{optT}\right) \times \beta + 1\right] \quad (4)$$

Where β is the attenuation coefficient that allows to take in to account the photoinhibition phenomenon.

4.2.9 Statistical analysis

All presented results are expressed as the mean ± SD (n=3). one-way analysis of variance (one-way ANOVA) followed by Tukey's post-hoc tests were performed to examine the statistical significance of variations among means between light exposure period and light intensity used for *P. galeata* and *M. aeruginosa*. Statistical analyses were performed by using SPSS statistics 25.0.0.

4.3 Results

The protein concentration of *P. galeata* and *M. aeruginosa* species after 2 and 8 days of exposure to light of different intensities presented in Figure 4-1. After 2 days exposure, protein concentration of each exposure conditions were nearly same as the initial values, 61.6±3.0 for *P.galeata* ($F_{6,14}$ = 1.250, p=0.34) and 54.5±0.70 for *M.aeruginosa* ($F_{6,14}$ = 3.960, p=0.016). However, it substantially increased after 8 days for *P.galeata* ($F_{6,14}$ =32.795, p < 0.0001), increased rapidly until 100 µmol•m^{-2}•s^{-1} intensity and remain increasing at slower rate until 600 µmol•m^{-2}•s^{-1}. For the *M. aeriginosa* ($F_{6,14}$ =34.824, p < 0.0001), the protein concentration was exhibited decreasing trend with the increasing light intensity, which is an almost an opposite trend of *P.galeata*. The protein concentration decreasing rate of *M.aeruginosa* was rapid until 100 µmol µmol•m^{-2}•s^{-1} light intensity and remain decreasing at slower rate until 600 µmol•m^{-2}•s^{-1}.

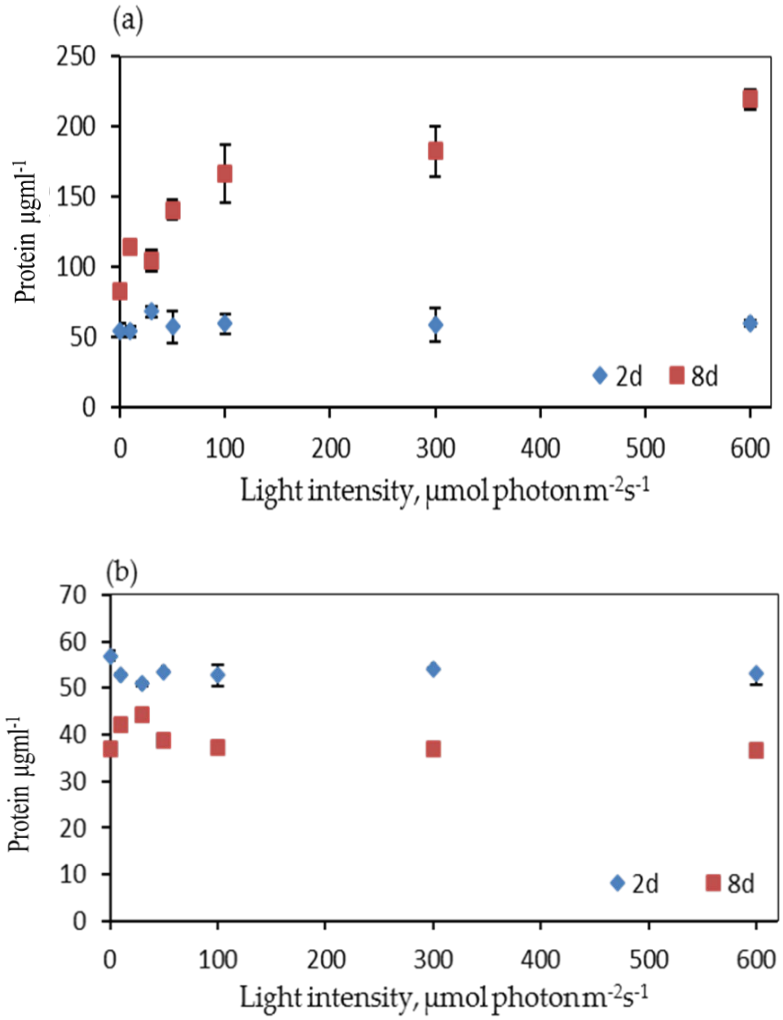


Figure 4-1: Protein concentration in relation to light intensity after 2 and 8 days for two species (a) *P. galeata* and (b) *M. aeruginosa*.

The variation of OD_{730} with respect to light intensity is presented in Figure 4-2. The *P. galeata* and *M. aeruginosa* both showed statistically significant difference between 2 day and 8 day exposure *P. galeata* ($F_{6,28} = 188.811$, $p < 0.0001$) and *M. aeruginosa* ($F_{6,28} = 145.041$, $p < 0.0001$). After 2 days, relatively constant OD_{730} values were observed under all the light intensities for both *P. galeata* and *M. aeruginosa*. The 8 day OD_{730} value of *P. galeata*, increased under 30 and 50 $\mu mol \bullet m^{-2} \bullet s^{-1}$ intensity and then decreased with higher light intensities over the 2 day. However, OD_{730} values after 8 days of *M. aeruginosa* was about double than OD_{730} value of *P. galeata*.

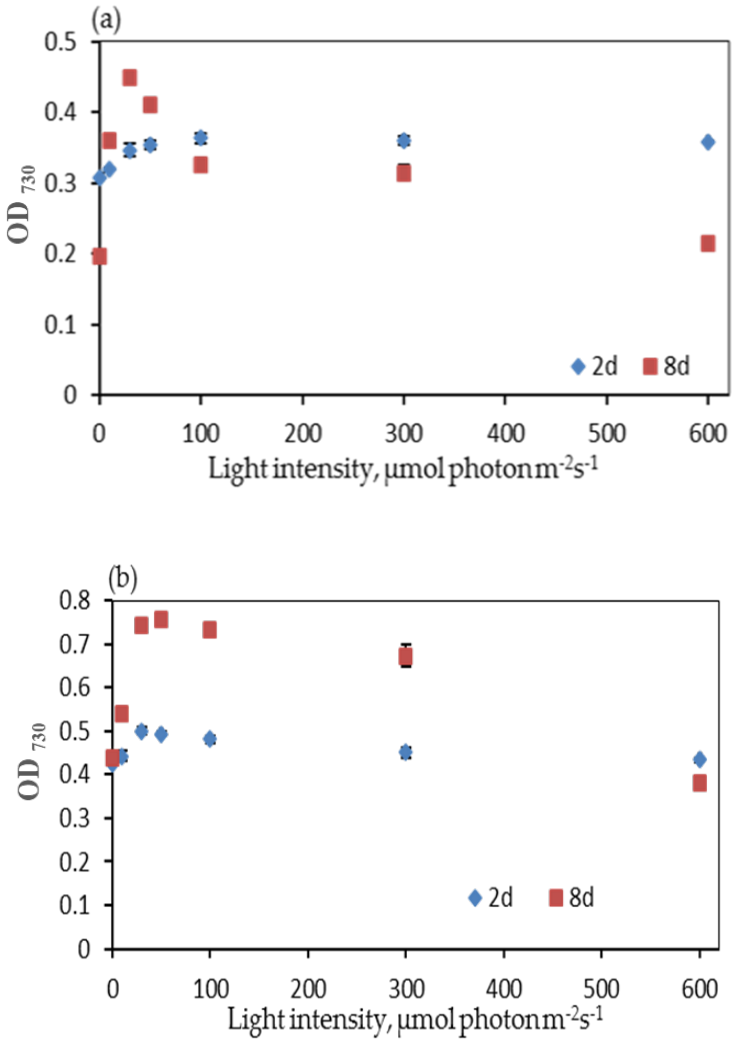


Figure 4-2: Optical density (OD_{730}) in relation to light intensity after 2 and 8 days for two species (a) *P. galeata* and (b) *M. aeruginosa*.

Chl-a concentration under different light intesntites of *P. galeata and M. aeruginosa* is presented in Figure 4-3. The content of Chl-a pigment of *P. galeata* and *M. aeruginosa* showed statistically significant difference after 8 days than 2 days (*P. galeata,* $F_{6,28} = 21.876$, $p < 0.0001$ and *M. aeruginosa,* $F_{6,28} = 9.036$, $p = 0.0001$). The 2day exposure, Chl-a content of both species showed slightly decreasing trend after 50 µmol•m^{-2}•s^{-1}. After 8 days, for *P. galeata*, Chl-a concentration increased at for 30 and 50 µmol•m^{-2}•s^{-1} light intensity however, decreased under high light intensities, than the 2 days. In contrast, after 8 days, Chl-a concentration of *M. aeruginosa*, also followed a same trend with *P. galeata* with different values. Chl-a gradually increased until 50 µmol•m^{-2}•s^{-1} of light intensity and started to degrade under higher light intensities.

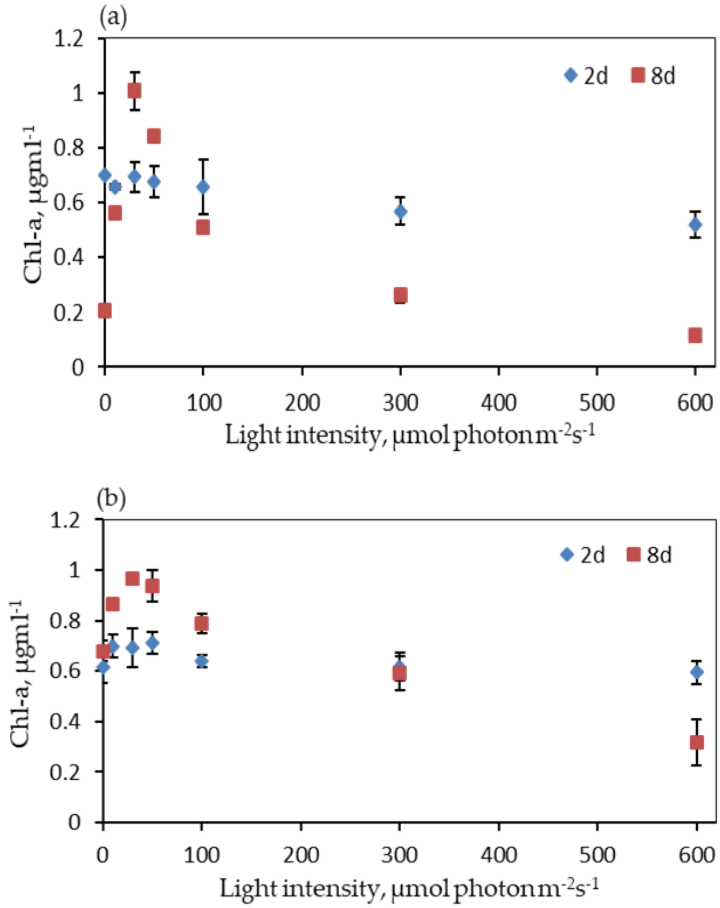


Figure 4-3: Chlorophyll a content in relation to culture light intensity for two species (a) *P.galeata* and (b) *M. aeruginosa* under different light intensity (0 ~ 600 µmol•m^{-2}•s^{-1}) after 2 and 8 days.

The H_2O_2 per protein (H_2O_2/protein) over different light intensities is shown in Figure 4-4. After 2days, *P. galeata* H_2O_2/protein was decreased for 30 and 50 µmol•m^{-2}•s^{-1} and increased gradually till 300 µmol•m^{-2}•s^{-1} and remained levelled under 600 µmol•m^{-2}•s^{-1}. After 8 days the H_2O_2/protein was steady until 100 µmol•m^{-2}•s^{-1} and followed a decreasing trend under 300 and 600 µmol•m^{-2}•s^{-1} intensity. The H_2O_2/protein of *M. aeruginosa* showed increasing trend until 100 µmol•m^{-2}•s^{-1} for both 2 days and 8 days exposure. Then, under the 300 and 600 µmol•m^{-2}•s^{-1} showed slightly increasing trend for 2 days exposure and decreasing trend for 8 days exposure. Both species *P. galeata* and *M. aeruginosa* showed statistically significant difference in H_2O_2/protein after 8 days over 2 days (*P. galeata*, $F_{6,28} = 9.036$, $p < 0.0001$; *M. aeruginosa,* $F_{6,28} = 10.864$, $p < 0.0001$).

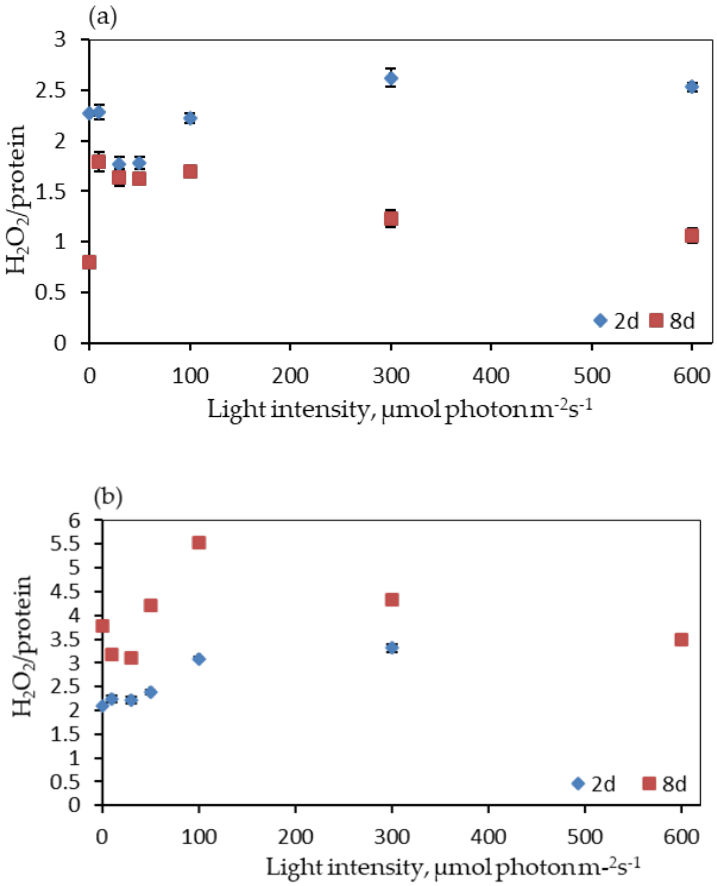


Figure 4-4： H_2O_2/protein in relation to culture light intensity for two species (a) *P.galeata* and (b) *M. aeruginosa* after 2, and 8 days.

The relationship between Chl-a concentration and H_2O_2 is shown in Figure 4-5. There is a clearer negative correlation between Chl-a concentration and H_2O_2 in case of *P. galeata* (R=0.94) and *M. aeruginosa* (R=0.71) after 2 days period only. The Chl-a concentration and H_2O_2 results were scattered beyond 2 days period. Both species *P. galeata* ($F_{6,28}$ = 40.569, $p < 0.0001$) and *M. aeruginosa* ($F_{6,28}$ = 16.026, $p < 0.0001$) showed statistically significant difference.

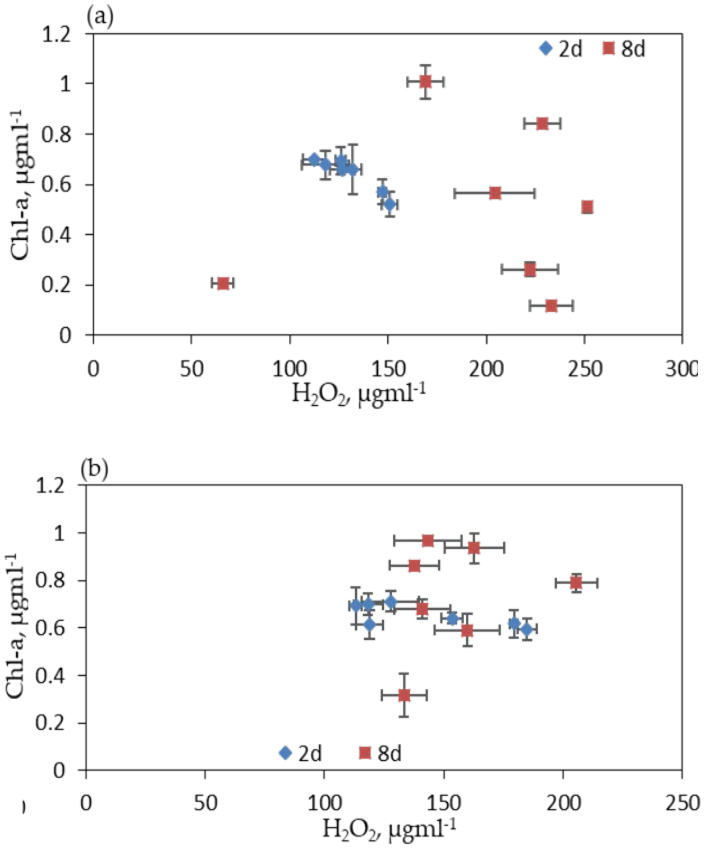


Figure 4-5: Chlorophyll a concentration and H_2O_2 for two species (a) *P. galeata* and (b) *M. aeruginosa* after 2, and 8 days.

Figure 4-6 shows the H_2O_2 per OD_{730} (H_2O_2/OD_{730}) variation with the light intensity of both species after 2 and 8 days. After 2 days, *P. galeata* H_2O_2/OD_{730} was remained same between light intensities. However, for *M. aeruginosa*, H_2O_2/OD_{730} was decreased for 10 and 30 µmol•m^{-2}•s^{-1} and increased with the further increasing light intensities. After 8 days, *P. galeata* H_2O_2/OD_{730} was increased significantly than 2 days exposure except for the 30 µmol•m^{-2}•s^{-1} ($F_{6,28}$ =69.894, $p < 0.0001$). On the other hand, *M. aeruginosa* H_2O_2/OD_{730} was decreased significantly from light intensity 30 µmol•m^{-2}•s^{-1} ($F_{6,28}$ =13.964, $p < 0.0001$). We identified a clearer positive correlation between H_2O_2/OD_{730} and light intensities for *P. galeata* after two days of exposure ($r = 0.74$) and after eight-day exposure ($r = 0.88$), as well as for *M. aeruginosa* after two ($r = 0.91$) and eight ($r = 0.54$) days of exposure.

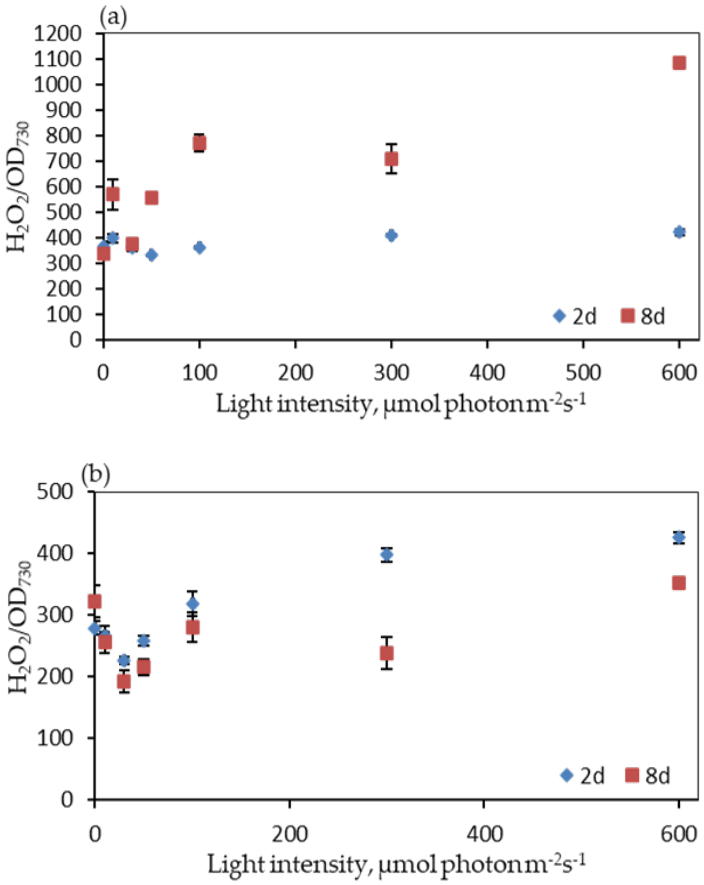


Figure 4-6: H_2O_2 by OD_{730} in relation to culture light intensity for two species (a) *P.galeata* and (b) *M. aeruginosa* under different light intensity (0 ~ 600 µmol•m^{-2}•s^{-1}) after 2 and 8 days.

The CAT activity among two cyanobacteria species are function of H_2O_2/protein showing in Figure 4-7. For *P. galeata* showed statistically significant difference ($F_{6,28}$ = 2.870, p = 0.026) whereas for *M. aeruginosa* showed statistically significant difference ($F_{6,28}$ = 2.881, p =0.026). The CAT activity became highest for 2 day period for highest H_2O_2 per protein values and after that its value decreased with decreasing H_2O_2 per protein value in case of *P. galeata.* In contrast, H_2O_2 per protein value increasing with days time period so that higher CAT activity were generated in *M. aeruginosa*

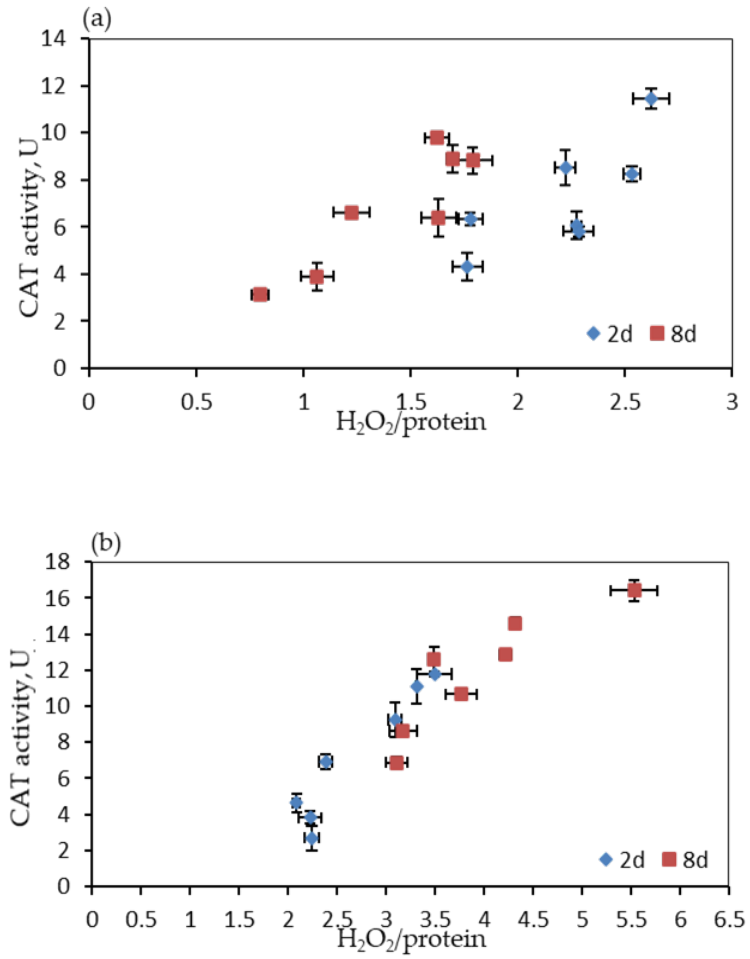


Figure 4-7: CAT activity in relation to H_2O_2 per protein for two species (a) *P.galeat*a and (b) *M. aeruginosa* under different light intensity (0 ~ 600 µmol•m^{-2}•s^{-1}) after 2 and 8 days.

The POD activity among two cyanobacteria species are also function of H_2O_2 per protein shown in Figure 4-8 *For P. galeata* showed statistically significant difference ($F_{6,28}$ = 16.452, p < 0.0001) and *M. aeruginosa* also showed statistically significant difference ($F_{6,28}$ = 3.640, p =0.009). In *P. galeata*, POD activity during 2 days became higher compared to 8 day experiment. In contrast, in *M. aeruginosa,* the POD activity was increased with increasing H_2O_2 per protein during 2 and 8days experiment.

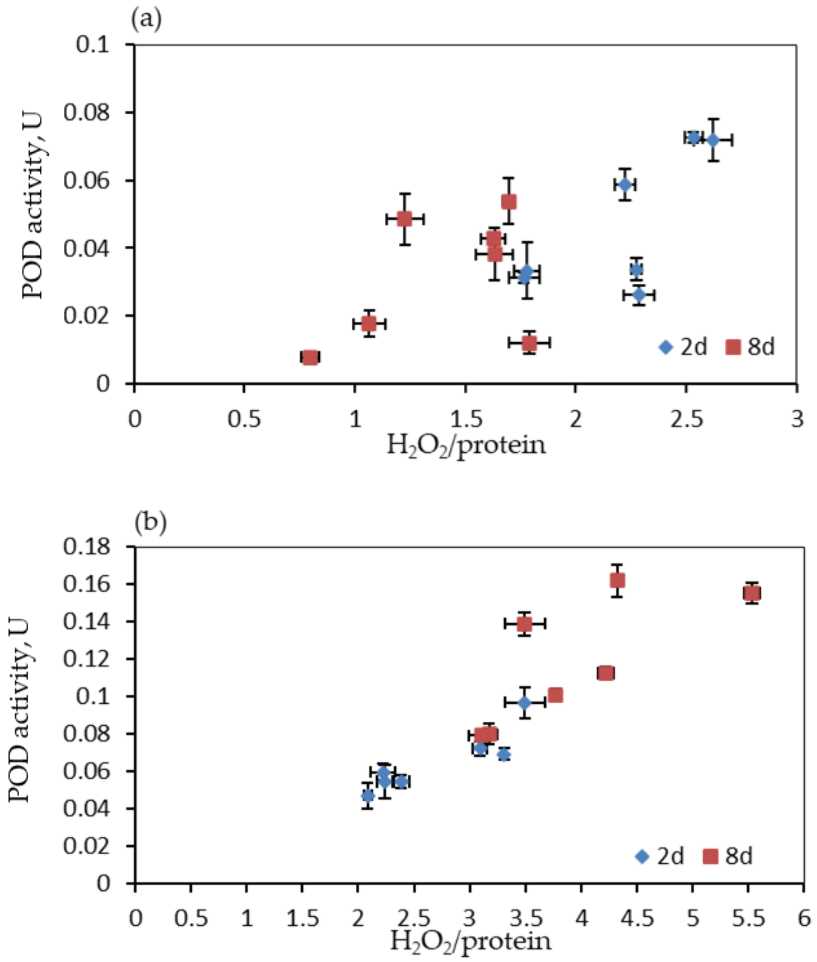


Figure 4-8: POD activity in relation to H_2O_2 per protein for two species (a) *P. galeata* and (b) M. aeruginosa under different light intensity (0 ~600 µmol•m^{-2}•s^{-1}) after 2 and 8 days.

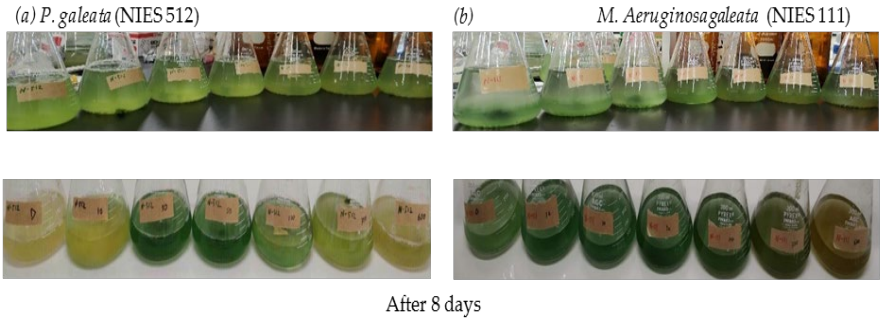


Figure 4-9: Photographs showing growth during 2 and 8 days period (a) *P. galeata* and (b) *M. aeruginosa* under different light intensity (0~600 µmol•m^{-2}•s^{-1}).

The trend was also observed in the color of the sample changed after 2 and 8 days period in different light intensities shown in Figure 4-9.

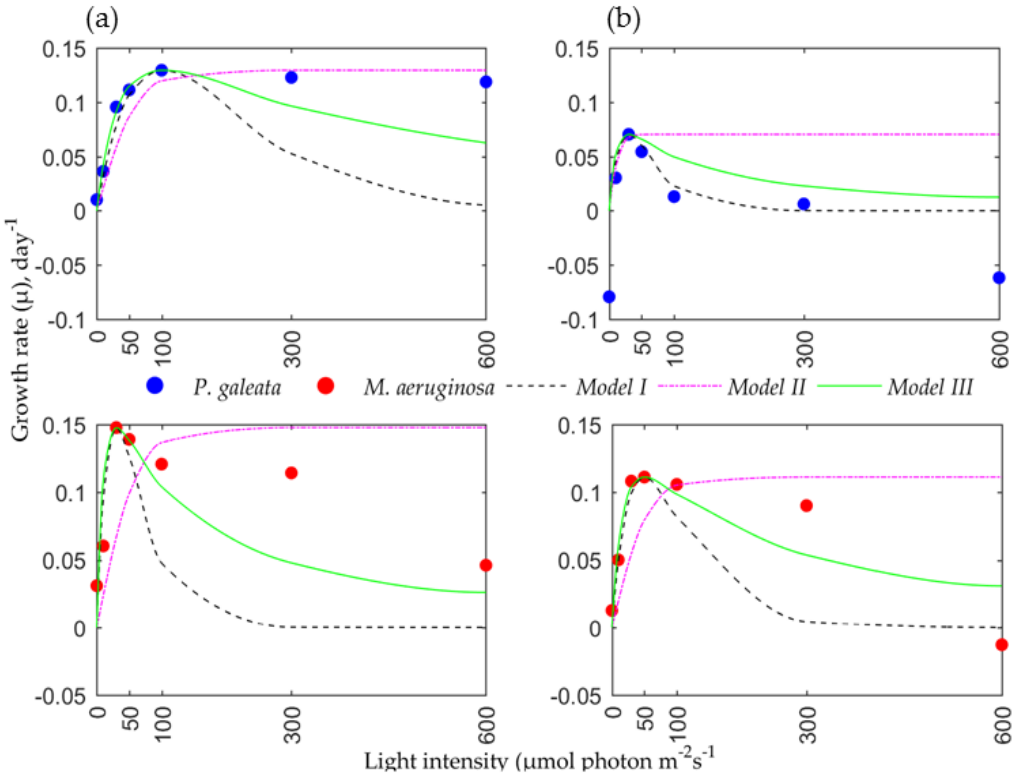


Figure 4-10: Growth rates of *P. galeata* and *M. aeruginosa* as a function of light intensity at different time interval (a) 2 days and (d) 8 days. The observed data are fitted with Model proposed by Steele (Model I), Platt and Jassby (Model II) and Peeters and Eilers (Model III).

The observed growth rates were fitted with the model proposed by Steele (Model I) (Steele 1965). Platt and Jassby (Model II) (Platt and Jassby 1976) and Peeters and Eilers (Model III) (Peeters and Eilers 1978). For observed 2 days growth rate of *P. galeata* fitted with Model II and 8 day growth rate were fitted with Model I (Figure 4-10). The observed 2 days data of *M. aeruginosa* were fitted with Model III and the 8 days data were fitted with model III. *P. galeata* has maximum growth rate of 0.13 day^{-1} attained with in first 2 days under light intensity of 100 $\mu mol \bullet m^{-2} \bullet s^{-1}$ and *M. aeruginosa* has maximum growth rate of 0.15 day^{-1} attained with in first 2 days under light intensity of 30 $\mu mol \bullet m^{-2} \bullet s^{-1}$ (Figure 4-11). The mean growth rates of *P. galeata* were 0.08 day^{-1} and *M. aeruginosa* was 0.12 day^{-1} achieved under light intensity 30 $\mu mol \bullet m^{-2} \bullet s^{-1}$.

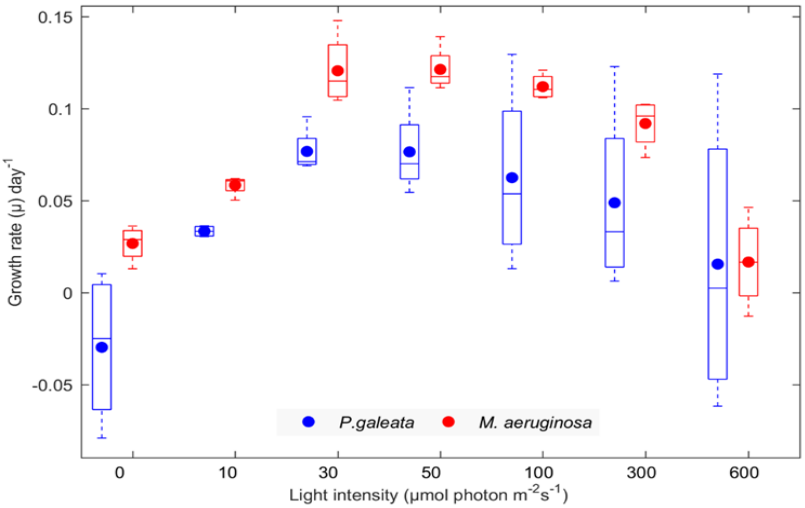


Figure 4-11: Comparison of growth rates of *P. galeata* and *M. aeruginosa* under different light intensity. Central lines indicate the median, and bottom and top edges of the box indicate the 25th and 75th percentiles respectively. The whiskers extend to the most extreme data points not considered outliers, circle shows the mean value.

4.4 Discussion

Two cyanobacteria species, *P. galeata* and *M. aeruginosa*, exhibited different responses under different light intensities and with increasing light conditions, particularly exceeding 50 µmol•m^{-2}•s^{-1}. *P. galeata* was negatively affected when light exceeded 100 µmol•m^{-2}•s^{-1} and *M. aeruginosa* was negatively affected. However, under two days of exposure, neither species was influenced even by the extreme light conditions (300 and 600 µmol•m^{-2}•s^{-1}), suggesting that under a short exposure to high light, both species would survive. However, the extended exposure (8 days) increased the stress on both species. Therefore, both species have the capacity to tolerate light stress for shorter durations but lose this tolerance after extended exposure periods. Light intensity between 30 to 50µmol•m^{-2}•s^{-1} can be considered the preferable light conditions for *P. galeata* growth and 10–100 µmol•m^{-2}•s^{-1} for *M. aeruginosa*. The findings showed similar trends as the previous results, as the growth of *Microcystis* and *Anabaena* species increased under low light (25 µmol•m^{-2}•s^{-1}), but decreased under high light (200

µmol•m^{-2}•s^{-1}) (Beardall 2017; Romo 1994; Venugopal et al. 2006). However, we confirmed that light intensity exceeding 200 µmol•m^{-2}•s^{-1} further intensifies stress on both cyanobacteria species. As the only parameter that was varied in the present experiment was light, this suggests that cells become stressed mainly due to photosystem-produced H_2O_2, even under 600 µmol•m^{-2}•s^{-1}, as in most cyanobacteria species, photoinhibition occurs when the light intensity exceeds 1000 µmol•m^{-2}•s^{-1} (Harel et al. 2004; Machová, Elster, and Adamec 2008; Whitelam and Cold 1983).

The reduced protein content, which reflects increased stress or vice versa for *M. aeruginosa* and most cyanobacteria species (Whitelam and Cold 1983), decreased more after the eight-day than the two-day exposure period, independent from the light intensity. This reveals that *M. aeruginosa* has a defense mechanism preventing cell damage from light. The decreasing protein content with increasing light intensity in *M. aeruginosa* is a result of decreased phycobiliprotein synthesis, which protects against the absorption of excess light energy and increased degradation of the protein by proteases (Pojidaeva et al. 2004; Rosales-Loaiza et al. 2008; Whitelam and Cold 1983). The increased OD_{730} of *M. aeruginosa*, except at 0 and 600 µmol•m^{-2}•s^{-1} (two- and eight-day differences in *M. aeruginosa* OD_{730} under 600 µmol•m^{-2}•s^{-1} minimum compared to *P. galeata*), evidences the survival and continuous cell proliferation of *M. aeruginosa*. *P. galeata*, which is relatively weak, strictly prefers 30 and 50µmol•m^{-2}•s^{-1}, experiencing high stress under lower or higher light intensities considering the Chl-a content and $OD_{730.}$ However, increasing protein content with increasing light stress suggests the deviated stress response of *P. galeata*. The increased protein content can be associated with the up-regulation of stress-related protein, as the oxidative stress was enforced due to the elevated H_2O_2 content with increasing light intensity (Whitelam and Cold 1983). However, further research focused on the up-regulation of stress protein is necessary to confirm this phenomenon.

The oxidative stress response mechanism helps to protect from extreme environmental conditions and trigger antioxidant defense systems responses in cyanobacteria (Liu et al. 2017). The balance between the oxidative stress and antioxidative enzymes is

disturbed by abiotic stress factors and cells will be subjected to oxidative stress (Chalanika De Silva and Asaeda 2017; Rastogi et al. 2010). In the present study, the H_2O_2 content of both species were enhanced under low light (0 and 10 μmol•m^{-2}•s^{-1}) and high light (100–600 μmol•m^{-2}•s^{-1}) intensities. After two days, the CAT and POD activities of *P. galeata* showed scattered trends with the oxidative stress, whereas for *M. aeruginosa*, they showed increasing trends with oxidative stress. However, when testing the regression relationships between H_2O_2/protein and antioxidant enzymes, CAT activity was found to have a strong relationship compared with the POD of both species (CAT: *M. aeruginosa*, r = 0.89 and *P. galeata*, r = 0.90; POD: *M. aeruginosa*, r = 0.75 and *P. galeata*, r = 0.45). This confirms that the CAT activity of both cyanobacteria species plays a more prominent antioxidant activity role than POD. Under higher stress, antioxidant balance is also lost.

The findings show that high levels of light exposure can be adopted as a non-chemical method for cyanobacteria control, similar to low light conditions. This is relevant for methods such as artificial mixing of water in lakes and reservoirs based on the hypothesis that low-light exposure suppresses cyanobacteria growth (Visser et al. 2016). However, we suggest that the control of cyanobacteria should involve a combination exposure of low and high light during mixing, together with rapid light intensity changes. Practical methods should be further studied for field implications of high-light exposure controlling of cyanobacteria (Visser et al. 2016), especially methods that would keep the water column illuminated, exceeding the tolerable levels of cyanobacteria. The growth performance of these two cyanobacteria species was fitted with one or more mathematical models tested, which confirmed the fit of the present data for the evaluation of growth responses of *P. galeata* and *M. aeruginosa* under low- and high-light conditions

CHAPTER 5

INTERACTION OF PLANTS AND CYANOBACTERIA UNDER DIFFERENT LIGHT INTENSITY

5.1 Introduction

The submerged macrophyte *Egeria densa* is an invasive and widespread species, which can suppress the growth of native macrophyte species and dominate the ecosystems (Mazzeo et al. 2003; Santos, Anderson, and Ustin 2011) by forming fragmented colonies (Dugdale et al. 2012; Wang et al. 2019). *Egeria densa* can be found in most subtropical and temperate regions as this species is more competitive in warm waters than in cold conditions (Curt et al. 2010; Riis et al. 2012), and with increasing global temperatures the invasiveness of invasive macrophytes is on the rise (Silveira and Thiébaut 2017). Although *E. densa* prefers low photosynthetically active radiation (PAR) intensities, it does not show stress symptoms until the light intensity is as high as 150 $\mu mol \bullet m^{-2} \bullet s^{-1}$ PAR (Riis et al. 2012; Rodrigues and Thomaz 2010; Tavechio and Thomaz 2003). There is some evidence for *E. densa* to have allelopathic (either negative or positive) interactions with other species (Espinosa-Rodríguez et al. 2016; Espinosa-Rodríguez, Sarma, and Nandini 2017).

Continuous supply of nutrients (e.g., phosphorus) to water bodies and increasing global temperature have promoted the presence of harmful cyanobacteria globally (Lürling et al. 2018; Wells et al. 2015a). As a consequence of the spread of harmful cyanobacteria, water bodies face many challenges such as bad odour and low aesthetic value, release of cyanotoxins, and clogging of water systems under cyanobacterial bloom situations (Howard 1994; Shi et al. 2017). *Microcystis aeruginosa* is a common harmful cyanobacterium found in water bodies (Amorim, Ulisses, and Moura 2017; Qu et al. 2018; Tanabe et al. 2018). The allelochemicals or microcystins of *M. aeruginosa* can be harmful to macrophytes as they negatively affect the physiology and morphology; under prolonged exposure to high concentrations of *M. aeruginosa* or its exudates, the growth of macrophytes is completely suppressed (Kang et al. 2015; Pflugmacher 2004; Saqrane et al. 2007; Zheng et al. 2013). The effect of cyanobacteria on *E. densa* growth

has not been widely investigated, although there is evidence that *E. densa* was negatively affected by high concentration *M. aeruginosa* under prolonged exposure (Amorim et al. 2017); however, the effect of *E. densa* on *M. aeruginosa* can be insignificant. Therefore, based on these findings, it can be hypothesised that under the conditions in which *E. densa* and *M. aeruginosa* coexist in an aquatic system, high concentrations of *M. aeruginosa* may suppress *E. densa* growth.

Although *M. aeruginosa* and *E. densa* have their preferable light intensity range (20–80 µmol•m^{-2}•s^{-1} PAR for *M. aeruginosa* and 80–100 µmol•m^{-2}•s^{-1} PAR for *E. densa*) (Renaud, Pick, and Fortin 2011; Rodrigues and Thomaz 2010; Salvador, Churro, and Valério 2016), they share an optimal growth temperature of 25 °C (Riis et al. 2012; Robarts and Zohary 1987; Yang et al. 2018). Since both species contain photosynthetic pigments, the light intensity can be a detrimental factor for both. Either lower or higher intensity than the optimal requirement could lead to reduced chlorophyll content and optical density, and increased oxidative stress in *M. aeruginosa* (Muhetaer et al. 2020). Similarly, unfavourable light intensities may reduce the macrophyte growth rate and cause oxidative stress. It is plausible that stress resistance of *E. densa* and allelopathy of *M. aeruginosa* would change in response to changing light conditions. Therefore, investigating the influence of light conditions on *M. aeruginosa* allelopathy would generate important information for understanding the conditions in which *E. densa* and *M. aeruginosa* coexist.

Currently, studies investigating the effect of different light conditions, ranging from dark to bright light, on *M. aeruginosa* on *E. densa* growth and their interactions are lacking. In the natural environment, these two species might coexist under different light conditions, at least in different water depths. Because of the different light preference ranges, the effect of the *M. aeruginosa* on *E. densa* can vary depending on light conditions. Also, high concentrations of *M. aeruginosa* in the water would affect the macrophyte because of the reduced light penetration in water, rather than

allelopathic interactions. Therefore, in the present study, we tested the influence of a low concentration of *M. aeruginosa,* which does not limit light penetration significantly, on *E. densa* under different light intensities. The biochemical, pigmentation, and chlorophyll fluorescence (ChF) responses of *E. densa* under controlled and *M. aeruginosa* exposed conditions were investigated.

5.2 Materials and methods

5.2.1 Plant material for culture and cuttings

Egeria densa stock was cultured in glass tanks with well washed river sand as the substrate. The cultures were kept in a temperature-controlled room (~25 °C) under 90–100 µmol•m^{-2}•s^{-1} PAR intensity provided by LED straight lights (Model LT-NLD85L-HN; OHM Electric INC, Japan) at a 12-h light/12-h dark regime. PAR intensity was measured using a quantum flux meter (Apogee, MQ-200, USA). The nutrients were provided with 5 ppm commercial nutrient solution (Hyponex concentrated nutrient solution, Hyponex, Osaka, Japan). The stock culture was maintained algae-free. The cuttings were obtained from stock and replanted in a separate tank which contained thoroughly washed river sand as the substrate and 10% Hoagland solution as the nutrient source. The cuttings were grown for three days under the same light and temperature conditions as the stock culture.

After three days, the cuttings were planted in 1-L Pyrex glass beakers by attaching them to a half cylindrical rubber cushion (Carboy Inc. Chiba, Japan). Six small holes were made in each rubber cushion and one *E. densa* cutting was fixed in each hole of the cushion (Figure 5-1). The beakers were filled with 10% Hoagland solution and a rubber cushion containing *E. densa* cuttings was fixed inside each beaker. The cuttings were carefully planted in the beakers to prevent algae contamination and mechanical damage to the cuttings. In order to prevent algae contamination, prior to

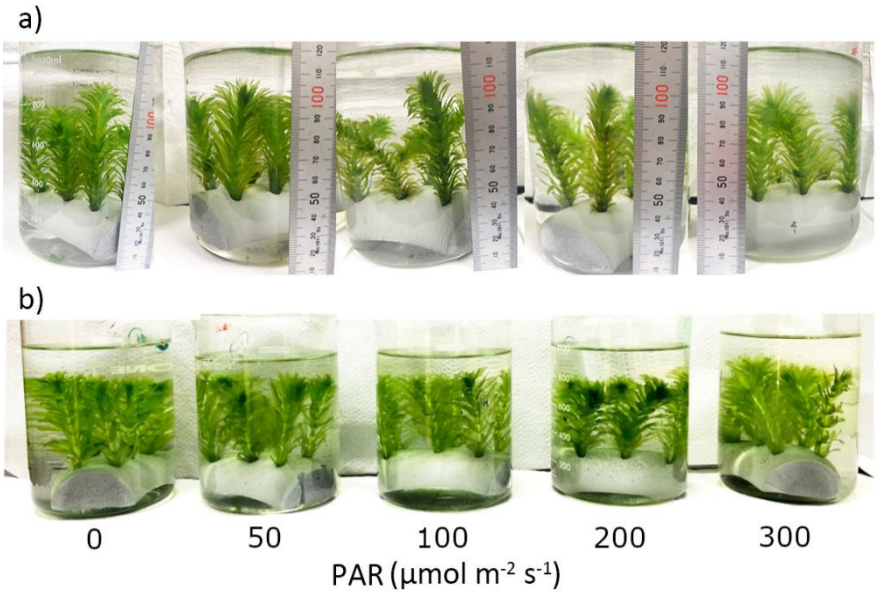


Figure 5-1: A representative image of *Egeria densa* growth at different photosynthetically active radiation (PAR) intensities exposed for seven days. *E. densa* growth a) without and b) with exposure to *Microcystis aeruginosa.*

the experiment, the beakers and rubber cushions were washed with detergent, disinfected with 70% ethyl alcohol, and then washed with distilled water three times. *Egeria densa* cuttings were washed two times with 10% Hoagland solution prior to attaching to the rubber cushion.

5.2.1 Cyanobacteria culture

The *M. aeruginosa* (N-111) strain obtained from the National Institute for Environmental Studies, Ibaraki, Japan was cultured in conical flasks containing BG-11 nutrient medium (1/3 flask volume). *Microcystis aeruginosa* was added to the flasks which were closed with porous stoppers (SILICOSEN, Shin-Etsu Polymer Co., Ltd, Japan). Flasks were kept inside an incubator at 20 °C, with the light intensity maintained at 25–30 µmol•m^{-2}•s^{-1} PAR intensity using dimmable, daylight LED panel lights (VBL-SL150, Valore, Kyoto, Japan) with a 12-h light/12-h dark regime. Each flask was manually shaken three times per day during the light phase. When the growing culture reached an optical density (OD_{730}) of ~1.2, the culture was multiplied by division. To measure the OD_{730}, 1 mL cyanobacteria samples were taken with a micropipette, placed

directly into a 1 mL quartz cuvette (T-9M-UV-10, TOSOH, Tokyo, Japan) and optical absorption at 730nm was measured with a spectrophotometer (UVmini-1240, Shimadzu, Kyoto, Japan).

5.2.2 experiment setup

Control study 1 - Egeria densa light exposure study

Egeria densa cuttings were acclimated under 90–100 µmol•m^{-2}•s^{-1} PAR intensity for three days. Then, the plant cuttings were subjected to different PAR intensities – 0, 50, 100, 200, and 300 µmol•m^{-2}•s^{-1} – with triplicate beakers under each light intensity and grown for seven days. The incubator conditions were the same during acclimation and during the experiment – a 12-h light/12-h dark regime at 25 °C. The water level of each beaker was maintained by replenishing evaporated water by adding distilled water. After seven days of exposure, the plant height was recorded, and five out of six plants were collected from each beaker, kept on ice, and transferred into a -80 °C freezer. The remaining plants within each beaker were subjected to darkness for 30 min, and chlorophyll fluorescence (ChF) parameters were quantified and photosynthetic pigments were extracted.

Control study 2 - Mycrosistis aeruginosa light exposure control study

This study was conducted to determine the optical density of *M. aeruginosa* under different light intensities. Beakers containing foam rubber cushions fixed to the bottom without plants were filled with 10% Hoagland solution. For each beaker, *M. aeruginosa* was added carefully maintaining an optical density (OD_{730}) of 0.04 ± 0.002. After reaching the optical density, three beakers for each light intensity (0, 50, 100, 200, and 300 µmol•m^{-2}•s^{-1}) were kept inside the incubator at 25 °C with a 12-h light/12-h dark regime for seven days. The water level of each beaker was maintained by adding distilled water every other day. After seven days, OD_{730} of the samples taken from each beaker was measured.

Egeria densa–Microcystis aeruginosa combined study

Beakers with six *E. densa* plants in 10% Hoagland medium were acclimated for three days as described above. After the acclimation, *M. aeruginosa* was added carefully maintaining the OD_{730} at 0.04 ± 0.002. Then three beakers for each light intensity (0, 50, 100, 200, and 300 $\mu mol \bullet m^{-2} \bullet s^{-1}$) were kept inside the incubator at 25 °C and with a 12-h light/12-h dark regime. After seven days, *M. aeruginosa* was sampled from each beaker and the OD_{730} was measured. Plants were sampled as described above: five plants per beaker were stored at -80 °C and the remaining plants were subjected to ChF measurement and extraction of photosynthetic pigments.

5.2.3 Measurement of *Egeria densa* pigments

The upper part of the plants (~150 mg fresh weight (FW) was used to extract photosynthetic pigments by adding N,N-dimethylformamide (5 mL) and incubating for 24 h in the dark at room temperature (25–27 °C). The optical absorptions of pigment extract were measured at 664, 647, and 480 nm wavelengths using a spectrophotometer (UVmini-1280). The concentrations of chlorophyll a (Chl a), chlorophyll b (Chl b), and total carotenoids (Car) were calculated using the equation provided by (Wellburn 1994)The pigment content was expressed as micrograms per gram of fresh weight ($\mu g\ g^{-1}$ FW).

Anthocyanin content was measured as described in (Nakata and Ohme-Takagi) with modifications. Plant samples (~50 mg) were collected from the upper portion of the plant, and then pulverised in liquid nitrogen and mixed with 2 mL of extraction buffer containing 45% (v/v) methanol and 5% (v/v) acetic acid in distilled water. The extract was centrifuged at 2,500× g for 15 min at 20 °C and the supernatant was collected. The optical absorption was measured at 637 and 530 nm using a spectrophotometer (UVmini-1280). Anthocyanin content was calculated considering one anthocyanin unit to be equivalent to one absorbance unit in 1 mL of extract (Teng et al. 2005).

5.2.3 H_2O_2 and antioxidant enzyme quantification in *Egeria densa*

For quantification of hydrogen peroxide (H_2O_2) content and antioxidant enzyme activity, ~200 mg of plant tissue was crushed in liquid nitrogen in the presence of polyvinylpyrrolidone. Then, 5 mL of 0.05 M phosphate buffer (pH 6.0) was added and the extraction mixture was centrifuged at 2,500× g for 15 min at 4 °C. The supernatant was collected and stored at -80 °C until further analysis.

H_2O_2 content was measured by combining 750µL of enzyme extract with 2.5 mL of 0.1% (w/v) titanium sulphate in 20% (v/v) H_2SO_4 as described by Satterfield and Bonnell (Satterfield and Bonnell 1955). The mixture was incubated at 25–27 °C for 30 min and the colour changes were quantified spectrophotometrically (UVmini-1240) at 410 nm. H_2O_2 concentrations were obtained from an H_2O_2 standard curve prepared with known concentrations of H_2O_2 and were expressed as µmol g^{-1} FW.

Guaiacol peroxidase activity (GPX) was measured by combining 40µL of 30 mmol H_2O_2 and 50µL of 25 mmol guaiacol and finally adding 100 µL of enzyme extract to initiate the reaction(Jennifer W. MacAdam, Sharp, and Nelson 1992). The absorbance at 420 nm was recorded spectrophotometrically (UVmini-1240) at every 10 s for 3 min. Based on the rate of absorbance increase, GPX activity was expressed as µmol min^{-1} g^{-1} FW using the extinction coefficient 26.6 $mmol^{-1}$ cm^{-1}.

Catalase (CAT) activity was measured by combining 100 µL of 10 mmol H_2O_2, 2 mL of 100 mM potassium phosphate buffer (pH 7.0), and finally adding 500 µL of extract supernatant to initiate the reaction. The absorbance was recorded spectrophotometrically (UVmini-1240) at 240 nm every 10 s for 3 min. Based on the rate of absorbance decrease, CAT activity was expressed as µmol min^{-1} g^{-1} FW using the extinction coefficient 40 $mmol^{-1}$ cm^{-1}.

Ascorbate peroxidase (APX) was measured according to Nakano and Asada(Nakano and Asada 1981) by combining 100 µL of extract supernatant, and 200 µL of 0.5 mmol ascorbic acid in 2 mL of 50 mM potassium phosphate buffer (pH 7.0). The assay was started with the addition of 60 µL of 1 mmol H_2O_2. The absorbance was recorded at 290 nm using a spectrophotometer (UVmini-1240) at every 10 s for 3 min. Based on the rate of absorbance decrease, APX activity was expressed in µmol min^{-1} g^{-1} FW using the extinction coefficient 2.8 $mmol^{-1}$ cm^{-1}.

5.2.4 Chlorophyll fluorescence of *Egeria densa*

Chlorophyll fluorescence parameters of *E. densa* were measured using the ChF imaging technique (Handy FluorCam – FC 1000-H, Photon systems Technology, Brno, Czech Republic). After adapting to dark for 30 min, *E. densa* plants were subjected to ChF measurements. After removal from the beakers, the plants were placed on a thick folded tissue soaked with 10% Hoagland solution placed under the FluorCam. The *E. densa* plants exposed to *M. aeruginosa* were washed quickly with 10% Hoagland solution to remove attached *M. aeruginosa* prior to the ChF measurement. The parameters measured were maximum quantum efficiency of photosystem II (Fv/Fm), non-photochemical quenching (NPQ), photochemical quenching (Qp), instantaneous chlorophyll fluorescence (Ft), and effective quantum yield of photosystem II (QY).

5.2.5 Shade conditions

The light received at the top, middle and bottom regions of the beakers of *M. aeruginosa–E. densa* combined experiment was measured using a submersible quantum flux meter (Apogee, MQ-510, USA). The PAR intensities of each region were measured under each light exposure condition (0, 50, 100, 200, and 300 µmol•m^{-2}•s^{-1}).

5.2.6 Data analysis

After checking for equality of variance, the significant difference between control and *M. aeruginosa* exposed plants was tested using independent sample Student's t-test. The comparisons between control and *M. aeruginosa* exposed plants were done for each light intensity separately. The comparisons within light conditions of control or *M. aeruginosa* exposed plants were tested with one-way analysis of variance (ANOVA) with post-hoc Duncan's test. *P* values lower than 0.05 were considered significant. Statistical analyses were performed using IBM SPSS Statistics, Version 25 (IBM, Armonk, NY, USA). All descriptive statistics and data visualisation were done using Microsoft Excel Version 2001(Microsoft, Washington, USA).

5.3 Results

5.3.1 H_2O_2 and Antioxidant activity of *Egeria densa*

The H_2O_2 content of the control plants remained relatively constant under varying light conditions and was significantly higher in the dark treated plants (ANOVA, $P < 0.01$, $F = 10.493$). H_2O_2 content fluctuated with the increasing light intensity in the plants exposed to *M. aeruginosa*. The H_2O_2 content of *M. aeruginosa* exposed plants was analysed by ANOVA. The ANOVA grouped the following PAR treatments: 100 and 200 $\mu mol \bullet m^{-2} \bullet s^{-1}$ PAR intensities; 200 and 300 $\mu mol \bullet m^{-2} \bullet s^{-1}$ PAR intensities; 0 and 300 $\mu mol \bullet m^{-2} \bullet s^{-1}$ PAR intensities; and 50 $\mu mol \bullet m^{-2} \bullet s^{-1}$ PAR intensity (ANOVA, $P < 0.01$, $F = 24.319$). The H_2O_2 content of *M. aeruginosa* exposed plants under 0 and 50 $\mu mol \bullet m^{-2} \bullet s^{-1}$ PAR conditions were relatively higher than that in the other light conditions. H_2O_2 content was highest in 50 $\mu mol \bullet m^{-2} \bullet s^{-1}$ PAR intensity and lowest in the 100 $\mu mol \bullet m^{-2} \bullet s^{-1}$ PAR intensity. H_2O_2 content of the *M. aeruginosa* exposed plants was significantly higher in all light conditions than that of the control plants (t-test, $P < 0.01$ for each condition). The difference in H_2O_2 content between *M. aeruginosa* exposed plants and control was highest (3-fold) in 50 $\mu mol \bullet m^{-2} \bullet s^{-1}$ PAR conditions and lowest in 100 $\mu mol \bullet m^{-2} \bullet s^{-1}$PAR intensity (Figure5-2).

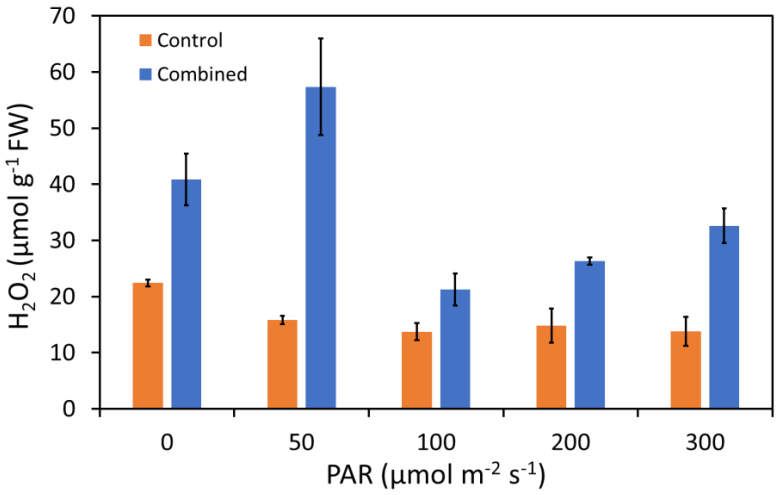


Figure 5-2: The H_2O_2 content of *Egeria densa* after seven days of exposure to different photosynthetically active radiation (PAR) conditions without *Microcystis aeruginosa* exposure (Control) and with *M. aeruginosa* exposure (Combined).

The GPX activity of *M. aeruginosa* exposed and control plants showed similar trends; the GPX activity increased with increasing light intensity until 100 µmol•m^{-2}•s^{-1} PAR intensity, reduced at 200 µmol•m^{-2}•s^{-1} PAR, and was highest under the 300 µmol•m^{-2}•s^{-1} PAR intensity (Figure 5-3). GPX activity was significantly different between each light condition in the plants from the control group (ANOVA, $P < 0.01$, F = 48.972). In *M. aeruginosa* exposed plants, the ANOVA grouped the following PAR treatments: 0 and 50 µmol•m^{-2}•s^{-1} PAR intensities; 100 and 200 µmol•m^{-2}•s^{-1} PAR intensities; and 300 µmol•m^{-2}•s^{-1} PAR intensity (ANOVA, $P < 0.01$, F = 90.161). GPX activity was significantly lower in plants with presence of *M. aeruginosa* than in the control plants under 50 and 100 µmol•m^{-2}•s^{-1} PAR intensities (t-test, $P < 0.01$ for 50 µmol•m^{-2}•s^{-1} PAR and $P < 0.05$ for 100 µmol m^{-2} s^{-1} PAR); higher under 300 µmol•m^{-2}•s^{-1} PAR intensity (t-test, $P < 0.01$); and similar under 0 and 200 µmol•m^{-2}•s^{-1} PAR intensity (t-test $P > 0.05$ for both conditions; Figure 5-3a).

The CAT activity of control plants was highest under the 50 µmol•m^{-2}•s^{-1} light intensity although it was not significantly different from that at 100 µmol•m^{-2}•s^{-1} light intensity. For control plants, the ANOVA grouped the following PAR treatments: 0, 200, and 300

µmol•m^{-2}•s^{-1} PAR intensities; 0, 100 and 300 µmol•m^{-2}•s^{-1} PAR intensities; and 50 and 100 µmol•m^{-2}•s^{-1} PAR intensities (ANOVA, $P < 0.05$, F = 8.295). The CAT activity of *M. aeruginosa* exposed plants gradually increased till 100 µmol•m^{-2}•s^{-1} PAR intensity and was highest under 100 µmol•m^{-2}•s^{-1} PAR intensity, although it was not significantly different from that of plants grown at 50 µmol•m^{-2}•s^{-1} PAR intensity. For *M. aeruginosa* exposed plants, the ANOVA grouped the following PAR treatments: 0 µmol•m^{-2}•s^{-1} PAR intensity; 200 and 300 µmol•m^{-2}•s^{-1} PAR intensities; and 50 and 100 µmol•m^{-2}•s^{-1} PAR intensities (ANOVA, $P < 0.01$, F = 24.516). CAT activity was lower in *M. aeruginosa* exposed plants than in the control plants under all light conditions, except for the 200 µmol•m^{-2}•s^{-1} light intensity. These differences were statistically significant under the 0, 50, and 300 µmol•m^{-2}•s^{-1} light intensities (t-test, $P < 0.01$ for 0 and 50 µmol•m^{-2}•s^{-1} light intensities and $P < 0.05$ for 300 µmol•m^{-2}•s^{-1} light intensity; Figure 5-3b).

The APX activity of controlled and *M. aeruginosa* exposed plants showed different trends with increasing light intensity. In the control plants, the APX activity was lowest at 300 µmol•m^{-2}•s^{-1} PAR and was significantly different from that of control plants at other light intensities (ANOVA, $P < 0.05$, F = 3.451). In plants exposed to *M. aeruginosa*, the APX activity was significantly lower at 50 and 100 µmol•m^{-2}•s^{-1} PAR than at other light intensities (ANOVA, $P < 0.01$, F = 10.079). The APX activity was significantly lower in *M. aeruginosa* exposed plants than control in 50 and 100 µmol•m^{-2}•s^{-1} PAR intensities (t-test, $P < 0.01$ for both light conditions) and was similar under other light intensities (t-test, $P > 0.05$ for all conditions). The APX activity in *M. aeruginosa* exposed plants was higher than that in control under 200 and 300 µmol•m^{-2}•s^{-1} PAR intensities; however, this was not significant (t-test, $P > 0.05$; Figure 5-3c).

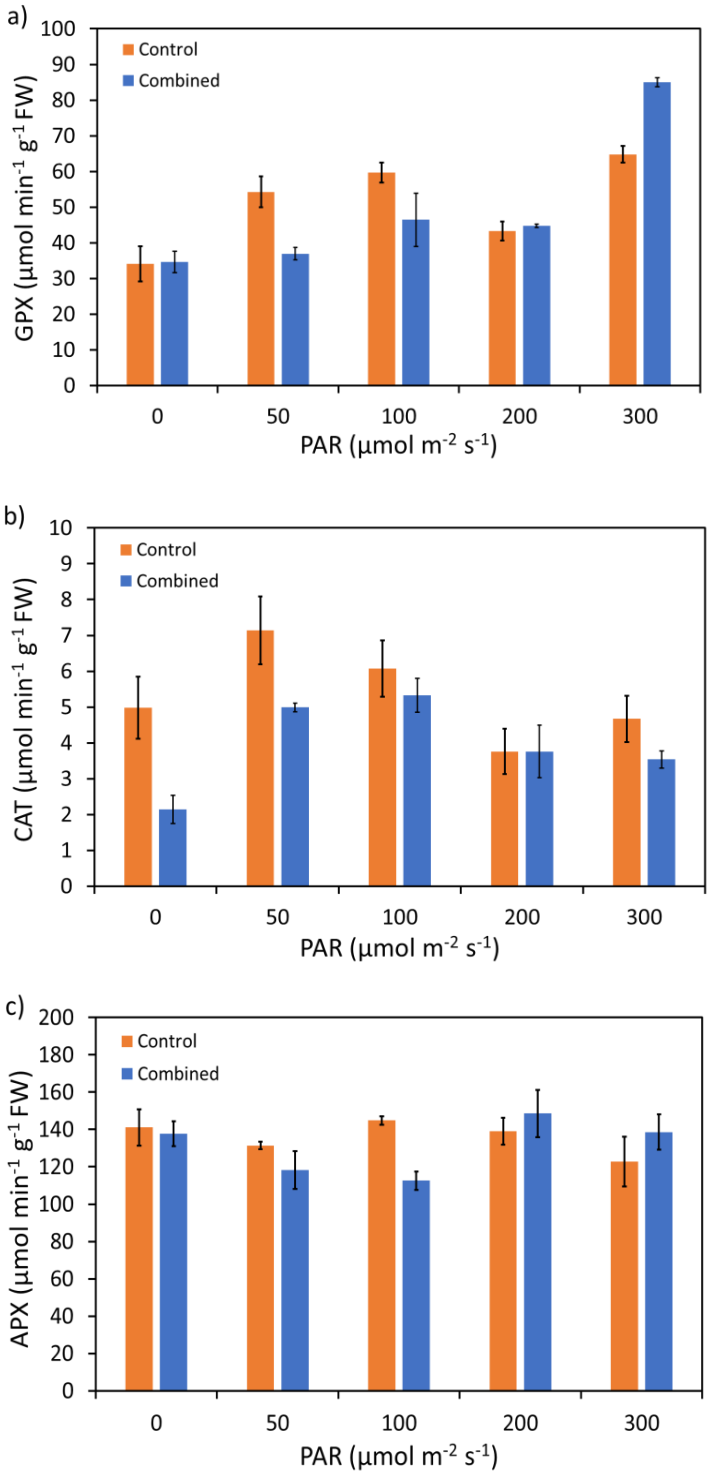


Figure 5-3: Antioxidant activities of Egeria densa after seven days of exposure to different photosynthetically active radiation (PAR) conditions without *Microcystis aeruginosa* exposure (Control) and with *M. aeruginosa* exposure (Combined). The a) GPX – Guaiacol peroxidase, b) CAT – Catalase and c) APX – Ascorbate peroxidase. Same number/s over the bars indicating observations are in the same ANOVA groups.

5.3.2 Chlorophyll fluorescence parameters of *Egeria densa*

The ChF parameters of *E. densa* under control and *M. aeruginosa* exposed conditions are shown in Figure 5-4. The ANOVA analysis of control plants, grouped the following PAR treatments: 0 µmol•m^{-2}•s^{-1}PAR intensity; 50 µmol•m^{-2}•s^{-1} PAR intensity; 100 and 300 µmol•m^{-2}•s^{-1} PAR intensities; and 100 and 200 µmol•m^{-2}•s^{-1} PAR intensities (ANOVA, $P < 0.01$, F = 21.961). For *M. aeruginosa* exposed plants, the ANOVA grouped the following PAR treatments: 0 and 50 µmol•m^{-2}•s^{-1} PAR intensities; 100 and 200 µmol•m^{-2}•s^{-1} PAR intensities; and 200 and 300 µmol•m^{-2}•s^{-1} PAR intensities (ANOVA, $P < 0.01$, F = 25.063). The Fv/Fm values were significantly higher in *M. aeruginosa* exposed plants than control at all light intensities (t-test, $P < 0.05$ under 0 µmol•m^{-2}•s^{-1} PAR intensity, and $P < 0.01$ under the remaining light intensities; Figure 5-4a).

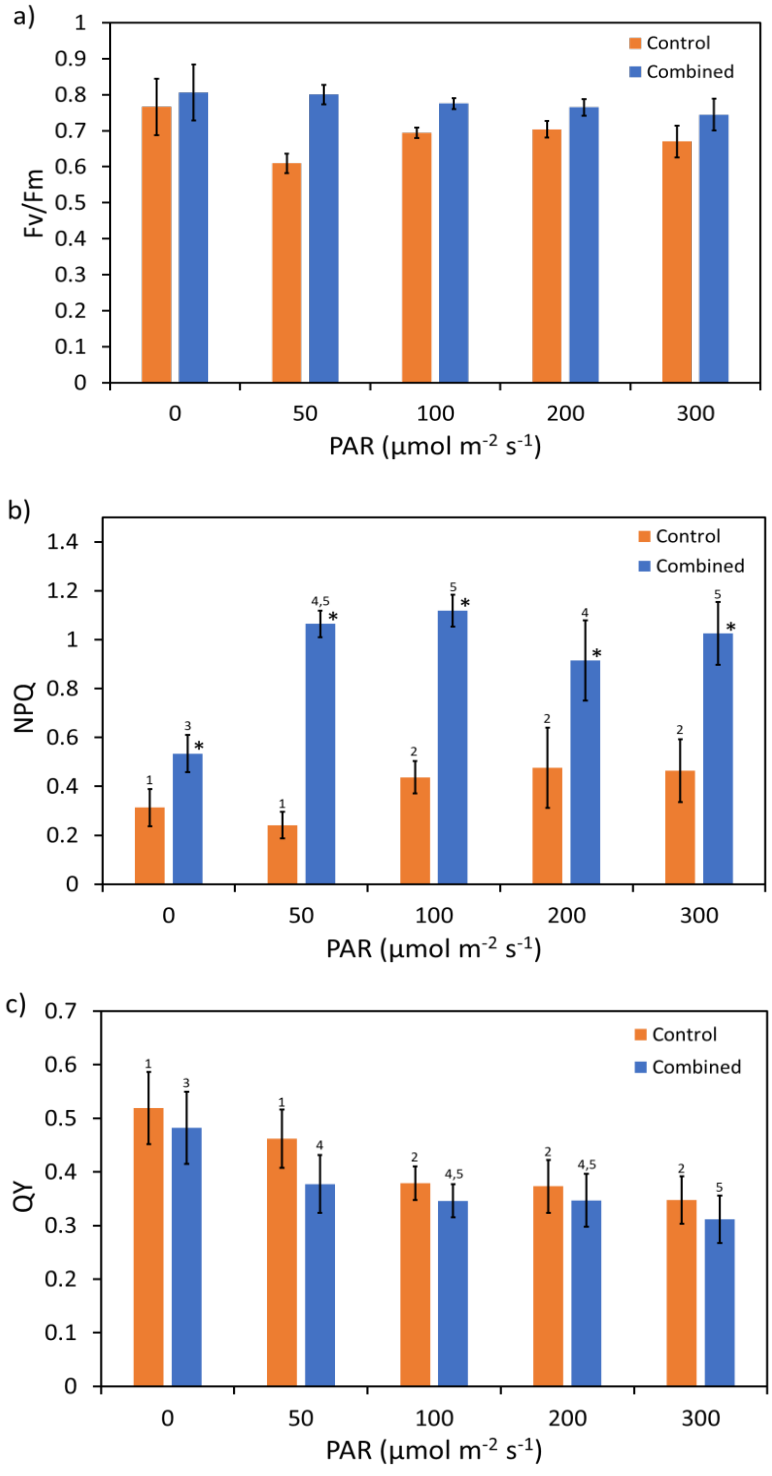
The NPQ of control plants had a similar pattern to Fv/Fm, and the ANOVA grouped the following PAR treatments: 0 and 50 µmol•m^{-2}•s^{-1}PAR intensities; and 100, 200 and 300 µmol•m^{-2}•s^{-1} PAR intensities (ANOVA, $P < 0.01$, F = 14.112). For *M. aeruginosa* exposed plants, the ANOVA grouped the following PAR treatments: 0 µmol•m^{-2}•s^{-1} PAR intensity; 50 and 200 µmol•m^{-2}•s^{-1} PAR intensities; and 50, 100, and 300 µmol•m^{-2}•s^{-1} PAR intensities (ANOVA, $P < 0.01$, F = 7.636). The NPQ values were significantly higher in *M. aeruginosa* exposed plants at all light intensities (t-test, $P < 0.01$ under each PAR intensity; Figure 5-4b).

The QY of control and *M. aeruginosa* exposed plants followed a similar trend and decreased with increasing light intensity. The QY values of *M. aeruginosa* exposed plants were lower than those of the control plants at all light intensities, and this difference was statistically significant under 50, 100, and 300 µmol•m^{-2}•s^{-1} PAR intensities (t-test, $P < 0.01$ for 50 µmol•m^{-2}•s^{-1} PAR intensity and $P < 0.05$ for 100 and 300 µmol•m^{-2}•s^{-1} PAR intensities; Figure 5-4c). For the control plants, the ANOVA test grouped the following PAR treatments: 0 and 50 µmol•m^{-2}•s^{-1} PAR intensities; and 100, 200, and 300 µmol•m^{-2}•s^{-1} PAR intensities (ANOVA, $P < 0.01$, F = 23.092). For *M.*

aeruginosa exposed plants, the ANOVA grouped the PAR treatments as follows: 0 µmol•m^{-2}•s^{-1} PAR intensity; 50, 100, and 200µmol•m^{-2}•s^{-1} PAR intensities; and 100, 200, and 300 µmol•m^{-2}•s^{-1} PAR intensities (ANOVA, $P < 0.01$, F = 16.317).

The Ft of control plants increased until 200µmol•m^{-2}•s^{-1} PAR intensity and slightly reduced in 300 µmol•m^{-2}•s^{-1} PAR intensity. For control plants, ANOVA analysis grouped the following PAR treatments: 0 and 50µmol•m^{-2}•s^{-1} PAR intensities; 100 and 300 µmol•m^{-2}•s^{-1} PAR intensities; and 200 and 300 µmol•m^{-2}•s^{-1} PAR intensities (ANOVA, $P < 0.01$, F = 22.986). The Ft of *M. aeruginosa* exposed plants varied with the light intensities and was highest under 0 and 100 µmol•m^{-2}•s^{-1} PAR intensities. For *M. aeruginosa* exposed plants, the ANOVA analysis grouped the following PAR treatments: 50, 200, and 300 µmol•m^{-2}•s^{-1} PAR intensities; 100 and 300 µmol•m^{-2}•s^{-1} PAR intensities; and 0 and 100 µmol•m^{-2}•s^{-1} PAR intensities (ANOVA, $P < 0.01$, F = 5.883). Ft values were significantly higher in *M. aeruginosa* exposed plants than in control plants at 0 µmol•m^{-2}•s^{-1} PAR intensity (t-test, $P < 0.01$); significantly lower at 200 and 300 µmol•m^{-2}•s^{-1} PAR intensities (t-test, $P < 0.01$); and similar at 50 and 100 µmol•m^{-2}•s^{-1} PAR intensities (Figure 5-4d).

The Qp of the control plants was highest at 0 and 50 µmol•m^{-2}•s^{-1} PAR intensities. For control plants, the ANOVA grouped the following PAR treatments: 0 µmol•m^{-2}•s^{-1} PAR intensity; 50 µmol•m^{-2}•s^{-1} PAR intensity; and 100, 200, and 300 µmol•m^{-2}•s^{-1} PAR intensities (ANOVA, $P < 0.01$, F = 45.226). Under the *M. aeruginosa* exposure conditions, the Qp value was highest at 0 µmol•m^{-2}•s^{-1} PAR intensity and was similar under remaining light conditions. For *M. aeruginosa* exposed plants, the ANOVA grouped the following PAR treatments: 0 µmol•m^{-2}•s^{-1} PAR intensity; and 50, 100, 200, and 300 µmol•m^{-2}•s^{-1} PAR intensities. Qp values were significantly lower in *M. aeruginosa* exposed plants than in control plants under
all light conditions (t-test, $P < 0.01$ for 0, 50, 200 and 300 µmol•m^{-2}•s^{-1} PAR intensities, and $P < 0.05$ for 100 µmol•m^{-2}•s^{-1} PAR intensity; Figure 5-4e).


a)
Fv/Fm
Control
Combined
PAR (µmol m-2 s-1)
b)
NPQ
Control
Combined
PAR (µmol m-2 s-1)
c)
QY
Control
Combined
PAR (µmol m-2 s-1)

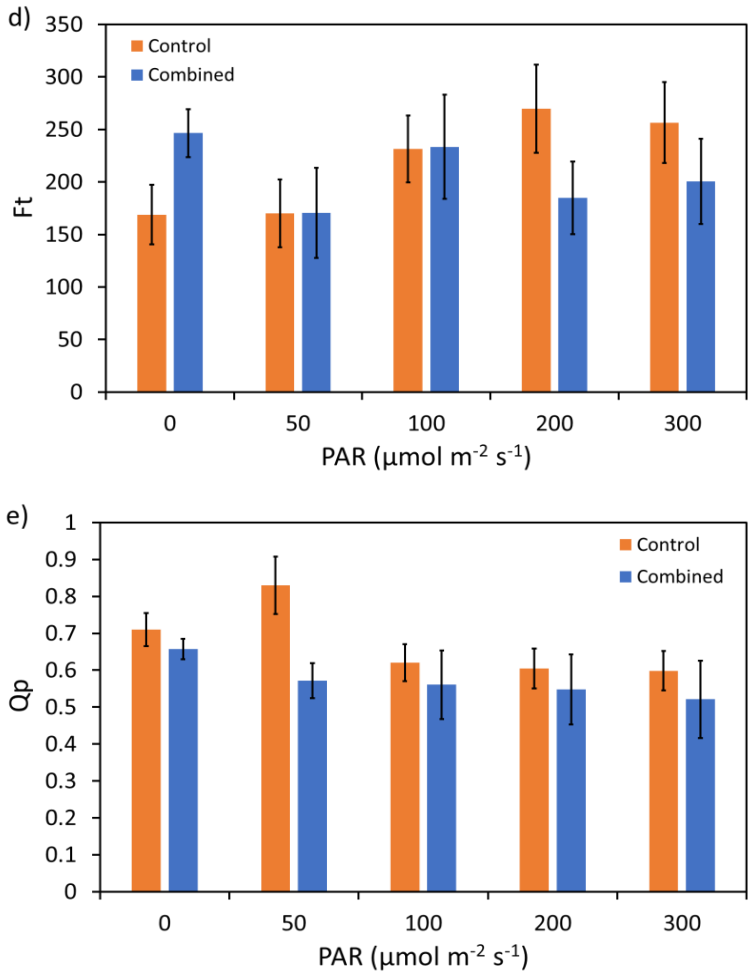


Figure 5-4: Chlorophyll fluorescence parameters of *Egeria densa* after seven days of exposure to different photosynthetically active radiation (PAR) without *Microcystis aeruginosa* exposure (Control) and with *M. aeruginosa* exposure (Combined). The a) Fv/Fm – maximum quantum yield, b) NPQ – non-photochemical quenching, c) QY – quantum yield, d) Ft – fluorescence yield, and e) Qp – photochemical quenching. Same number/s over the bars indicating observations are in the same ANOVA groups.

5.3.3 Chlorophyll a, b, and carotenoid content of *Egeria densa*

The Chl a, Chl b, and Car content of both control and *M. aeruginosa* exposed plants had similar trends; the pigment content gradually increased until 100 µmol•m^{-2}•s^{-1} PAR intensity for both control and *M. aeruginosa* exposed plants (Figure 5-5). For the Chl a content of control plants, the ANOVA grouped the following PAR treatments: 0 and 300 µmol•m^{-2}•s^{-1} PAR intensities; 200 and 300 µmol•m^{-2}•s^{-1} PAR intensities; and 50 and 100 µmol•m^{-2}•s^{-1} PAR intensities (ANOVA, $P < 0.01$, F = 23.940). For the Chl b

content of control plants, the ANOVA grouped the following PAR treatments: 0, 200, and 300 µmol•m^{-2}•s^{-1} PAR intensities; 0, 50, and 200 µmol•m^{-2}•s^{-1} PAR intensities; and 100 µmol m^{-2} s^{-1} PAR intensity (ANOVA, $P < 0.01$, F = 8.013). For the Car content of control plants, the ANOVA grouped the following PAR treatments: 0 µmol•m^{-2}•s^{-1} PAR intensity; 300 µmol•m^{-2}•s^{-1} PAR intensity; 50 and 200 µmol•m^{-2}•s^{-1} PAR intensities; and 100 µmol m^{-2} s^{-1} PAR intensity (ANOVA, $P < 0.01$, F = 76.197). For the Chl a of *M. aeruginosa* exposed plants, the ANOVA grouped the following PAR treatments: 0 and 200 µmol•m^{-2}•s^{-1} PAR intensities; 0 and 50 µmol•m^{-2}•s^{-1} PAR intensities; 50 and 300 µmol•m^{-2}•s^{-1} PAR intensities; and 100 and 300 µmol•m^{-2}•s^{-1} PAR intensities (ANOVA, $P < 0.01$, F = 9.882). For the Chl b content of *M. aeruginosa* exposed plants, the ANOVA grouped the following PAR treatments: 0, 50, and 200 µmol•m^{-2}•s^{-1} PAR intensities; and 100 and 300 µmol•m^{-2}•s^{-1} PAR intensities (ANOVA, $P < 0.01$, F = 8.883). For the Car content of *M. aeruginosa* exposed plants, the ANOVA grouped the following PAR treatments: 0, 50, and 200 µmol•m^{-2}•s^{-1} PAR intensities; 200 and 300 µmol•m^{-2}•s^{-1} PAR intensities; and 100 µmol•m^{-2}•s^{-1} PAR intensity (ANOVA, $P < 0.01$, F = 11.554). Chl a content was lower in *M. aeruginosa* exposed plants than control plants at all light intensities, except for 300 µmol•m^{-2}•s^{-1} PAR, and this difference was statistically significant at 0, 50, and 200 µmol•m^{-2}•s^{-1} PAR intensities (t-test, $P < 0.01$ under each light intensity). Chl b content was significantly lower in *M. aeruginosa* exposed plants than control at all light intensities, except for 300 µmol•m^{-2}•s^{-1} PAR (t-test, $P < 0.01$ for 0, 50, and 200 µmol•m^{-2}•s^{-1} PAR intensities, and $P < 0.05$ for 100 µmol•m^{-2}•s^{-1} PAR intensity), which was significantly higher than the control at 300 µmol•m^{-2}•s^{-1} PAR (t-test, $P < 0.01$). Car content was lower in *M. aeruginosa* exposed plants than control at all light intensities, and this difference was statistically significant at 50, 100 and 200 µmol•m^{-2}•s^{-1} PAR intensities (t-test, $P < 0.01$ under each light conditions).

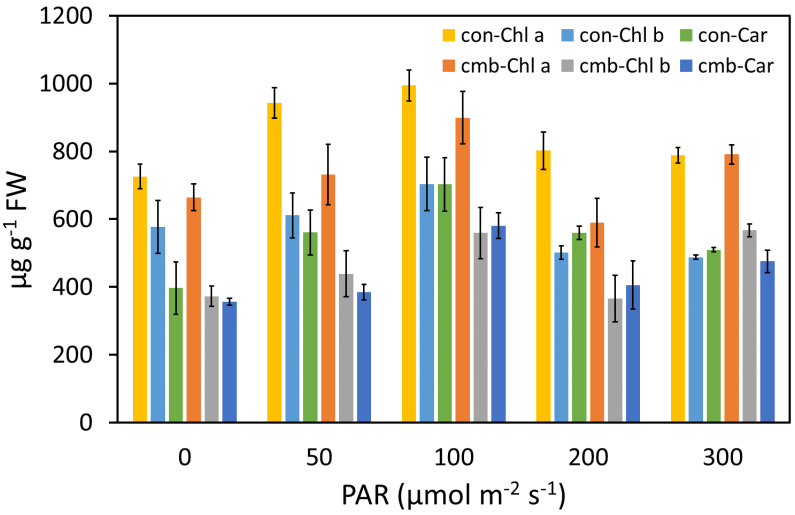


Figure 5-5: Chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoid (Car) content of *Egeria densa* after seven days of exposure to different photosynthetically active radiation (PAR) without exposure to *Microcystis aeruginosa* (con) and with exposure to *Microcystis aeruginosa* (cmb). Data are means ± standard deviation (n = 3).

5.3.4 Anthocyanin content of *Egeria densa*

Anthocyanin content of both control and *M. aeruginosa* exposed plants showed a similar trend with increasing light intensity (Figure 5-6). In both control and *M. aeruginosa* exposed conditions, anthocyanin content gradually increased until 100 µmol•m^{-2}•s^{-1} PAR intensity and then decreased under higher light intensities (200 and 300 µmol•m^{-2}•s^{-1} PAR). For control plants, the ANOVA grouped the following PAR treatments: 0 µmol•m^{-2}•s^{-1} PAR intensity; 50 and 200 µmol•m^{-2}•s^{-1}PAR intensities; 200 and 300 µmol•m^{-2}•s^{-1} PAR intensities; and 100 and 300 µmol•m^{-2}•s^{-1} PAR intensities (ANOVA, $P < 0.01$, F = 9.543). For the *M. aeruginosa* exposed plants, the ANOVA grouped the following PAR treatments: 0, 200, and 300 µmol•m^{-2}•s^{-1} PAR intensities; 50 and 300 µµmol•m^{-2}•s^{-1} PAR intensities; and 100 µmol•m^{-2}•s^{-1} PAR intensity (ANOVA, $P < 0.01$, F = 10.238). *Microcystis aeruginosa* exposed plants had a higher anthocyanin content than control plants, and this difference was statistically significant at 0, 50 and 100 µmol•m^{-2}•s^{-1} PAR intensities (t-test, $P < 0.01$ for 0 and 50 µmol•m^{-2}•s^{-1} PAR intensities and $P < 0.05$ for 100 µmol•m^{-2}•s^{-1} PAR intensity).

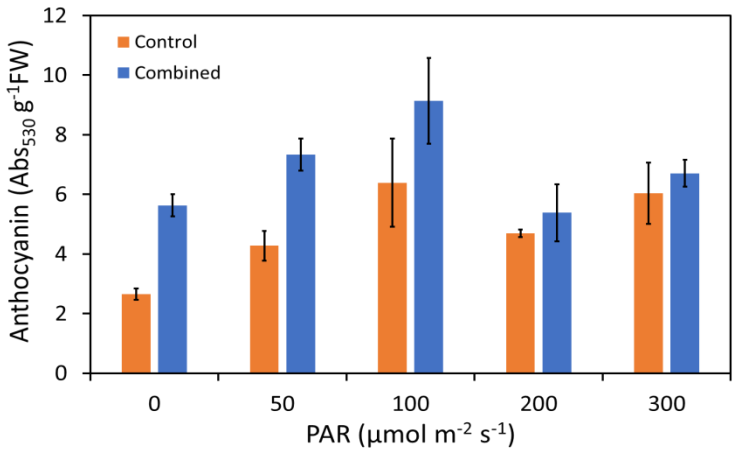


Figure 5-6: Anthocyanin content of *Egeria densa* after seven days of exposure to different photosynthetically active radiation (PAR) without exposure to *Microcystis aeruginosa* (Control) and with exposure to M. aeruginosa (Combined). Same number/s over the bars indicating observations are in the same ANOVA groups.

5.4 Discussion

The exposure to a low concentration of *M. aeruginosa* for seven days affected *E. densa* physiology and photosynthetic capacity, as shown by all measured parameters except for GPX. Although the presence of *M. aeruginosa* either increased or decreased these parameters, the changes in PAR intensity had a stronger effect on these *E. densa* parameters. Consequently, photosynthetic parameters of *E. densa* were not significantly different between control and plants exposed to *M. aeruginosa* at different PAR intensities. The lack of *M. aeruginosa* effect on *E. densa* biochemistry may be due to low *M. aeruginosa* density (OD_{730} = 0.04) maintained during this experiment and that water was not mixed to improve the cyanobacteria growth. Therefore, the altered parameters of *E. densa* should be the result of *M. aeruginosa–E. densa* interaction.

The Fv/Fm value is known to reduce under most stress conditions including light, salt, drought, and is therefore used to evaluate plant stress status(Banks 2018; Guidi, Lo Piccolo, and Landi 2019; Yan et al. 2015). Similarly, chlorophyll fluorescence parameters can be used to evaluate plant photosystem efficiency under stress(Banks

2018). In the present study, the Fv/Fm of *E. densa* slightly increased when exposed to *M. aeruginosa* for each light condition (including dark treatment), indicating that the presence of *M. aeruginosa* improved plants photosynthetic efficiency. However, *M. aeruginosa–E. densa* combined treatment plants had similar QY values, increased NPQ, and decreased Qp when compared to control, under each light intensity, indicating that the photon energy dissipated as heat without being utilised for photosynthesis (Müller, Li, and Niyogi 2001; Tietz et al. 2017). On the other hand, under *M. aeruginosa* exposure, the Ft values of plants were significantly higher in the darkness, unchanged under low light conditions (50 and 100 $\mu mol \bullet m^{-2} \bullet s^{-1}$) and decreased under high light exposure. As decreased Ft indicates stress, *M. aeruginosa* exposure increased plant stress under high light (Zarco-Tejada et al. 2003). In contrast, the increased plant Ft in the darkness may suggest that *M. aeruginosa* exposure reduced plant stress or promoted photosynthetic efficiency of *E. densa,* which is also reflected in the NPQ values.

The H_2O_2 content of the *M. aeruginosa–E. densa* combined experiments was higher than in control under each light condition; however, under 50 $\mu mol \bullet m^{-2} \bullet s^{-1}$ PAR intensity, the H_2O_2 content of the plants was extremely high. In a previous laboratory study, the optimal light conditions for *M. aeruginosa* were lower than 50 $\mu mol \bullet m^{-2} \bullet s^{-1}$ PAR intensity, and those light intensities exceeding 50 $\mu mol \bullet m^{-2} \bullet s^{-1}$PAR caused stress on *M. aeruginosa* (Muhetaer et al. 2020). According to Rodrigues and Thomaz and our preliminary studies (unpublished data), 80–100 $\mu mol \bullet m^{-2} \bullet s^{-1}$ PAR intensity is optimal for *E. densa*. Therefore, under darkness and 50 $\mu mol \bullet m^{-2} \bullet s^{-1}$ exposure conditions, the H_2O_2 content of *E. densa* may have increased because of the low light stress and the presence of *M. aeruginosa* allelopathy. Consequently, the allelopathic effect of *M. aeruginosa* on *E. densa* was the highest under the optimal light conditions for *M. aeruginosa* (50 $\mu mol \bullet m^{-2} \bullet s^{-1}$).

Anthocyanins can be plant stress indicators as they have antioxidant properties and play a role in plant protection from stress (Altangerel et al. 2017; Landi, Tattini, and Gould

2015) under the *M. aeruginosa* exposure, anthocyanin content was significantly higher than the control up to 100 µmol•m^{-2}•s^{-1} PAR intensity, which confirms the significant effect of *M. aeruginosa* on *E. densa* in this light range. The anthocyanin content of *E. densa* was more responsive than H_2O_2 content when plants were not exposed to *M. aeruginosa* (Pearson correlation: Control, Anthocyanin-H_2O_2, R = -0.737, $P < 0.01$; Plants exposed to *M. aeruginosa*, Anthocyanin-H_2O_2, R = -0.121, $P > 0.05$). Under the influence of *M. aeruginosa*, the content of all antioxidants (GPX, CAT, and APX) was lower than control until 100 µmol•m^{-2}•s^{-1} PAR intensity, while the anthocyanin content was higher. Therefore, the anthocyanin may play an antioxidant role in *E. densa* under 100 µmol•m^{-2}•s^{-1} PAR intensity, and under higher light conditions, the antioxidants took over the role of anthocyanin.

The photosynthetic pigment content *M. aeruginosa* exposed plants was significantly lower than control until 200 µmol•m^{-2}•s^{-1} PAR intensity. Under the 300 µmol•m^{-2}•s^{-1} PAR intensity, photosynthetic pigment content was similar in *M. aeruginosa* exposed and control plants, indicating that *M. aeruginosa* does not affect *E. densa* as much under higher light conditions. The contents of the photosynthetic pigments Chl a and Chl b were significantly lower in *M. aeruginosa* exposed *E. densa* plants than in control plants in a previous study at 40 µmol•m^{-2}•s^{-1} PAR intensity at very high cell densities (Amorim et al. 2017). Our results indicate that *E. densa* chlorophyll content is affected even by the very low starting density of *M. aeruginosa* (OD_{730} = 0.04). However, in the study of, the total carotenoids content was higher in *M. aeruginosa* exposed plants than in control plants, whereas in our study, it was not different under each light condition, which may be due to differences in exposure conditions between these two studies.

CHAPTER 6

CONCLUSION AND FURTHER RESEARCH

Phototropic species, Cyanobacteria has its range of comfort illumination, extreme lights both low and high are disadvantages to them. The three species studied in this research, despite the biological, physical, morphological and behavioural characteristics, all showed increased oxidative stress under PAR intensity exceeding 200 $\mu mol \bullet m^{-2} \bullet s^{-1}$. When the duration of exposure concerned, the findings are suggesting that cyanobacteria showing the excess formation of H_2O_2 under excess light within a short period when exposed to extreme light. However, the simultaneous elevation of the antioxidant enzymes preventing from oxidative stress up to a certain level; however, prolonged or extremely high light can exceed the oxidative stress than the antioxidant activity. This is leading to oxidative stress and as a result of oxidative stress, discoloration reduced optical density and reduced pigmentation of the cyanobacteria resulted. In the diurnally varying light conditions research conducted for a single day, found that cyanobacteria could exhibit proportional antioxidant activities with the oxidative stress. However, the present single day experiment suggests the existence of oxidative stress even after the absence of light, and antioxidant levels are reduced. interaction of cyanobacteria with other species, also influenced by the light intensity. Cyanobacteria can increase the oxidative stress on macrophytes and in conditions which favourable light intensities for cyanobacteria the influence is greater than unfavourable conditions.

In searching for effective control measures, the combination of light stress together with existing measures (bubbling or mixing) can be used to suppress the growth further. However, further research is recommended including investigating, recovery of cyanobacteria after high light exposure, macrophyte allopathy on cyanobacteria under different lights and influence of cyanobacterial circadian rhythm on light exposure.

Based on the current preliminary research, we found that cyanobacteria can be recovered from highlight exposure when the conditions become favourable for their

growth (Figure 6-1 and 6-2). Therefore, further studies should be considering the high light adaptation ability and recovery after high light exposure of cyanobacteria.

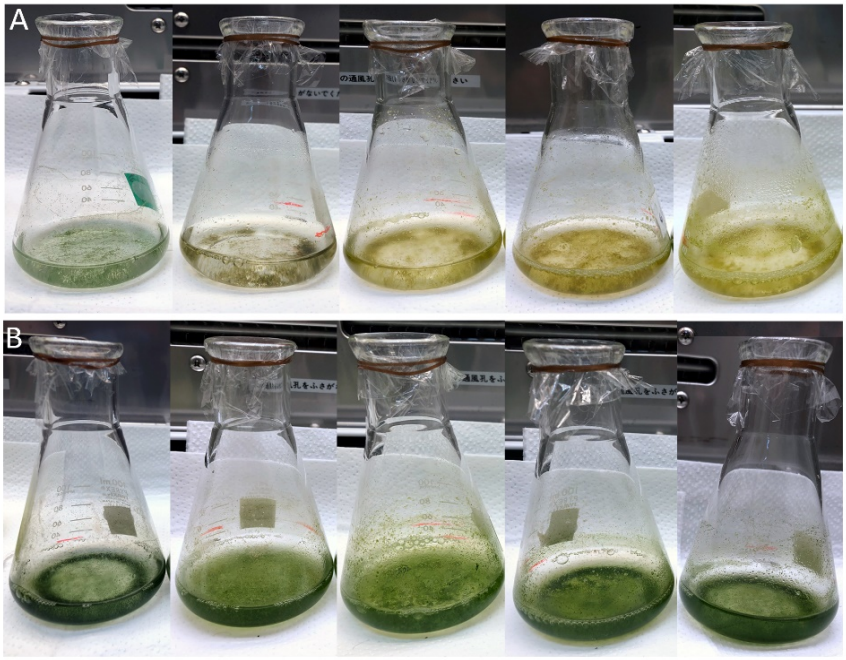


Figure 6-1: A – From left to right, 0 days (starting), 2 days, 4 days, 6 days and 8 days (12 hour light: 12 hour dark) exposure of 1000 µmol•m^{-2}•s^{-1} PAR. B – after 8 days recovery under 30 µmol•m^{-2}•s^{-1} PAR (12 hour light: 12 hour dark) after exposing to 1000 µmol•m^{-2}•s^{-1} PAR from left 0 days (starting), 2 days, 4 days, 6 days and 8 days.

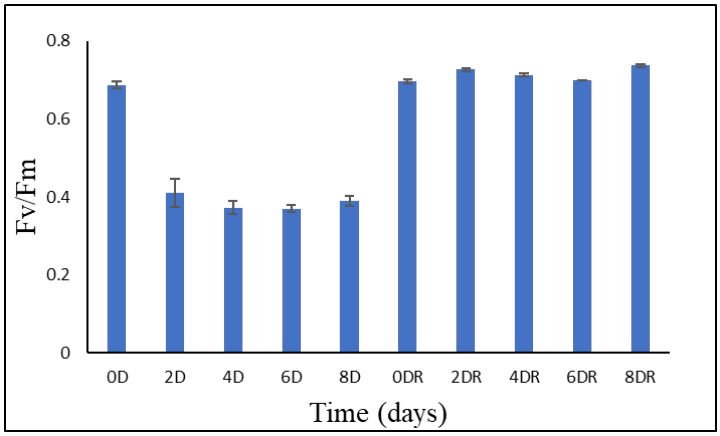


Figure 6-2: Fv/Fm of *Mycrosistis argunosa* after highlight exposure for different days and after 8 days recovery. X-axis, numbers represent the number of days exposed to 1000 µmol•m^{-2}•s^{-1} PAR. Letter D represents 'days,' and letter R represents '8 days recovery'. Y axis represents the Fv/Fm values.

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