# Application of hydrogen peroxide as an oxidative stress marker in plant and cyanobacteria

# (植物およびシアノバクテリアにおける酸化ストレスマーカーとしての過酸化水素の応用)

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**Dedicated to my beloved parents**

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# Abstract

Hydrogen peroxide ($H_2O_2$) is the two-electron reduction product of $O_2$. Under a stressful environment, endogenous reactive oxygen species (ROS) production, including superoxide, hydroxyl radicals, and $H_2O_2$ concentration exceeds its scavenging capacity. Reactive oxygen species are essential for growth regulation and signaling mechanisms in photosynthetic organisms. Accumulation of excessive ROS inside cells causes harmful impacts such as disrupting cellular homeostasis, causing membrane lipid peroxidation, protein oxidation, enzyme inhibition, and DNA and RNA damage. The $H_2O_2$ in plant and cyanobacteria cells are relatively stable and can easily be measured. $H_2O_2$ measurement is suitable with minimum losses compared with other ROS, such as the superoxide radical (($O_2^{\cdot-}$) and the hydroxyl radical ($OH^{\cdot-}$). $H_2O_2$ has been extensively used to quantify ROS damage or stress levels in many plant and cyanobacteria studies. Therefore, quantification of $H_2O_2$ can play a vital role in measuring oxidative stress more accurately. We are trying to develop a rapid method for evaluating environmental stressors in plants by measuring $H_2O_2$ concentration. In riparian vegetation management, this method can be used, and the stress intensity can be measured within a short period of time. This will reduce the cost and time consumed by other methods like the observation of plant traits. To evaluate the application of $H_2O_2$ quantification in stressed conditions reliably and accurately, individual experiments were conducted in plants and cyanobacteria.

To quantify $H_2O_2$ concentration quickly and easily, a simple and easy method is needed. If we can get similar results using two methods, it will be more appropriate than using one individual method. The spectrophotometric methods of modified ferrous oxidation xylenol orange (eFOX) assay and a Titanium sulfate ($Ti(SO_4)_2$) assay can be used for this purpose. In spite of some interferences, both methods are widely accepted for their sensitivities and reliability. Leaf samples were collected from a riparian vegetation zone on sunny days of *Ambrosia trifida*, *Solidago altissima*, *Artemisia princeps*, and *Sicyos angulatus*. The $H_2O_2$ concentration on plant leaves was evaluated in two groups: Nonfrozen: As soon as the samples arrived in the laboratory after collection, they were prepared for analysis; and Frozen: After being transported to the laboratory, the samples were kept in the refrigerator at -80$^o$C for 25 days. Frozen samples were prepared after 25 days in order to compare $H_2O_2$ concentration with those of nonfrozen samples. Due to higher sensitivity, the eFOX assay is capable of measuring even lower fluctuations of $H_2O_2$ concentration more accurately in comparison to the $Ti(SO_4)_2$ assay. There was a substantial decreasing correlation observed due to chilling stress from nonfrozen to frozen samples in eFOX and $Ti(SO_4)_2$. $H_2O_2$ concentration measurement was not affected by sample weight using either method regardless of whether the sample was frozen or nonfrozen. Each species showed a significantly high correlation between the eFOX and $Ti(SO_4)_2$ assays in nonfrozen conditions for each species. These findings indicate that in riparian plants, the ideal condition for measuring $H_2O_2$ concentration is nonfrozen. It is also found that both methods can be used to measure $H_2O_2$ concentration to quantify both biotic and abiotic oxidative stress on a large scale.

The foliar $H_2O_2$ concentration is also used to assess environmental stress to find the spatial distribution of plants in riparian vegetation communities. Samples were collected from Ara River, Hii river, and Arakawa-Tarouemon. *Salix spp*., *Robinia pseudoacacia*, *Ailanthus altissima* with *Juglans mandshurica*, *Phragmites australis*, *Phragmites japonica*, and *Miscanthus sacchariflorus*, *Sicyos angulatus*, and *Solidago altissima* were selected for this study. Leaf tissues were collected to analyze $H_2O_2$ concentration. Meanwhile, riparian soil was sampled to measure total nitrogen (TN), total phosphorus (TP), and moisture content. In this study, we found a unique significant interaction between soil moisture content and $H_2O_2$

concentration, both positively or negatively correlated relationships, when compared with other parameters, such as TN or TP concentrations or TN: TP in riparian soil. The generalized additive model (GAM) confirms that only $H_2O_2$ significantly correlated with soil moisture than other parameters like TN or TP concentrations or TN: TP. The species-specific distribution zones can be explained by the $H_2O_2$ concentration in the plant for gravelly and sandy channels on a theoretical range of soil moisture. Hence, the present study suggests that foliar $H_2O_2$ concentration can be a useful benchmark for the distribution potentiality of riparian vegetation management.

In the cyanobacteria, $H_2O_2$ concentration measurement can give a threshold level for growth a nd control measurement. To understand the cyanobacterial elimination process, three laborato ry experiments were performed. 1. One-day exposure: A laboratory experiment was conducte d to evaluate the combined effect of diurnal light intensity in different temperatures on *Microc ystis aeruginosa* and *Phormidium ambiguum*. 2. Long-term exposure: This study aims to suppr ess *Phormidium ambiguum and Pseudanabaena foetida* using light intensities and fluctuating t emperatures. 3. Nutrient depletion: The effect of combined stresses, photoinhibition, and nutr ient depletion on the oxidative stress of cyanobacteria was measured in laboratory experiments to develop the biomass prediction model of *Phormidium ambiguum*.

1. One-day exposure: In order to acclimatize the *M. aeruginosa* and *P. ambiguum* cells, the sufficient cells were transferred to three incubators for 23 days at 30°C, 20°C, and 10°C. The light intensities changed diurnally hourly by 100 µmol $m^{-2}$ $s^{-1}$ from 6:00 to 18:00, and maximum light intensity of 600 µmol $m^{-2}$ $s^{-1}$ was reached at 11:00 to 13:00 after 23 days of incubation before decreasing parallel until 18:00. Hydrogen peroxide ($H_2O_2$), protein concentration, chlorophyll-a (Chl-a) content, and catalase (CAT) activity were measured at intervals of three hours - 6:00, 9:00, 12:00, 15:00, 18:00, and 21:00. $H_2O_2$, Chl-a, and CAT increased in both *M. aeruginosa* and *P. ambiguum* until 15:00, 3 hrs after the peak of photosynthetically active radiation (PAR), then decreased. CAT activity was directly proportional to $H_2O_2$ concentration at each temperature. $H_2O_2$ concentration and protein content were highest at 20°C with *M.aeruginosa*, although values were similar between 20°C and 30°C with P. *ambiguum*. The temperature effect was significantly larger in *M. aeruginosa*, compared with *P. ambiguum* on $H_2O_2$ concentration, protein concentration, and Chl-a content. In the summer season, *M. aeruginosa* has a higher competitive advantage over *P. ambiguum*. Cyanobacterial blooms can be treated with these findings.

2. Long-term exposure: Three incubators were used to acclimate *P. ambiguum* and *P. foetida* cultures at 30°C, 20°C, and 10°C for 24 days each. The starting day samples were collected on the 24 days of acclimatization, and the second samples were collected seven days later for the five light intensities, 10, 30, 50, 200, and 600 µmol $m^{-2}$ $s^{-1}$ in each temperature. Optical density ($OD_{730}$), hydrogen peroxide ($H_2O_2$) concentration, protein content, chlorophyll-a (Chl-a), and catalase (CAT) were measured from the samples. $H_2O_2$ concentration increased during the seven-day exposure compared to the starting day until 200 µmol $m^{-2}$ $s^{-1}$ light intensity in each temperature for both species. Chl-a showed a decreasing trend after 50 µmol$m^{-2}s^{-1}$ in each temperature, then affected the protein content in *P. ambiguum* and *P. foetid*. High light intensity and lower temperatures (20°C and 10°C) introduce oxidative stresses that can reduce algal biomass. CAT activity increases proportionately with $H_2O_2$ or biomass-contained values, $H_2O_2$/protein. Due to the temperature effect low growth rate was observed for *P. ambiguum* and *P. foetida* at 10°C compared to 30°C. Hence, lowering temperatures and increasing light intensities are necessary to treat algal blooms.

3. Nutrient depletion: *Phormidium ambiguum* was exposed to various photosynthetically active radiation (PAR) intensities and phosphorous concentrations with fixed nitrogen concentrations. The samples were subjected to stress assays by detecting the $H_2O_2$ concentration and antioxidant activities of catalase (CAT) and superoxide dismutase (SOD). $H_2O_2$ concentrations decreased to 30 $\mu molm^{-2}s^{-1}$ of PAR, then increased further with higher PAR intensities. Regarding phosphorus concentrations, $H_2O_2$ concentrations ($nmolL^{-1}$) generally decreased with increasing phosphorus concentrations. SOD and CAT activities were proportionate to the $H_2O_2$ $protein^{-1}$. No $H_2O_2$ concentrations detected outside cells indicated the biological production of $H_2O_2$, and the accumulated $H_2O_2$ concentration inside cells was parameterized with $H_2O_2$ concentration $protein^{-1}$. With over 30 $\mu molm^{-2}s^{-1}$ of PAR, $H_2O_2$ concentration $protein^{-1}$ had a similar increasing trend with PAR intensity, independently of phosphorous concentration. Meanwhile, with increasing phosphorous concentration, $H_2O_2$ $protein^{-1}$ decreased in a similar pattern regardless of PAR intensity. Protein content decreased with gradually increasing $H_2O_2$ up to 4nmol $H_2O_2$ $mg^{-1}$protein, which provides a threshold to restrict the growth of cyanobacteria.

High light intensities, temperatures alterations, and nutrient depletion can be a countermeasure against algal blooms over the aeration circulation, and vertical mixing process. The aeration circulation and vertical mixing have limitations and cannot eradicate cyanobacteria for more than three weeks after the treatment.

Different methods were used to measure $H_2O_2$ concentrations in leaves, and highly correlation between methods implies the efficacy of measuring the oxidative stress of plants rapidly. By measuring the soil moisture and nutrient concentration from the soil, we grasped the environment suitable for the growth of each species and showed that it matches the environment where plant species actually grow. We can also demonstrate the control measure of native and invasive plant species in Japan.

# Chapter 1: Introduction

## 1.1 General discussion about Hydrogen peroxide ($H_2O_2$)

Hydrogen peroxide is formed when oxygen is reduced by two electrons. Despite its potential for reactivity, it is not a free radical (Halliwell *et al*. 2000). Compared to superoxide, $O_2^{\bullet}$, and certainly to the hydroxyl radical ($^{\bullet}OH$), $H_2O_2$ is relatively "safe": in the absence of transition metals, it is stable and unreactive, even at concentrations much higher than a biological system would ever generate. Additionally, this confers greater mobility within tissues and can serve not only as a substrate but also as a molecule that can communicate with reactive oxygen species (ROS). Fenton reaction occurs when $H_2O_2$ combines with molecules containing Ferus ($Fe^{2+}$) or other transition metals. (Becana *et al*. 1998). The "evil" result of this reaction is the homolysis of $H_2O_2$ to 2 $^{\bullet}OH$, and $H_2O_2$ toxicity is most commonly associated with that action. An identical fragmentation pattern occurred in intact chloroplasts when oxidant scavenging systems were inhibited (Ishida *et al*. 1999), or when cold-sensitive maize leaves were exposed to low temperatures (Kingston-Smith *et al*. 1999), as a consequence of the fact that chloroplasts contain as much as 80% of the Fe in a plant, and are a good source of radical oxygen species (ROS) generally. A catalase or an ascorbate peroxidase enzyme can remove $H_2O_2$ enzymatically to reduce its toxicity, or by complexing Fe(III) and Fe(II) with compounds such as tannic acid and proanthocyanidins, thus preventing $^{\bullet}OH$ generation (Toda 2005; Andrade *et al*. 2006).

### 1.1.1 What are the relevant tissue levels of $H_2O_2$?

It is surprising that there are such wide differences between estimates for $H_2O_2$ in plant tissues spanning nearly seven orders of magnitude and no apparent consensus on the size of a physiologically significant change caused by stress or treatment. There are so many good methods for assaying $H_2O_2$ in solutions, some of them quite specific. At the low end, Hernández *et al*. (2001) reported tissue levels ranging from 10 to 150 pmol/ gFW in the pea leaf apoplast with the difference (salt-induced) being sufficient to cause oxidative lesions. At the other extreme, He *et al*. (2005) reported concentrations in *Poa pratensis* leaves as high as 1.3% of the dry weight, which, based on data in their report, was 60 µmol/gFW or 100 mM on a leaf water basis. In maize, Tewari et al. (2004) reported $H_2O_2$ concentrations of 20 µmol/gFW, rising to 75 µmol/gFW in N-deficient conditions. Ben Amor *et al*. (2006), in an interesting study of the coastal halophyte, *Cakile maritima* also reported tissue $H_2O_2$ contents on the high end, as much as 45 µmol/gFW. Veljovik-Jovanovic *et al*. (2002) were the first to recognize possible interferences by plant constituents with $H_2O_2$ assay protocols, and suggested that leaf levels should generally be less than 0.1 µmol/gFW. In contrast, an analysis of field grown plants, adjusting for possible interferences and $H_2O_2$ metabolism after harvest, suggested values between 1-5 µmol/gFW might be reasonable (Cheeseman 2006). A similar confusion exists regarding what an "oxidative burst" means based on tissue-level $H_2O_2$ concentrations. The rapid turnover of $H_2O_2$ in plants and after tissue harvest complicates this issue (Cheeseman 2006). In response to acute ozone exposure, (200 ppb/ 2 hr), Chen and Gallie (2005) reported tobacco (cv. 'Xanthi') leaf $H_2O_2$ levels had increased 4x (in the 100 nmol/gFW range, using plants grown with as little prior ozone exposure and potential irradiance stress as possible), but increased another four to five-fold after 24 hr recovery. Karpinski *et al*. (1997) reported an

oxidative burst in *Arabidopsis* with exposure to excess irradiance in this case, ten-fold higher than their growth irradiance of 200 µmol $m^{-2}$ $s^{-1}$ that increased the leaf content from about 5 µmol/gFW to less than 7 µmol/ gFW.

### 1.2 Mechanism of generation of $H_2O_2$ in plants and cyanobacteria

The energy coming from the sun reaches the earth as light, and since light also has the character of particles, when light collides with a molecule in the body, the electrons in the orbit of that molecule absorb the energy of the light and are excited. However, the excited state does not last long and immediately returns to the ground state, and the energy obtained at this time is lost as heat or fluorescence. Therefore, if the electrons excited by this light energy can be captured well, the obtained energy can be effectively used instead of being lost as heat or fluorescence. Plants and cyanobacteria capture these electrons through the electron transport chain on the thylakoid membrane of chloroplasts. Condensing pigments (pigments such as chlorophyll and carotenoids) that collect radio waves exist on the tyracoid film, and when light hits the condensing pigments lined up like roof tiles, the excited state of electrons is efficiently generated. It is sent to the internal reaction mechanism. In the reaction mechanism, we will proceed to the photochemical system (Koda et al., 2003; Beecraft et al., 2019). Figure 1.2.1 shows the simplified electron transfer of the thylakoid membrane.

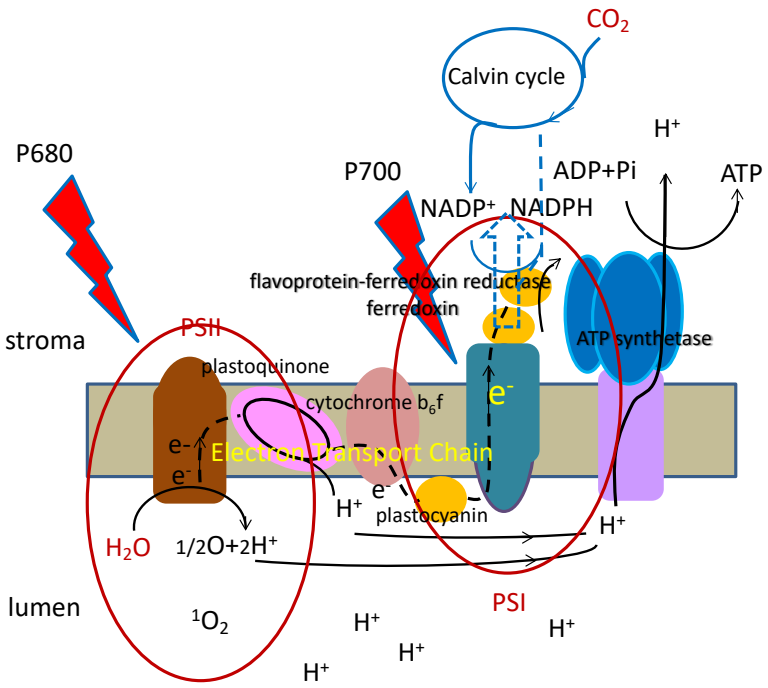


Figure 1.2.1 Electron transfer mechanism of thylakoid membrane

In the photochemical system, light energy is used to extract electrons from water to generate oxygen, and at the same time, it produces reduced power and chemical energy. This reducing power generates oxygen and generates superoxide radicals. The generated active oxygen is converted to hydrogen peroxide by superoxide dismutase (SOD) on the thylakoid membrane. After that, antioxidant enzymes such as ascorbate peroxidase (APX) work, and the generated

hydrogen peroxide is rapidly reduced to water (Asada, 1999). In this reaction, ascorbic acid (ASA) is used as an electron donor, and after the reaction, monodehydroascorbic acid (MDA) is oxidized, but the electrons generated by the oxidation of water reduce this, so APA is regenerated. NS. This series of flow is called "water-water cycle", and when the number of electrons extracted from water is excessive, that is, the reducing power is excessive, the route of "superoxide radical-> hydrogen peroxide-> water" is followed. It has the function of returning electromagnetic waves to the original state of water and preventing excessive reduction (Ogata, 2002; Asada, 2003). The series of reaction formulas for the water-water cycle is as follows. (Asada, 1999, Koda et al., 2003)

1. $2H_2O \rightarrow 4e^- + 4H^+ + O_2$ (Occurrence of $O_2$)
2. $2O_2 + 2e^- \rightarrow 2 \cdot O_2^-$ (Generation of superoxide radical)
3. $2 \cdot O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ (Hydrogen peroxide production by SOD)
4. $H_2O_2 + 2ASA \rightarrow 2H_2O + 2MDA$ (Reduction reaction by antioxidant enzyme)
5. $2MDA + 2e^- + 2H^+ \rightarrow 2ASA$ (Reduction reaction of MDA)

計 $2H_2O + O_2 \rightarrow O_2 + 2H_2O$

Figure 1.2.2 shows a schematic diagram of oxygen being reduced and converted to water. agreement)

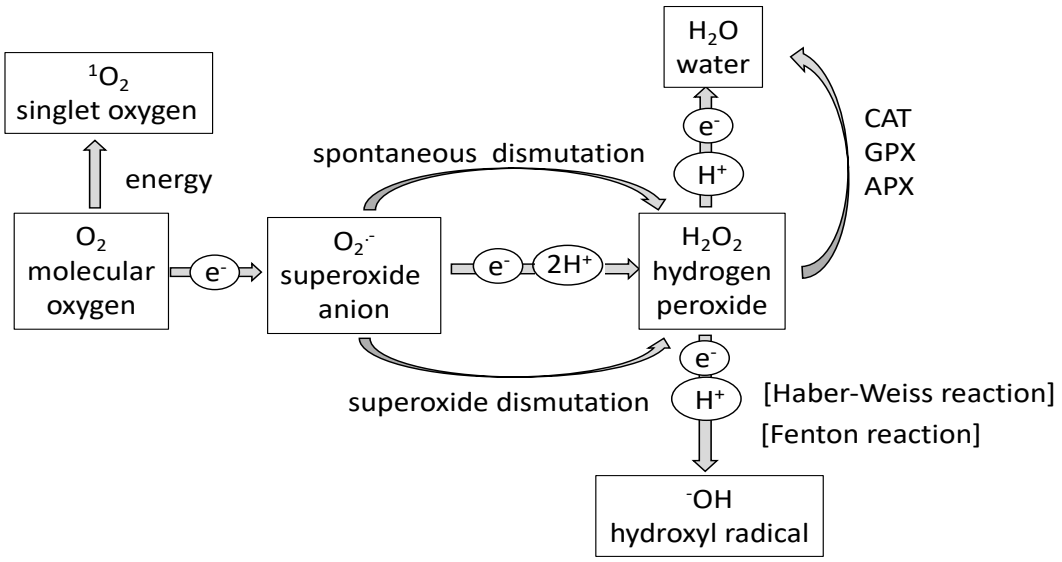


Figure 1.2.2 Flow from the conversion of oxygen to water

When the environment stresses, the activity of the carbon dioxide fixation system of the carbyne-benzene circuit decreases (Choudhury et al., 2013). For example, the activity of carbon dioxide-fixing enzymes decreases when exposed to environmental stress due to water temperature (Chandani et al., 2018). When the activity of the carbon dioxide fixation system decreases, the activity of the carbyne-benzene circuit itself also decreases, so the reduction reaction in the circuit also decreases, and the reducing power and electrons generated in the photosystem become excessive and become over reduced (Nishiyama et al., 2006). Furthermore, since oxygen is generated in the photosystem, oxygen is reduced by the reducing

power that should be originally used for reduction in the carbyne-benzone circuit, superoxide radicals are generated, and hydrogen peroxide is generated (Asaeda et al., 2017).
Hydrogen peroxide is produced even under normal conditions without much stress, but it is immediately detoxified by the water-water cycle. However, when environmental stress is applied, hydrogen peroxide is excessively produced, and if it exceeds a certain amount that can be detoxified by antioxidant enzymes, biological components such as proteins, lipids, and nucleic acids are damaged, and Lead to death (Selman et al., 2002; Schuurmans, et al., 2018). From the above, it is considered that environmental stress can be quantified by measuring the concentration of hydrogen peroxide in the body. Figure 1.2.3 shows the flow of reducing power under normal and stress conditions.

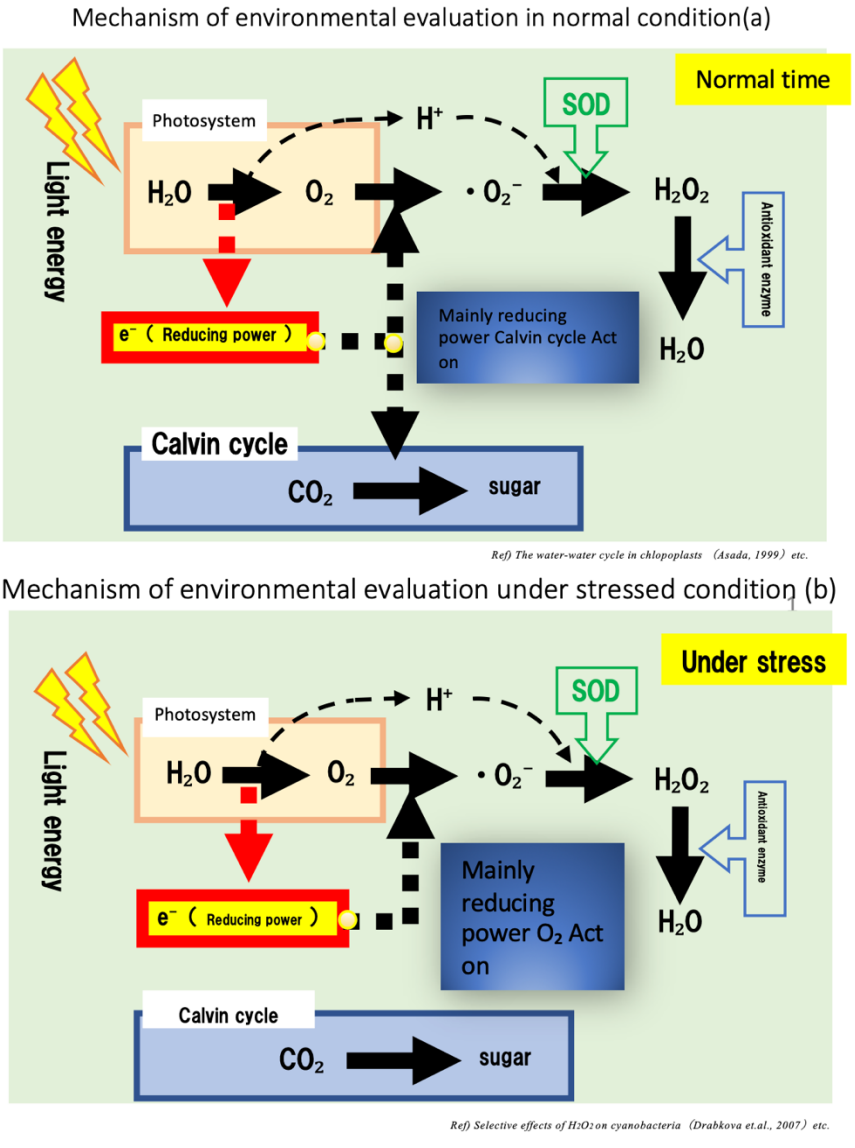


Figure1.2.3 $H_2O_2$ generation in both normal and stressed conditions

## 1.3 Riparian vegetation

Rivers and streams are interconnected by riparian zones. Vegetation along the riparian zone is known as riparian vegetation. In order for the riparian ecosystem to function properly, it plays an important role. Rivers and streams provide the best habitat for aquatic species to grow and reproduce in the riparian zone (Allan et al., 1993). A healthy stream is a mix of riparian plants and aquatic species that depend on natural hydrologic regimes for survival. As a result of human disturbances, the riparian zones are undergoing great damage, which may disrupt the plants and habitats in those areas (Vitousek et al., 1997). Lakes and waterways, bounded by riparian plant communities and landforms, provide important transportation corridors. Riparian zones exhibit wide ranges of physical fluctuations that appear in community characteristics that are distinctly expressed by a broad array of life history actions and by chronological and analytical patterns (Vitousek et al., 1997). Therefore, riparian zones are among the biosphere's most composite ecological systems. Assemblages of communities, physical drainage networks, and processes shaping stream channels all contribute to the vulnerability of biotic virtue found in natural riparian zones. Biotic and abiotic factors interact intensely to form riparian communities. No matter when they occur or when they have occurred (Jankovsky 1994).

### 1.3.1 Importance of riparian zone

Riparian zones act as sources of linkage in the ecosystems within a landscape. Landscapes are composed of (often overlapping) patches of different types of vegetation, soil and available nutrients, moisture and light (Rosell et al., 2005). Disturbances in the landscape, such as fire, pest outbreaks or even the death of a single tree, create these patches, which provide different types of habitats. This diversification of environment is important, but so is relatedness between them. Riparian zones grant wildlife to travel between habitat “islands” by providing transportation corridors, and help to circulate nutrients between different ecosystems (Trombulak & Frissell, 2000). Since riparian zones are wetter than the surrounding landscape, they also often resist destruction by fire, and recover more quickly. This helps the landscape as a whole to recover.

## 1.4 Literature review on environmental stressors in plants

Riparian vegetation is naturally adapted to abiotic conditions characterized by fluctuating water, sediment, and nutrients (Gurnell et al., 2004). Riparian plants are diverse in species, structure, and regeneration strategies (Maingi et al., 2006). Therefore, riparian habitats are regarded as biodiversity corridors for restoration (Corbacho et al., 2003; Heuner et al., 2015), providing an ecotone between the terrestrial and aquatic ecosystems. Despite the importance of riparian vegetation, riparian degradation often occurs due to various natural disturbances and human activities reducing species diversity.This degradation affects the composition and the plant community structure (Mligo 2016). The magnitude, frequency, and duration of flood events have a distinct impact on the creation of the riparian environment. This impact occurs through inundation frequency, level, duration (Casanova et al., 2000), and flow velocity during the inundated period, driving erosion in other fluvial landforms and deposition of the transported sediment. The sediment moisture content of riparian zones is associated with the stratigraphy

of alluvium, groundwater, hyporheic flows, and the position in the flood or plain corridor. However, it generally decreases with elevation, depending on the sediment particle size. These conditions help a riparian species to grow at the elevation of its preferred riparian soil moisture zone. Due to frequent flood disturbances the sediment accumulations cannot develop properly. As a result, its nutrient level may decrease (Rashid and Asaeda 2015). The sediment nutrient level may also determine which species can distribute at specific-zones (Asaeda et al, 2015). The sediment nutrient level in riparian soil, especially TN or TP concentration, can filter and alter its biogeochemistry. For instance, wetland plants take up 16% to 75% of TN (Reddy et al., 1987). TN and TP contents of plant biomass have organ-specific differences. Plant's N: P ratios <10 and >20 often (not always) correspond to N- and P-limited biomass production in short term periods respectively, although it can vary in the long term (Güsewell et al., 2003).

The species distribution depends on previously experienced abiotic stresses. However, when subjected to flood disturbance, river habitat conditions change frequently and often suddenly, which is related to the source of deposited sediment and fluvial dynamics. Therefore, it is difficult to evaluate the spatial distribution of each species. There may be an effect with the presence and abundance of dominant factors in preference to promote or inhibit under these complex and constantly changing stressful environments.
In the current system, only long-term monitoring of plant traits, such as growth rate and biomass, is commonly employed to obtain whether the plants can grow or die, or whether their colonies can be established (Riis et al., 2012; O'Hare et al., 2018). One of the reasons is that there is no other method to evaluate the plant's physiological/ecological condition in a short period of experiments. There are various types of effective stresses affecting the plant physiology/ecology in nature. In flume experiments, we need to keep a steady condition of other factors for a suitably long period, even if we look at the effect of drag force, only. In fields, environmental conditions frequently change and it is difficult to keep the same condition. Hence, measuring $H_2O_2$ concentration can give us a rapid and cost-effective way to obtain oxidative stress of plants.

Living organisms and biological systems play a significant role against stress to prevent or repair damage. When plants are subjected to environmental stress through metabolic and physiological adjustments, ROS is generated in different organelles depending on the stressor types (e.g., anoxia, drought). Some ROS is scavenged relatively quickly by antioxidants, and the homogeneity of ROS in tissues is maintained by balancing the ROS and antioxidants. The balance flips over when oxidative stress surpasses the scavenging capacity of the antioxidants. During exposure to different types of environmental stressors, $H_2O_2$ is generated (Sharma et al., 2012; Dumon 2019). The above studies focus various environmental factor. Each factors can be stressful or not. A suitable and reliable way must be developed. The quantification of $H_2O_2$ can be used in large scale.

$H_2O_2$ quantification is difficult due to a variety of factors. The addition of chemicals, such as salicylic acid, increases $H_2O_2$ concentration in tomato leaves from 0.15 µmol/gFW to 0.25 µmol/gFW (Chen et al., 1993). The concentration of $H_2O_2$ in pear fruit tissue was increased from 0.35 mol/gFW to 0.8 mol/gFW in response to potassium cyanide with a $TiSO_4$ assay (Brennan and Frenkel, 1977). *Bruguiera parviflora*'s $H_2O_2$ concentrations rose from 0.067 µmol/gFW to 0.089 µmol/gFW under greenhouse hydroponic conditions (Parida & Das, 2005). In addition, the results of $H_2O_2$ concentration vary due to the sensitivity of the methods applied and interference from other redox-active compounds (Queval et al., 2008). For example, Apple leaves were estimated to contain 20–70 nmol/g FW of $H_2O_2$ using the $TiSO_4$ assay (Okazaki et

al. 1998), while 5–25 nmol/g FW was found using the Bioxytech $H_2O_2$-560 colorimetric assay (Vilaplana et al. 2006). In pears, 0.5–0.8 μmol/g FW of $H_2O_2$ was quantified utilizing the $TiSO_4$ assay, while 6–11 nmol/g FW was observed using the Bioxytech $H_2O_2$-560 kit (Lentheric et al., 2003). The estimation of $H_2O_2$ concentration in living tissues has been achieved using a variety of techniques (Halliwell and Gutteridge, 2015). $H_2O_2$ levels are commonly measured with peroxidase assays (Demmano et al., 1996), Amplex Red $H_2O_2$ detection kit (Orozco-Cárdenas and Ryan, 2002), 3,3-diaminobenzidine (DAB) (Thordal-Christensen et al., 1997), DCFDA (Di chloro dihydro fluorescein diacetate) (Yao et al., 2002), fluorescence (Jimenez et al., 2002) and chemiluminescence (Jones and Lee, 2019). As with superoxide detection, many methods used to measure $H_2O_2$, such as DAB and DCFDA, are poor specificities, thus measuring generalized oxidative stress rather than a specific ROS (Halliwell & Whiteman, 2004). As a result, it is necessary to use more appropriate methods to determine $H_2O_2$ concentration in plant tissues. The spectrophotometric reading is a very popular method to detect $H_2O_2$ concentration. Various researchers have developed spectrophotometric techniques from time to time (Jana & Choudhuri, 1981; Patterson et al., 1984; Zhou et al., 2006). The determination of $H_2O_2$ in plant tissues, particularly leaves, has been described in numerous studies. Among the major spectrophotometric assays is one using a Titanium $H_2O_2$ color complex ($Ti(SO_4)_2$ assay) (Brennan & Frenkel, 1977), and another using ferrous ions being oxidized by $H_2O_2$ to ferric ions (Gay et al., 1999). In spite of some interference between the eFOX and $Ti(SO_4)_2$ assays during the measurement process (Patterson et al., 1984; Veljovic-Jovanovic et al., 2002; Queval et al., 2008), their correlation in quantifying plant leaves is unknown. Hence, the correlation between the eFOX and $Ti(SO_4)_2$ assays can be used as a new approach to quantify $H_2O_2$ easily and readily.

## 1.5 Cyanobacterial bloom management using $H_2O_2$ as an indicator

### 1.5.1 Freshwater ecosystems

Lakes, ponds, rivers, streams, springs, bogs, and wetlands are among the freshwater ecosystems. Most terrestrial organisms require freshwater to survive. A fresh water resource contains less than 0.5 parts per thousand dissolved salts (Dodds 2002). Rain and snow are the ultimate sources of freshwater. 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 $km^3$. Humans have access to freshwater in lakes, rivers, soil moisture, and shallow groundwater basins. There are approximately 200 000 $km^3$ of usable freshwater resources in the world, which is less than 1 % of all freshwater and 0.01 % of all water on earth (UNEP 2002).

### 1.5.2 Physical environment in fresh waters

Biota in an ecosystem is largely influenced by the intensity and quality of the light (spectral quality). The amount of light a water body receives depends on its geographical location, season, and weather conditions. The water column also controls light penetration (attenuation) by dissolved and particulate matter (Boavida 1999).

Another important factor is the temperature, which is also affected by the light intensity or the season. The water column of deeper freshwater ecosystems can stratify, due to the warming of the surface water (epilimnion) and the cooling of the deeper layers (hypolimnion). Metalimnion

refers to the boundary between epilimnion and hypolimnion, which has sharp temperature changes (thermocline). A lake's chemical cycling relies on these two layers, and therefore its biota relies on them as well (Teneva et al. 2005). Epilimnion becomes colder in colder seasons, and its density reaches hypolimnion's density before cooling continues.

### 1.5.3 Chemical environment in fresh waters

Biological or chemical reactions produce oxygen as dissolved oxygen (DO). By diffusion and mixing, oxygen mixes with water primarily from the atmosphere. Anoxic conditions can occur when biological demands for oxygen exceed the Oxygen supply (Tanabe et al. 2018). Hypolimnion can experience this during summer and winter when stratified lakes are isolated from the atmosphere. Oxygen depletion in rivers can also be caused by excessive organic loading. Under anoxic conditions, only a few specialized bacteria and macro organisms can survive (Sinetova and Los 2016).

### 1.5.4 Cyanobacteria Bloom

### 1.5.4.1 Reproductive characteristics

Between May and October, cyanobacteria proliferate abnormally under strong solar radiation. Cyanobacteria reproduce by dividing individual cells, since they are single-celled aquatic organisms. Breeding and splitting rates are usually higher than 1 per day, and can reach 3 to 5 times per day at their highest. The number of cells in a liter of water can exceed 1 million when the concentration is high.

### 1.5.4.2 Movement of cyanobacteria

Studies on the living rules of cyanobacteria have revealed that in the morning and at noon, cyanobacteria floated on top of the water, gathered in large numbers, dispersed in the afternoon, sunk to the surface at night, followed by a repeat of the process on the following day. As a result of studies on cyanobacteria, it has been shown that they maintain their floating and sinking positions by adjusting the size of the gas nucleus inside the cell. As soon as the sun comes up in the morning, the photosynthetic reaction of the cyanobacteria is intensified and the gas nucleus becomes larger, so at the surface of the water, it floats atop the water. In the event that the photosynthetic reaction is strengthened further, the pressure within the nucleus will rise, and finally, the weaker gas nucleus will rupture due to the increased pressure inside. Cyanobacteria are buoyant after the bubble ruptures. Drop and settle below the water surface. Cyanobacteria can rest underwater since the 10 cm sunlight intensity is only a few percent of incident sunlight (Garcia- Pichel et al. 2020).

### 1.5.4.3 Toxicity of cyanobacteria

Besides destroying the landscape, making the water smelly, and causing the water to be contaminated with microcystin (MC-LR), cyanobacteria pose a threat to public health. 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. There have been reports of dermatitis caused by cyanobacteria in Hawaii and Japan. There have been numerous reports of gastroenteritis, hepatitis, headaches, dizziness, dysentery, and other symptoms caused by drinking cyanobacteria in the United States, Australia, the United Kingdom, and other countries. 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. Based on Paerl (Paerl et al. 2001), there is between 40 and 210 ug of toxic content per 100 mg of cyanobacteria in the water body of the bloom, indicating that cyanobacteria are extremely toxic.

### 1.5.5 General cause of cyanobacteria blooms

### 1.5.5.1 Internal factors - biological characteristics of cyanobacteria

Cyanobacteria can ingest inorganic carbon and nutrients excessively when exposed to high temperatures, low light intensity, and ultraviolet light, and they benefit from a low nitrogen-phosphorus ratio. After two months of cultivation in the pond waters, a large amount of plankton has produced and converted energy, and in addition to the rich nitrate, the other two nutrients, phosphates and silicates, have also 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. Nitrate and ammonia nitrogen accumulate in this process, which is called biological nitrogen fixation. Cyanobacteria that prefer high nitrogen and low phosphorus have unique growth characteristics.

### 1.5.5.2 External factors- environmental conditions

The presence of a large number of cyanobacteria and hydrometeorological condition. Cyanobacteria growth: cyanobacteria + light + temperature + suitable environment.

The specific description is as follows:

1) An algal phase ecosystem is primarily affected by temperature in continuous high temperatures and high temperatures weather. A wide range of temperatures is suitable for most algae to survive, but a narrow range is best for their growth (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. When the temperature of the water increases, the growth rate of cyanobacteria increases as well. It is found that some beneficial single-cell algae grow at the same rate as cyanobacteria under normal temperature conditions. 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. Due to the growth of other algae species, under normal temperatures it is unlikely that cyanobacteria will explode on a large scale, as a result of the growth of other algae species. The advantage of cyanobacteria in terms of growth rate will only become apparent when the hot season begins. Thus it must be

emphasized that the temperature is a significant factor that determines whether or not cyanobacteria are spread (Wells et al. 2015).

2) There is an abundance of organic matter in the water, which is rich in nitrogen and low in phosphorus. As far as aquaculture water is concerned, there are two types of sources of nutrients such as nitrogen and phosphorus.

## 1.5.6 The harm of cyanobacteria

### 1.5.6.1 Consumption of dissolved oxygen in water

As the cyanobacteria blooms in aquaculture water, the oxygen production by phytoplankton during photosynthesis is severely hindered. As a result, the oxygen in the aquaculture water body is severely reduced and the atmosphere in the air is blocked from entering the aquaculture water body. As a result, there is a serious shortage of dissolved oxygen in aquaculture water. When hypoxia or subanoxic conditions persist long enough in an aquaculture system, the water continues to deteriorate, causing the accident to become worse directly or indirectly (Huntington et al. 2006).

## 1.5.7 Available way to eradicate cyanobacteria around the world

The methods that can be used to diminish cyanobacteria can be divided into two categories. direct methods and indirect methods.

1) Direct way

There are three major categories of direct methods that can be categorized into: chemical methods, physical methods, and biological methods. As a chemical method, it is possible to put in an algaecide or to carry out salt treatment to remove the algae. 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. As good as biological methods can be, they can also cause damage to the biological chain as a result of causing certain organisms to multiply and cause new problems to arise as a result of the damage they have caused.

2) Indirect way

a. Countermeasures for watersheds. In order to prevent the occurrence of eutrophication in lakes, it is important to control the amount of lake pollution sources that enter the lakes in order to reduce the input of nutrient load into the lakes. For long-term eutrophication control, it is necessary to reduce nutrient inputs to water bodies. Among these measures are wastewater phosphorus and nitrogen removal technologies, sewage interception engineering, and wastewater resource utilization. There is a serious problem with water pollution in China at the moment. 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. In the short term, it may be hard to see the effects of the changes.

b. Lakeside countermeasures. Two aspects of the countermeasures in the lake include dredging, removing organic matter that has accumulated in the river or lake for a long time, and removing 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 new siltation. Aquatic plants are purified, benign algae are reproduced, and so on. For this method to be utilized, it is necessary to consider the recovery of aquatic plants and benign algae, in order to prevent decay in water and the formation of new pollution sources as a result of the use of this method. 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. The total amount of phosphorus excluded by the algae separation technology is a drop in the amount of phosphorus in Taihu Lake as compared to the amount excluded by the algae separation technology.

c. Lake emergency measures. This consists mainly of rectifying membranes, aeration, etc., which are the most common methods of controlling the growth of algae through changing the distribution of nutrient salts in the water by means of flow. The Japanese have conducted a lot of research in this field and found that the method is somewhat practical.

### 1.5.8 Literature review on the effect of light intensities, temperatures, and nutrient depletion to eradicate Cyanobacteria

Cyanobacteria are directly dependent upon light. Stress may be caused by moderate light changes (Welkie et al. 2019). Cyanobacteria rely almost exclusively on light for energy, so responding to various light intensities is essential (Asaeda et al., 2022a). A lot can be learned about photosynthesis and biotechnological applications of cyanobacteria based on how they respond to various light intensities (Flombaum 2013; Oliver 2016; Leblanc Renaud et al. 2011). The light intensity changes within a day also affect the habitat preference of cyanobacteria (Saha et al. 2016). Through an elaborate electron transport pathway, cyanobacteria carry out photosynthesis and respiration with the help of solar energy (Lea-Smith et al., 2006). Excess solar energy produces reactive oxygen species (ROS), including superoxide radicals (Latifi et al., 2009; Raja et al., 2017), and damages cellular components, such as the D1 protein. This protein is responsible for recovering the damaged photosynthesis apparatus (Gill and Tuteja 2010; Nishiyama, Y., Murata, 2014; Weerakoon et al., 2018). Antioxidant activities detoxify superoxide radicals into the water through superoxide dismutase (Asada et al., 1999; Ma and Gao 2010; Rastogi et al., 2010; Sharma et al., 2012), and photoinhibition occurs. Thus, excessive solar radiation inhibits the proliferation of cyanobacteria. In the ecology of cyanobacteria in water, even though solar radiation intensity is highest at the surface,

photoinhibition plays an important role in reducing cyanobacterial biomass (Harrisn and Smith 2013; Salonen et al., 1999).
Compared to other algae species, *M. aeruginosa*, *P. ambiguum,* and *P. foetida* can proliferate at high temperatures, making cyanobacteria prevalent in summer blooms (Drake et al., 2010). The preferable growth temperature for *Microcystis spp*., *P. ambiguum*, and *P. foetida* is 24°C to 34°C (Ganf, 1974, Bryanskaya et al., 2008; Chamizo et al., 2020). It is suggested that changing water temperature can effectively control cyanobacterial blooms (Chu et al. 2007). However, there has been little documentation of the species-specific effect of temperature change on their growth.

Hiroyuki et al (2009) suggest that Water temperature changes during summer may be an important environmental factor for the alternating succession of *Microcystis* species. a cardinal temperature model for *M. aeruginosa* with the inflection point (optimal temperature) located at 27.5°C. The model describes 98% of the variability of experimental data from 5°C to 35°C (You et al 2017). cyanobacteria dominate phytoplankton assemblages in temperate freshwater environments during the warmest periods of the year, particularly in eutrophic systems (Paerl, 1988, Paerl et al., 2001, Paerl and Huisman, 2008, Paul, 2008). Harmful cyanobacteria such as Microcystis have been found to have an optimal temperature for growth and photosynthesis at, or above, 25 °C (Konopka and Brock, 1978, Takamura et al., 1985, Robarts and Zohary, 1987, Reynolds, 200, Paerl and Huisman, 2008). Furthermore, the cellular toxin content of multiple genera of cyanobacteria increases with increasing temperature to a maximum above 25 °C (Van der Westhuizen and Eloff, 1985, Codd and Poon, 1988).

Toxicity and the toxin production rate increased with light intensity up to an intensity of about 40 μmol $m^{-2}$ $s^{-1}$ and decreased at higher light intensities, while the ratio of toxin to protein was constant at intensities of more than 40 μmol $m^{-2}$ $s^{-1}$ (Utkilen et al 1992) A fluence rate of 145 μmol $m^{-2}$ $s^{-1}$ harvested in the late logarithmic growth phase yielded the maximum quantity of toxin (Thomas et al., 1986). Low light intensity can promote the persistent floatation of microcystis colony. High light intensity can induce high growth rate and large size of colonies (Xu et al., 2023) Although the individual effect of temperatures and light intensities has been studied (Tan et al., 2020; Asaeda et al., 2020, 2021), detailed information on their combined effects is required. Thus, for short and long-term treatment is needed for cyanobacterial blooms. Cyanobacteria often occur in places where eutrophication has progressed, where nutrients such as nitrogen and phosphorus are abundant, and proliferate by photosynthesis (Hirose et al., 2009). Insolation has also been considered an important factor in its proliferation due to photosynthesis (Fadel et al., 2017). Similarly, the shortage of nutrient conditions, including phosphorous (P) and nitrogen (N), is identified as a dominant stressor that suppresses the growth of cyanobacteria (Asaeda, and Barnuevo 2019). Phosphorus is an important macronutrient to plankton growth in many ways. Phosphorus makes rigid structures in cell walls, membranes, and nucleic acids by making covalent links between monomers. Phosphorus is also involved in cell metabolism directly by storing energy as polyphosphate bodies in plankton cells (Asaeda et al., 2019). The absolute concentrations of P and N and the stoichiometric ratio of these elements often play an important role in plankton growth in lakes. N:P mass ratio varies between 240 and 0.5, depending on the variation of P concentration in lakes (Asaeda et al., 2020). When the mass ratio of N:P exceeds 10, P is considered as the limiting factor. On the other hand, when N:P less than 10, N becomes the limiting factor on phytoplankton growth, including cyanobacteria in freshwater bodies. Hence, both surplus and deficiency of nutrients could cause significant alternations in cyanobacteria biomass and

cellular stress. The combined effect of various abiotic stresses on the production rate is often reported (Jana 1982). Some combinations inhibit growth due to the contradicting impacts of stressors; however, a significant reduction of biomass is also reported as caused by simultaneous exposure to multiple stressors compared to a single stress source. Hence, excessive radiation stress combined with a shortage of P and N nutrients could generate huge cellular stress and cyanobacterial growth inhibition.

## 1.6 General research objective

Measurement of $H_2O_2$ help us to provide useful information for oxidative damage of plants, macrophytes, and cyanobacteria. Various factors influence the measurement of $H_2O_2$. We need to find an easy way to quantify $H_2O_2$. Therefore the main objective is to find a correlation between eFOX and $Ti(SO_4)_2$ assays using riparian plant species. If the correlation significantly works, any of the methods can be used for quantification of $H_2O_2$. The species-based objective is given as follows

### 1.6.1 The objective of quantifying $H_2O_2$ in plants

There are several objectives for measuring $H_2O_2$ in plants. These are as follows: 1) As $H_2O_2$ varies depending on various methods, there is in need to choose a suitable way to measure it. Therefore, it will be a new approach to quantify $H_2O_2$ by evaluating the correlation between eFOX and $Ti(SO_4)_2$ assays using riparian plant species. 2) Causal observation of plant traits, such as growth rate and biomass, commonly used in vegetation monitoring, are not necessarily appropriate evaluation methods. Hence, measurement of $H_2O_2$ plant can pave the way to know physiological process more accurately and easily which can be used as an application in ecohydraulics instead of considering only dynamic model. 3) Riparian plant species prefer specific elevation sites to grow. So, determining the relationships between species-specific habitat conditions and the $H_2O_2$ concentration in riparian plant leaves to understand the feasible $H_2O_2$ level for the species to grow, in order to explore the conditions as an index of plant distribution. 4) High solar radiation, soil moisture content, soil macronutrient especially TN or TP concentration, and TN: TP can become stressful for plants when subjected to overabundance than its critical values. Hence quantification of $H_2O_2$ concentration considering high solar radiation, soil moisture, soil TN or TP concentration, and TN: TP can provide the most dominant stressors for riparian plants.

Macrophytes can be affected by environmental factors like water velocity, temperatures, solar radiation and a few other factors. Quantification of $H_2O_2$ with the $Ti(SO_4)_2$ methods may provide us to know the physiological condition of Macrophytes under various stressors.

### 1.6.2 Objective of measuring $H_2O_2$ concentration in cyanobacteria

Cyanobacterial blooms have become a major problem throughout the world. Therefore, it is necessary to give proper attention to eliminate it. $H_2O_2$ Hydrogen peroxide can be a plausible biomarker in cyanobacterial bloom treatment. Therefore, the aim of measuring $H_2O_2$ is (1) to investigate diurnal light intensity changes in different temperatures to observe daily physiological changes in cyanobacteria. 2) to observe the long-term combined effect of light intensities in temperature alterations. 3) to diminish cyanobacteria using nutrient depletion in different light intensities.

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# Chapter 2

# Hydrogen peroxide measurement can be used to monitor plant oxidative stress rapidly using modified ferrous oxidation xylenol orange and Titanium sulfate assay

## 2.1 Background of the study

There are various factor that affect $H_2O_2$ quantification. The addition of chemicals, such as salicylic acid, increases $H_2O_2$ concentration in tomato leaves from 0.15 µmol/gFW to 0.25 µmol/gFW (Chen et al., 1993). The concentration of $H_2O_2$ in pear fruit tissue was increased from 0.35 mol/gFW to 0.8 mol/gFW in response to potassium cyanide with a $Ti(SO_4)_2$ assay (Brennan and Frenkel, 1977). *Bruguiera parviflora*'s $H_2O_2$ concentrations rose from 0.067 µmol/gFW to 0.089 µmol/gFW under greenhouse hydroponic conditions (Parida & Das, 2005). In addition, the results of $H_2O_2$ concentration vary due to the sensitivity of the methods applied and interference from other redox-active compounds (Queval et al., 2008). For example, Apple leaves were estimated to contain 20–70 nmol/g FW of $H_2O_2$ using the $Ti(SO_4)_2$ assay (Okazaki et al. 1998), while 5–25 nmol/g FW was found using the Bioxytech $H_2O_2$-560 colorimetric assay (Vilaplana et al. 2006). In pears, 0.5–0.8 µmol/g FW of $H_2O_2$ was quantified utilizing the $Ti(SO_4)_2$ assay, while 6–11 nmol/g FW was observed using the Bioxytech $H_2O_2$-560 kit (Lentheric et al., 2003). $H_2O_2$ levels are commonly measured with peroxidase assays (Demmano et al., 1996), Amplex Red $H_2O_2$ detection kit (Orozco-Cárdenas and Ryan, 2002), 3,3-diaminobenzidine (DAB) (Thordal-Christensen et al., 1997), DCFDA (Di chloro dihydro fluorescein diacetate) (Yao et al., 2002), fluorescence (Jimenez et al., 2002) and chemiluminescence (Jones and Lee, 2019). As with superoxide detection, many methods used to measure $H_2O_2$, such as DAB and DCFDA, are poor specificities, thus measuring generalized oxidative stress rather than a specific ROS (Halliwell & Whiteman, 2004). As a result, it is necessary to use more appropriate methods to determine $H_2O_2$ concentration in plant tissues.
The determination of $H_2O_2$ in plant tissues, particularly leaves, has been described in numerous studies. Among the major spectrophotometric assays is one using a Titanium $H_2O_2$ color complex ($Ti(SO_4)_2$ assay) (Brennan & Frenkel, 1977), and another using ferrous ions being oxidized by $H_2O_2$ to ferric ions (Gay et al., 1999). The modified ferrous oxidation xylenol orange (eFOX) assay has gained considerable acceptance due to its sensitivity, stability, and adaptability to high-throughput techniques. In spite of some interference between the eFOX and $Ti(SO_4)_2$ assays during the measurement process (Patterson et al., 1984; Veljovic-Jovanovic et al., 2002; Queval et al., 2008), their correlation in quantifying plant leaves is unknown.

## 2.2 Objective of the study

The main objectives of this study are as follows; 1. As soon as leaf tissues are collected (frozen or nonfrozen state), determine the optimal environmental condition for analysis. 2. Establish a new approach to quantifying $H_2O_2$ by evaluating the correlation between eFOX and $Ti(SO_4)_2$ assays using riparian plant species.

## 2.3 Material and methods

### 2.3.1 Plant leaves collection

Leaves of riparian plants, such as *Ambrosia trifida*, *Solidago altissima*, *Artemisia princeps,* and *Sicyos angulatus*, were collected for this study. Sampling was conducted at the Arakawa-

Taroemon (350 56' 52.2"N, 139032' 13.1"E to 35° 59' 7.3" N, 139° 30' 58.2"E) on a sunny day on June 1st, August 2nd, and September 9th, 2022 (Figure 2.4.1). The collected samples were transported to the laboratory after finishing the sampling. Fully expanded leaves from the middle part of plants in each replicate were collected. Plant leaves were divided into two groups to evaluate the best conditions for obtaining maximum $H_2O_2$ concentrations. Nonfrozen samples: The collected samples were prepared for analysis soon after arriving in the laboratory, which was described later. Nonfrozen samples were kept at a normal temperature (25°C ±3 °C) until arrival in the laboratory. Frozen samples: The collected samples were kept at -80°C for 25 days after being transported to the laboratory. Frozen samples were kept in dry ice (~ -70°C) until arrival in the laboratory. After 25 days, samples were prepared to compare $H_2O_2$ levels with their nonfrozen counterparts.

### 2.3.2 Preparation of frozen and nonfrozen samples

Approximately 40 to 50 mg of the plant leaf was weighed and placed into a 15 ml centrifuge tube with a combination of beads (3mm and 10mm) (BMS Inc.). For each leaf, triplicate was performed. The centrifuge tube was frozen with liquid nitrogen and ground to a powder using Shake master (BMS Inc.). A volume of 5ml potassium phosphate buffer (pH 6, 50mM) was added, and a small amount of polyvinylpyrrolidone (PVP) was used to prevent the effect of the phenolic compound. The mixture was centrifuged (Kokusan H60-R, Japan) twice at 5500 rpm for 10 min, and the supernatant was collected as an extract to analyze $H_2O_2$ in both methods. The extract was transferred to a -80°C refrigerator until analysis.

### 2.3.3 Preparations of the standard curve with the eFOX and $Ti(SO_4)_2$

Commercially available 30% $H_2O_2$ (w/w) was diluted with potassium phosphate buffer (pH 6, 50mM) to prepare a known $H_2O_2$ concentration of 1, 2, 4, 5, 10, 20, 25, 50, and 100 µmol/L to measure with both methods. The ultra-pure (Millipore Milli-Q) water was used throughout the experiment where necessary.

### 2.3.4 Determination of $H_2O_2$ Content with eFOX assay

The modified ferrous-xylenol orange assay was used to measure $H_2O_2$ (Queval et al., 2008; Cheeseman 2006). The supernatant (100 µL) was added to 1 mL of the assay solution containing 250 µM ferrous ammonium sulfate, 100 µM sorbitol, 100 µM xylenol orange, 25 mM $H_2SO_4$ (sulfuric acid), and adding 1% ethanol to the reagent increased its sensitivity to $H_2O_2$ by 50% (Fujifilm wako pure chemical corporation, Japan), which was deoxygenated with gaseous nitrogen to prevent artifacts. Reaction mixtures were incubated at room temperature for 15 min. The absorbance was measured at 560 nm by spectrophotometry (UVmini-1240, Shimadzu, Japan). $H_2O_2$ content was calculated by a standard curve using a series of diluted solutions of commercial, high-grade 9.8 M $H_2O_2$ (30%) (w/w) (Dautania et al. 2014; Asaeda et al. 2022b)

#### 2.3.4.1 The eFOX assay's advantages and disadvantages

The eFOX method is inexpensive, sensitive, stable, and adaptable to high-throughput techniques. Due to the higher sensitivity of the eFOX assay, a very low amount of tissue-level $H_2O_2$ concentration can be measured accurately. The eFOX also reduces the concentrations of interferents. The eFOX can measure a wide range of $H_2O_2$ concentrations. For instance, 0.67 µmol/gFW (Cheeseman 2006) to 160 µmol/gFW (Yu et al., 2013).

Sometimes overestimation occur as a result of higher sensitivity. It shows sensitivity to ascorbate peroxidase, lipid peroxidase which can interfere with the quantification of $H_2O_2$ concentration.

### 2.3.5 Determination of $H_2O_2$ Content with $Ti(SO_4)_2$ assay

The Brennan and Frenkel (1977) method was used to measure $H_2O_2$. The titanium (II) metal ion in the acidic solution forms a peroxide complex with $H_2O_2$, a yellow-colored compound. In a 10 ml round centrifuge tube, 750 µL of extraction was taken. 2.5 mL of 0.1% of $Ti(SO_4)_2$ in 20% $H_2SO_4$ solution was added. The mixture was centrifuged at 10000rpm for 15 min at room temperature. 1 mL of the supernatant was transferred into a 1 mL spectrophotometer cell. The absorbance was measured at 410 nm. For the blank, a mixture of 750 µL of 0.05 M phosphate buffer (pH 6.0) and 2.5 mL of 0.1 $Ti(SO_4)_2$ in 20% $H_2SO_4$ was used.

#### 2.3.5.1 The $Ti(SO_4)_2$ assay's advantages and disadvantages

Titanium(IV) complexes with peroxide, resulting in 410 nm ligand-to-metal charge transfer bands that give the specific intense color. The color formation of this specific charge transfer band is extremely selective since it can only be formed by peroxide complexation. In addition, color formation is not a result of redox reactions. As a result, ozone or nitrogen dioxide do not affect the reagent. In combination, titanium(IV)-peroxide complexes are used in colorimetric analysis of solutions because of their selectivity, stability, and color intensity. The lack of reversibility allows a robust measurement of the final color. The $Ti(SO_4)_2$ method is also suitable for the quantitative colorimetric determination of hydrogen peroxide in the presence of organic hydroperoxides. With $Ti(SO_4)_2$ assay, we can determine 0.35 µmol/gFW (Brennan and Frenkel, 1977) to more than 100 µmol/gFW (Clapp et al., 1989) accurately.

The $Ti(SO_4)_2$ determination is adversely affected only by hydroperoxides which tend to liberate $H_2O_2$ in an acidic medium (e.g. hemiperacetals). Ascorbic acid present in leaf extracts interfere with $Ti(SO_4)_2$ assay. Phenolic compounds also interfere the measurement process (Veljovic-Jovanovic et al., 2002).

### 2.3.6 Statistical analyses

IBM SPSS Statistics (Version 28.0 IBM Corporation, USA) software was used to execute the statistical analysis. Pearson's correlation analysis was employed to assess correlations between the two methods.

## 2.4 Results

The correlation between the two methods and a wide range of $H_2O_2$ concentrations is highly linear through standard curves in Fig. 2.4.2. Compared to $Ti(SO_4)_2$, the eFOX method exhibits a higher level of sensitivity in terms of absorbance. This clearly indicates that the eFOX assay is capable of measuring even lower fluctuations of $H_2O_2$ concentration more accurately. A significant decreasing trend was observed from nonfrozen to frozen samples in the eFOX ($r= 0.879$, $p<0.001$) and $Ti(SO_4)_2$ ($r= 0.837$, $p<0.001$) assays, which is shown in Fig. 2.4.3 (a, b). Meanwhile, the sample weight does not affect the quantification procedure of $H_2O_2$ concentration in eFOX (nonfrozen $r=-0.017$, $p=0.959$; frozen $r=0.061$, $p=0.851$) and $Ti(SO_4)_2$ (nonfrozen $r=0.477$, $p=0.117$; frozen $r=0.37$, $p=0.236$) assays (Fig. 3). Each species is independent of sampling weight in frozen and nonfrozen conditions for eFOX and $Ti(SO_4)_2$ (Table 1). The maximum reduction rate (%) in both methods between frozen and nonfrozen samples for each species (Table 2). Fig. 2.4.4 also exhibits a highly substantial correlation in the decreasing rate between eFOX and $Ti(SO_4)_2$ in frozen and nonfrozen samples ($r=0.832$,

$p$<0.001). From the above findings, it is clear that nonfrozen samples give higher $H_2O_2$ concentration than frozen samples. Therefore, the study was conducted on a large scale to find the correlation between eFOX and $Ti(SO_4)_2$ with the nonfrozen samples. The 2nd and 3rd time collection (August 2nd, and September 9th, 2022) was performed to expand the volume of nonfrozen samples to analyze with both methods. Each species of nonfrozen leaves showed a high substantial correlation between eFOX and $Ti(SO_4)_2$ (*Ambrosia trifida* r=0.767, $p$<0.001; *Solidago altissima* r=0.583, $p$<0.001, *Artemisia princeps* r=0.672, $p$<0.001; and *Sicyos angulatus* r=0.828, $p$<0.001) (Fig. 2.4.5).

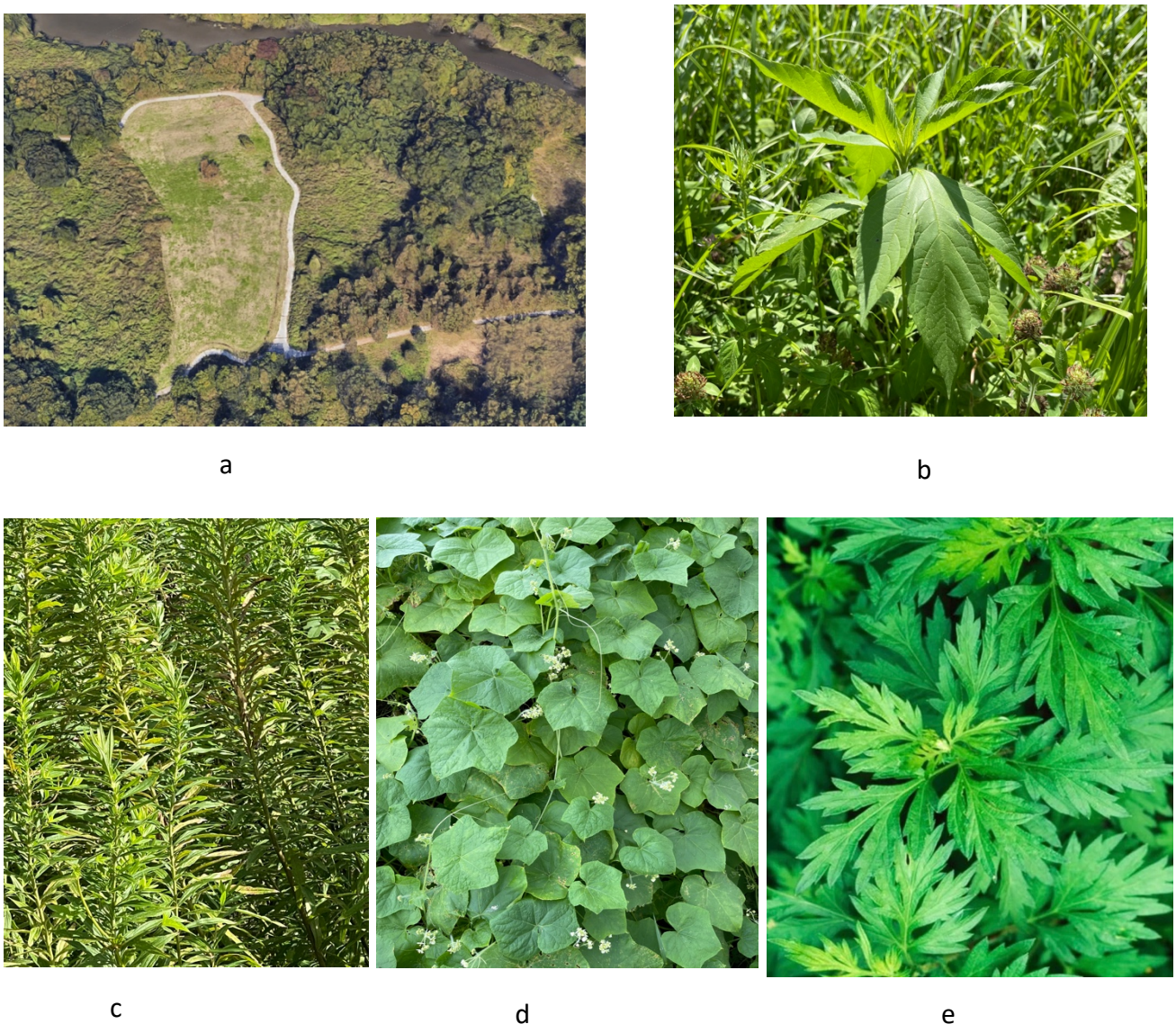



Figure 2.4.1 Study site (a) and collected riparian plant species were *Ambrosia trifida* (b), *Solidago altissima* (c), *Artemisia princeps* (d), and *Sicyos angulatus* (e) in nonfrozen samples

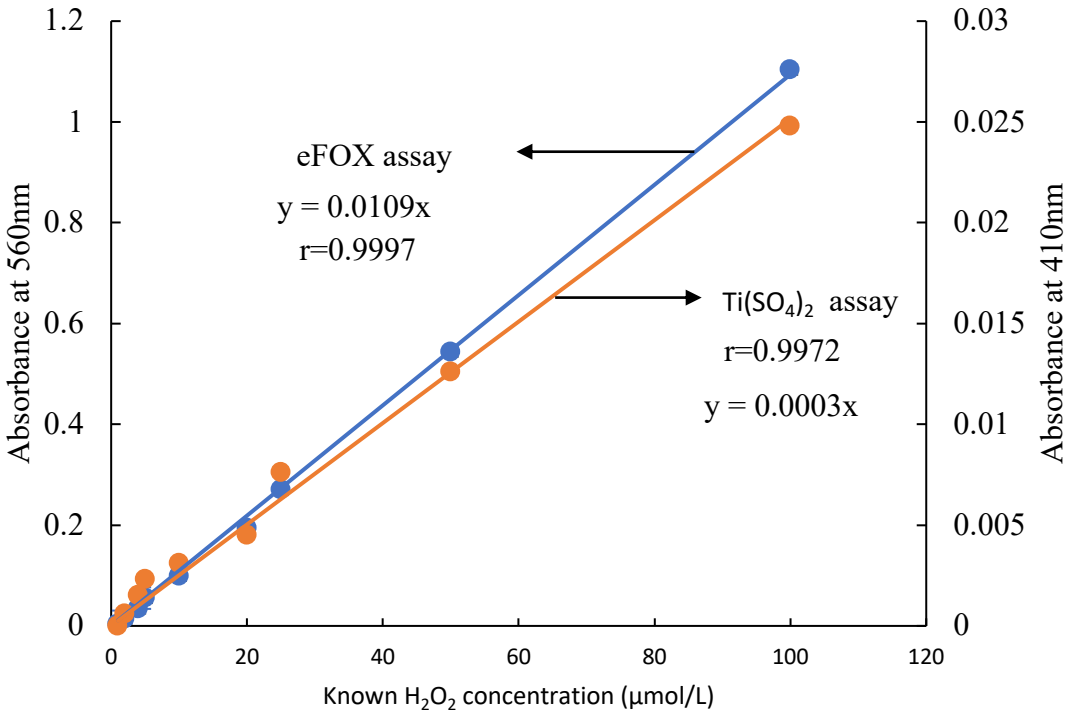
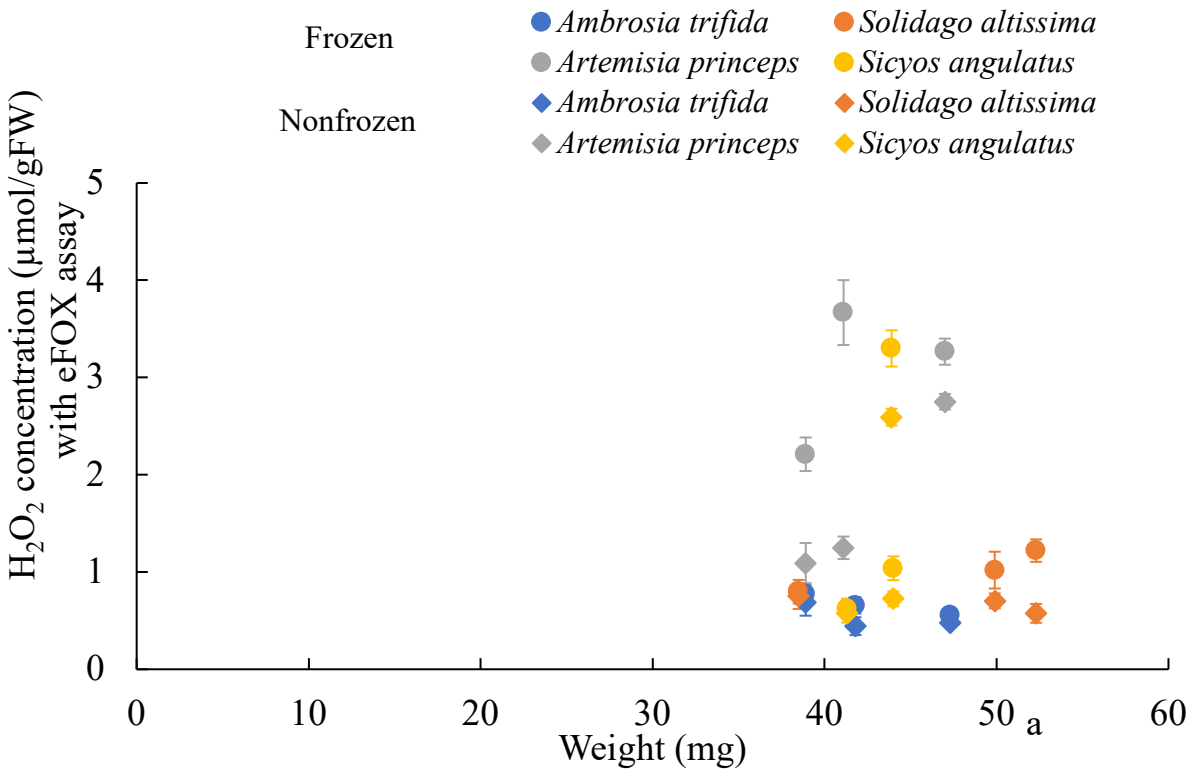



Figure 2.4.2 Standard curve for eFOX (a) and $Ti(SO_4)_2$ (b) using the known concentration of $H_2O_2$. Vertical bars indicate eFOX and $Ti(SO_4)_2$ standard deviation.



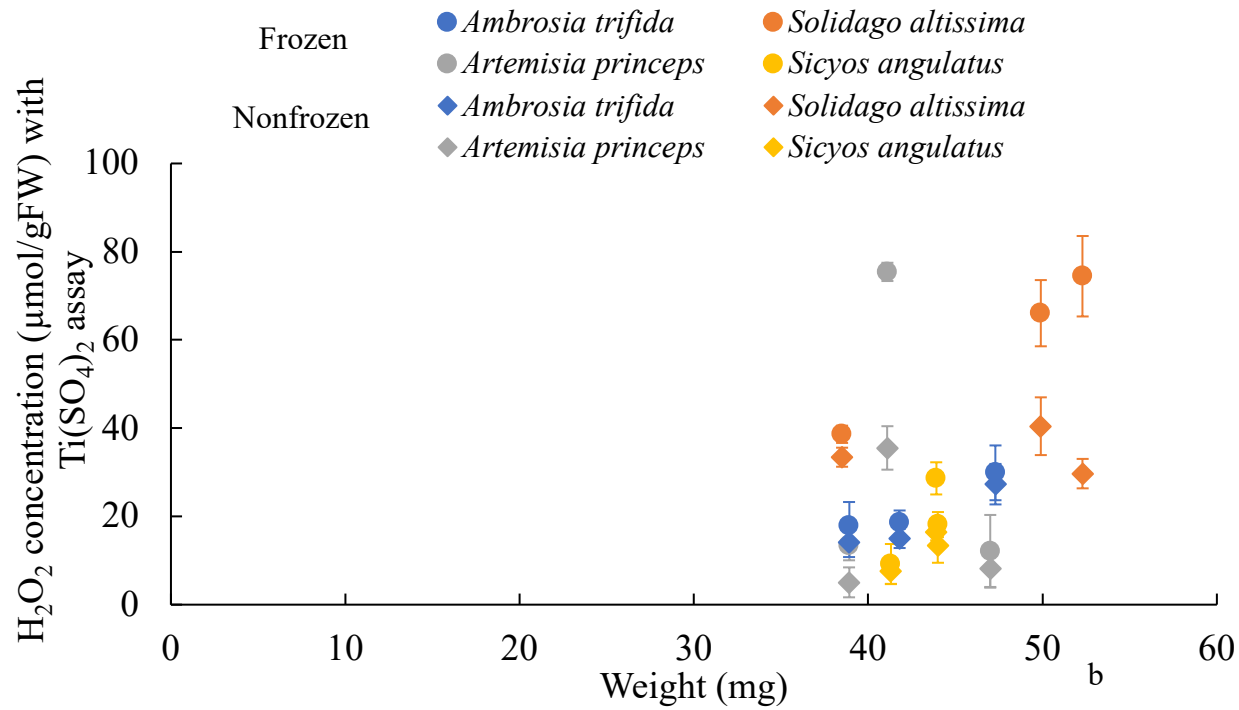




Figure 2.4.3 Decreasing trend between frozen and nonfrozen samples in both methods. Vertical bars indicate standard deviation (eFOX (a) and $Ti(SO_4)_2$ (b)).

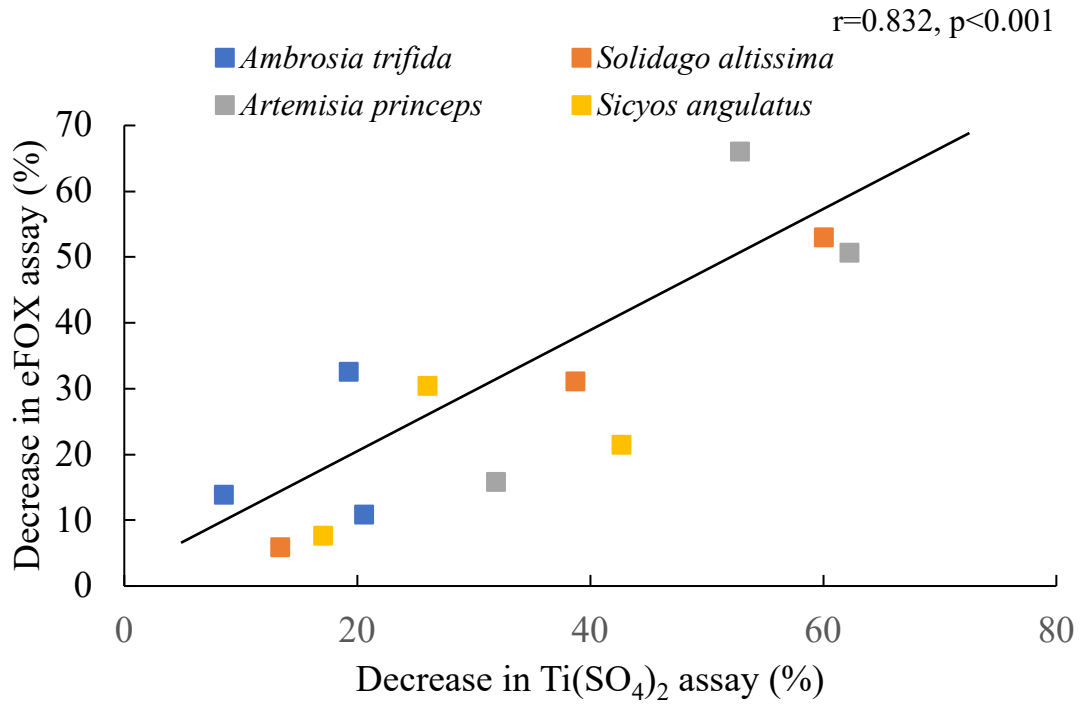
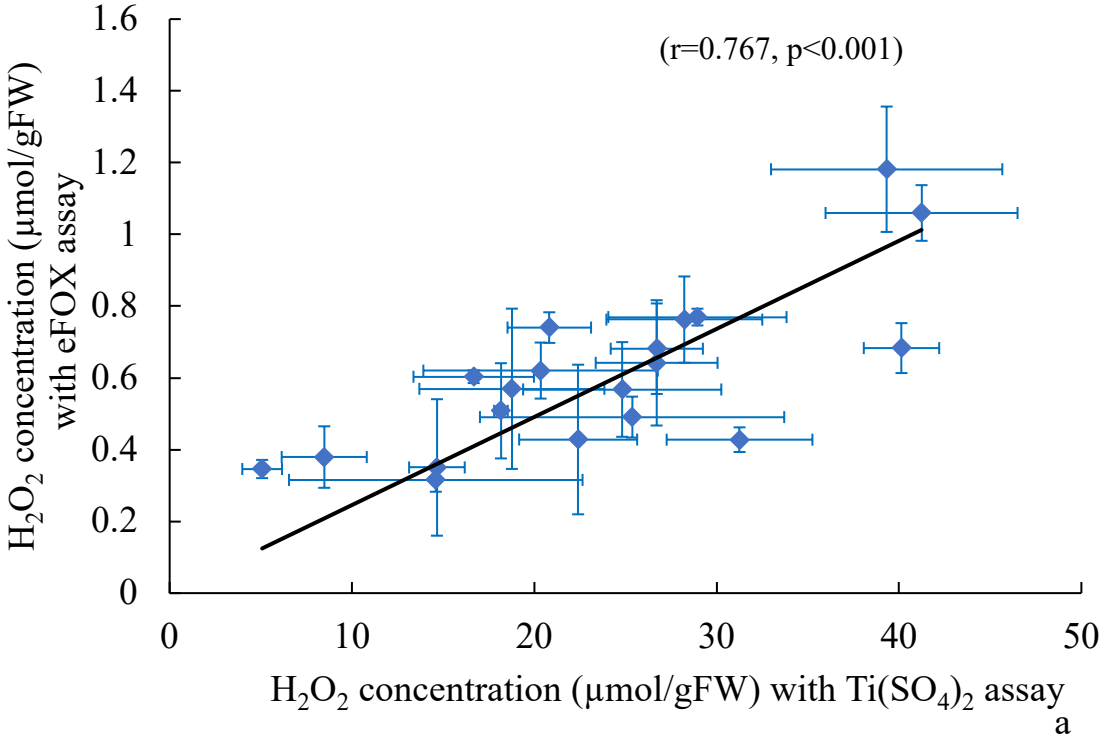
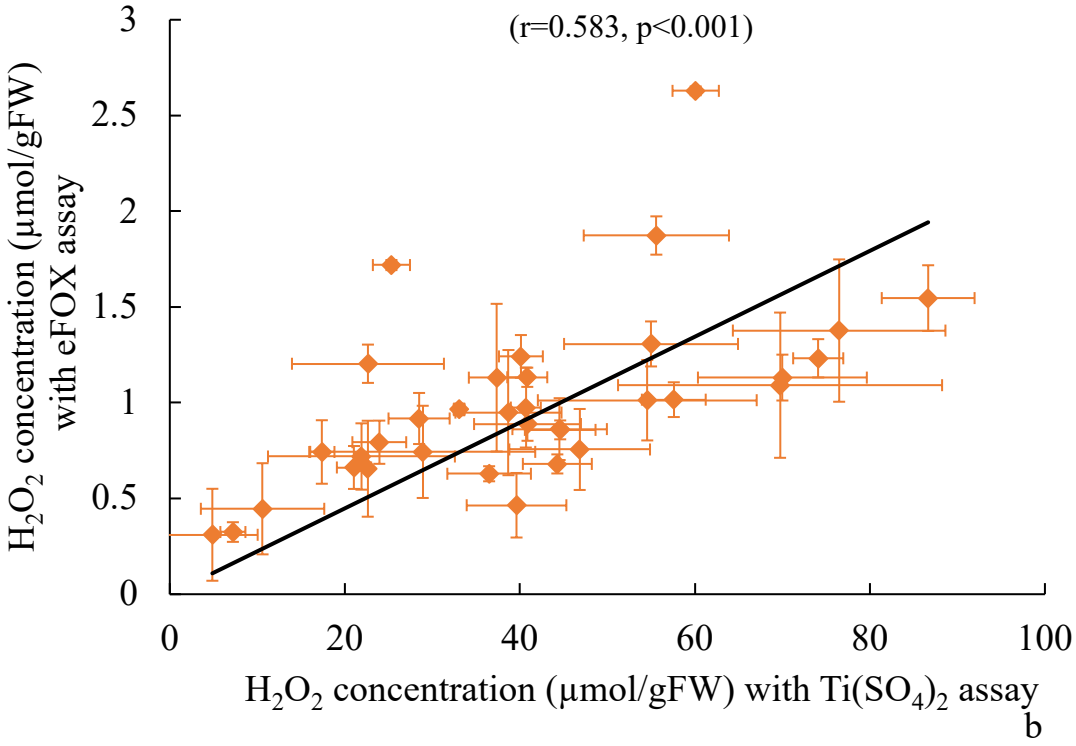


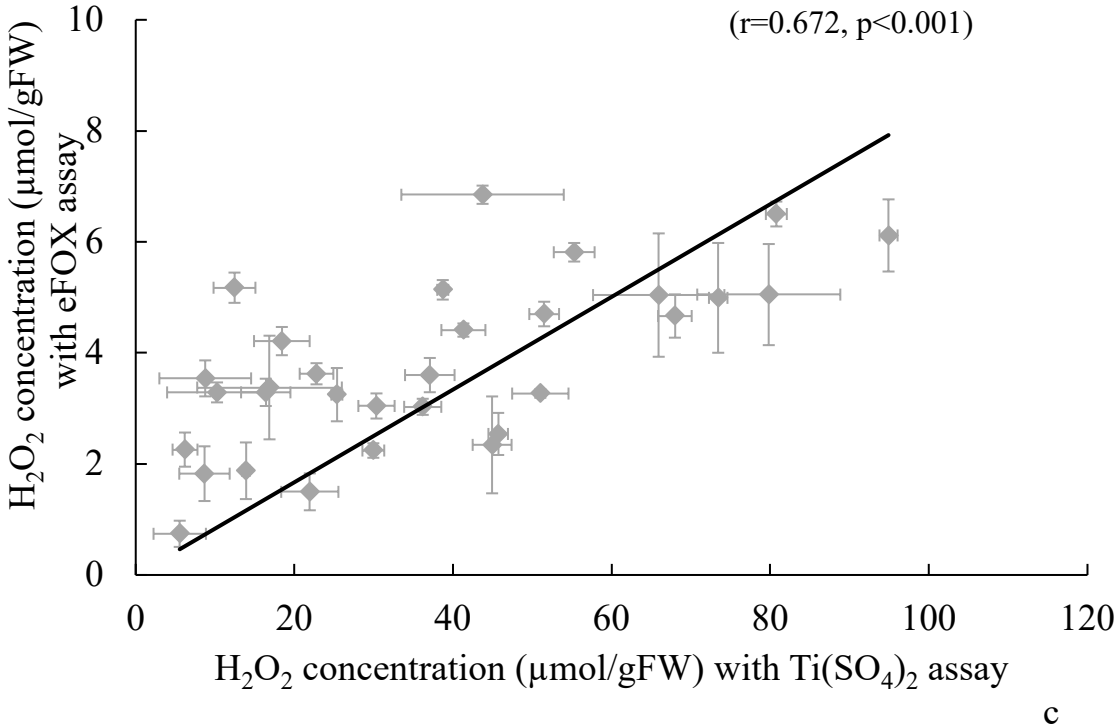

Figure 2.4.4 Percentage decrease correlation with both methods comparing frozen and nonfrozen samples.




a


(r=0.583, p<0.001)
$H_2O_2$ concentration (µmol/gFW) with eFOX assay
$H_2O_2$ concentration (µmol/gFW) with $Ti(SO_4)_2$ assay
0
0.5
1
1.5
2
2.5
3
0
20
40
60
80
100

b


(r=0.672, p<0.001)
$H_2O_2$ concentration (µmol/gFW) with eFOX assay
$H_2O_2$ concentration (µmol/gFW) with $Ti(SO_4)_2$ assay
0
2
4
6
8
10
0
20
40
60
80
100
120

c

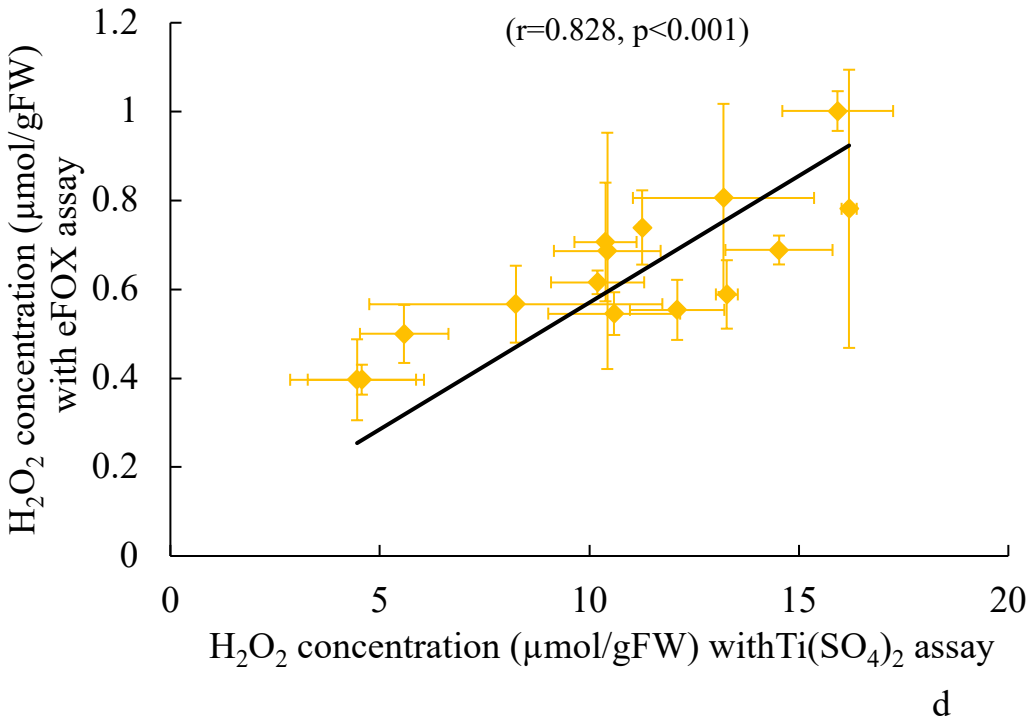



Figure 2.4.5 Correlation between $Ti(SO_4)_2$ and eFOX in *Ambrosia trifida* (a), *Solidago altissima* (b), *Artemisia princeps* (c), and *Sicyos angulatus* (d) in nonfrozen samples. Vertical and horizontal bars indicate eFOX and $Ti(SO_4)_2$ standard deviation, respectively.

Table 1: Relationship between sampling weight and frozen along with nonfrozen conditions utilizing both methods

| | eFOX assay | | $Ti(SO_4)_2$ assay | |
|---|---|---|---|---|
| Species name | Frozen | Nonfrozen | Frozen | Nonfrozen |
| *Ambrosia trifida* | r=-0.670, *p*=532 | r=-0.977, *p*=0.137 | r=0.958, *p*=186 | r=0.957, *p*=0.187 |
| *Solidago altissima* | r=-0.812, *p*=0.397 | r=0.946, *p*=0.21 | r=0.30, *p*=0.806 | r= 0.302, *p*= 0.804 |
| *Artemisia princeps* | r=0.984, *p*=0.115 | r=0.496, *p*=0.67 | r=-0.162, *p*=0.896 | r=-0.273, *p*=0.824 |
| *Sicyos angulatus* | r=0.528, *p*=0.646 | r=0.593, *p*=0.596 | r=0.931, *p*=0.239 | r=0.824, *p*=0.383 |

Table 2: Maximum reduction rate (%) in each species for both methods

| Species name | Maximum reduction rate (%) in eFOX assay | Maximum reduction rate (%) in $Ti(SO_4)_2$ assay |
|---|---|---|
| *Ambrosia trifida* | 32.47 | 20.62 |
| *Solidago altissima* | 52.91 | 60.10 |
| *Artemisia princeps* | 65.94 | 52.90 |
| *Sicyos angulatus* | 30.28 | 26.10 |

Table 3: $H_2O_2$ concentrations in leaves of various plant species

| Species name | $H_2O_2$ concentration (µmol/gFW) | Technique | Notes | Reference |
|---|---|---|---|---|
| *Litchi chinensis* Sonn. | 0.64 | Glucose peroxidase | Fully expanded leaves | Zhou et al., 2006 |
| *Spinacia olearacea* | 0.54 | Titanium (IV)-PAR complex. | Fully expanded leaves | Patterson et al. 1984 |
| *Glycine max* L | 3.47 | Ferrous oxidation/xylenol orange | Fully expanded leaves | Cheeseman, 2006 |
| *Calendula officinalis* | 6 | Glycolate oxidase | Young leaves | Chaparzadeh et al. 2004 |
| *Arabidopsis thaliana* | 5 | Luminol chemiluminescence | Young leaves | Karpinski et al., 1997, 1999 |
| *Zea mays L.* | 75 | $Ti(SO_4)_2$ assay | Fully expanded leaves | Tewari et al., 2004 |
| *Nicotinia tabacum* | 5 | Titanium-peroxide complex assay | Fully expanded leaves | Mur et al. (2005) |
| Rice (*Oryza sativa* L.) | 0.26 | Titanium (IV) sulphate- TiCl4 complex | Young leaves | Li et al. (2000) |
| Wheat (*Triticum aestivum* L.) | 0.58 | Titanium (IV) sulphate- TiCl4 complex | Old leaves | Li et al. (2000) |
| Banana (*Musa acuminata Colla*) | 0.72 | Glucose peroxidase | Fully expanded leaves | Zhou et al. (2006) |

## 2.5 Discussion

Plants suffer from oxidative stress when too much ROS, especially $H_2O_2$, is present in the tissues (Sharma et al., 2012; Choudhury et al., 2017). Therefore, quantitative detection of $H_2O_2$ is very important for riparian vegetation plant species. $H_2O_2$ content in plants is difficult to determine due to the variable sensitivity of methods and interfering compounds (Cheeseman, 2006; Queval et al., 2008), such as the high concentrations of ascorbic acid present in leaf extracts interfere with peroxidase coupled assay and luminometric methods (Veljovic-Jovanovic et al., 2002). Using peroxidase-coupled oxidation, values of approximately 50 nmol/ gFW were obtained (Veljovic-Jovanovic et al., 2002; Bouche´ et al., 2003), whereas luminol chemiluminescence with catalase pre-treatment yielded values of around 5 µ mol/ gFW (Karpinski et al., 1997, 1999). Methods should be developed where the above restrictions can be overcome as well as quantify $H_2O_2$ concentrations with minimal losses. The correlations with eFOX and $Ti(SO_4)_2$ can be of great interest. A broad range of $H_2O_2$ concentrations was obtained by different researchers due to various techniques and interferences (Table 3). This study also demonstrated a vast range of $H_2O_2$ employing both methods. Among different species, *Ambrosia trifida* shows the minimum (eFOX 0.31± 0.03 µmol/gFW; $Ti(SO_4)_2$ 5.07± 0.65 µmol/gFW) and *Artemisia princeps* exhibits the maximum (eFOX 6.85± 0.16µmol/gFW; $Ti(SO_4)_2$ 94.92± 1.14 µmol/gFW) $H_2O_2$ concentrations (Fig. 5). From the standard curve, it can

be observed that eFOX shows more sensitivity than the $Ti(SO_4)_2$ assay, achieving lower values more accurately. For example, in the case of eFOX $H_2O_2$ concentration measurement of 10µmol/L, it gives 0.092 absorbances at 560nm, whereas the same $H_2O_2$ concentration in $Ti(SO_4)_2$ is 0.0031 at 410nm. This is the key point across results in the same samples of both methods of riparian species. The standard curve absorbance ratio of eFOX: $Ti(SO_4)_2$ is almost 1:36. This ratio is also a factor for obtaining different results in individual methods.

During lowering temperatures, plant leaves experience stress resulting in chilling injury, which induces ROS accumulation and impairs the normal functioning of the plants (Apel and Hirt, 2004; Ruelland et al., 2009). Due to chilling stress, $H_2O_2$ causes oxidative injuries. The temperature plays an important role in abiotic stresses as well as crop productivity (Miura and Tada 2014). In tomato plants, the chilling injury is a result of low temperatures reducing water and nutrient uptake, but it is not caused by nonfreezing temperatures (above 10 °C) (Gu et al., 2008). The chilling injury also decreases tomato fruit quality (Sevillano *et al*. 2009). The two main mechanisms involved in chilling injuries are temperature-dependent and time-dependent. Generally, environmental stress frequently reduces plant growth through the overproduction of ROS, which damages various macromolecules and cellular structures (Apel and Hirt, 2004), cellular membranes, photosynthetic apparatus, and enzymes (Lukatkin, 2003) and leads to the death of cells (Upadhyaya *et al*., 2007; Liu *et al*., 2010; Goud and Kachole, 2011). Low temperatures also result in reductions in catalase activity, which can be found in leaves of both chilling-sensitive and non-sensitive species (Lukatkin 2005; Kacperska 2008), and in avocado fruit (Sharon and Kahn 1979). The function of catalase is to degrade $H_2O_2$, which is relatively unreactive $H_2O_2$ (Yang and Poovaiah 2002). It is possible for $H_2O_2$ to generate hydroxyl radicals or singlet oxygen. As a result of catalase inactivation, $H_2O_2$ can accumulate, resulting in the production of free radicals and cell damage (Yang and Poovaiah 2002, Rao 2011). Pea leaves $H_2O_2$ content was markedly reduced (38.31%), while mung bean leaf $H_2O_2$ decreased (25.40%) by chilling injury (20$^o$C to 4$^o$C) (MacRae and Ferguson 1985). In the present study, the chilling injury was also observed due to keeping samples in the refrigerator (-80$^o$C) for 25 days before the preparation of frozen samples. As a result, each selected species undergoes a reduced rate of $H_2O_2$ concentration measurement (Fig. 4). Therefore, it is anticipated that preparing the samples soon after collection is the best way to get more accurate values of $H_2O_2$ concentration.

Although there are some interferences between the eFOX and $Ti(SO_4)_2$ assays, present study results demonstrated that there is a high correlation for both methods in *Ambrosia trifida*, *Solidago altissima*, *Artemisia princeps,* and *Sicyos angulatus* in nonfrozen samples. It can be considered that frozen samples yield less $H_2O_2$ concentration than nonfrozen samples, which is mainly caused by chilling injury. Thus, the nonfrozen samples are in favorable conditions to get a considerable amount of $H_2O_2$ concentration, and the high correlation in both methods signifies that a wide range of $H_2O_2$ concentrations is measured effectively.

**2.6 Conclusion**

The correlation between eFOX and $Ti(SO_4)_2$ assays can pave a simple and acceptable way to measure $H_2O_2$ concentration on a large scale in nonfrozen conditions. The weight of samples has no impact on $H_2O_2$ concentration measurement regardless of whether the sample was frozen or nonfrozen. The best condition to get minimum loss of $H_2O_2$ is the nonfrozen state. The significant relationship between the eFOX and $Ti(SO_4)_2$ assays in riparian plants indicates that these methods' correlation can be adapted to measure $H_2O_2$ effectively. Hence, these two methods can be parallelly measured in the same samples. In order to quantify oxidative stress caused by biotic and abiotic stressors, both methods' correlations are highly recommended.

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# Chapter 3

# Measurement of foliar $H_2O_2$ concentration can be an indicator of riparian vegetation management

## 3.1 Background of the study

Riparian vegetation is naturally adapted to abiotic conditions characterized by fluctuating water, sediment, and nutrients (Gurnell et al 2004). Riparian plants are diverse in species, structure, and regeneration strategies (Leibowitz 2003; Maingi 2006). Therefore, riparian habitats are regarded as biodiversity corridors for restoration (Corbacho et al 2003; Heuner et al 2015; González et al., 2017), providing an ecotone between the terrestrial and aquatic ecosystems (Malason 19993; Hrivnal et 2020). The sediment moisture content of riparian zones is associated with the stratigraphy of alluvium, groundwater, hyporheic flows, and the position in the flood or plain corridor (Timm et al., 2004). Causal observation of plant traits, such as growth rate and biomass, commonly used in vegetation monitoring (Barko et al., 1991; Riis et al., 2012), is not necessarily appropriate evaluation methods. The traits may have been developed under different abiotic conditions than the ones at the monitoring time. Thus, false correlations with the prevailing abiotic conditions may occur. Therefore, an immediate monitoring system is needed to gain information on the possible suitable conditions that can explain the spatial distribution of each species.

## 3.2 Objective of the study

The main objective of this study is to empirically determine the relationships between species-specific habitat conditions and the $H_2O_2$ concentration in riparian plant leaves to understand the feasible $H_2O_2$ level for the species to grow, in order to explore the conditions as an index of plant distribution.

## 3.3. Methodology

### 3.3.1 Study site

Field sampling was conducted in the gravelly sites of the Arakawa River and the sandy sites of the Hii River to compare differences depending on sediment particle size (Fig. 3.5.9). Both sites were in the anastomosing section. The Arakawa River is located at the center of Japan, originating from the Chichibu Mountains, flowing over 173 km before draining into Tokyo Bay. The middle section of the river consists of gravel channels typical of Japanese rivers.
Sampling was conducted at the Kumagaya Oaso gravel bar, 84 km upstream of the river mouth ($36^o$ 8’ 20” N, $139^o$ 20’ 35” E). The channel slope was approximately 1/500, and the mean sediment particle size at the sampling site was approximately $D_{50}$=50mm ($D_{25}$=10mm, and $D_{75}$=99mm). The land use in the basin upstream of the sampling site was mostly agriculture and forests. Historically, the riparian zone was occupied by non-vegetated gravel areas until 50 years ago. However, vegetation coverage has gradually increased since then due to river regulation, and now half of the area is covered with woody or herbaceous plant species. More details of the Arakawa sampling site are given in other works of the authors (Asaeda 2011; Asaeda and Sanjaya 2017).
The Hii River is located in the Shimane prefecture (Western region of Japan). The river originates from the Chugoku Mountains and flows first into Shinji Lake, then into Naka Lake before draining into the Sea of Japan. It extends about 153 km and drains a basin of about 2017

$km^2$. Eighty percent of the basin area is covered with forest, and the downstream 20% is covered chiefly with rice fields and residential areas. The sampling site was located 14 km upstream of Shinji Lake (35°21'57" N, 132°47'20" E), where the channel slope was approximately 1/600, and the bed was entirely composed of sand, $D_{50}$ = 0.5 mm ($D_{25}$ = 0.1 mm and $D_{75}$ = 0.8 mm). This sand had been deposited in the past after iron mining activities upstream.

The riparian topsoil-to-groundwater distance increases as the distance from the river channel increases, while the overall soil moisture level decreases. The sediment's rhizospheric zone was not saturated with water, and oxygen could penetrate sufficiently except in the zone close to channels (Guilloy 2011). All sampling points were taken on the riparian slopes where the ground surface was moderately covered with vegetation without being completely shaded. Both rivers and riparian zones are subject to intense flooding events. These floods are generally short and are caused by heavy rainfall. Once every 10 years, a major flood occurs. In all cases, however, the water level returns relatively quickly to the normal condition, that is, within less than a week. Except for these events, the water surface level is relatively stable. The soil moisture pattern in the riparian zone, which decreases with elevation from the ordinary water level, has not changed significantly for decades, as evapotranspiration rate balances with precipitation rate in most seasons. These conditions make the selected field sites excellent for studying the relationship between soil moisture and plant stress.

### 3.3.2 Plant and soil sampling

Sampling was conducted between 10:00 and 14:00 on days with normal, fine weather conditions for the respective sites and more than a month after the most recent flood in Ara river and Hii river. The sampling dates were more than a year later than flooding in the respective rivers in all cases.

Dominant plant species of each site were sampled for this study. The major tree species at the Arakawa site were *S. gilgiana*, *S. subfragilis*, *R. pseudoacacia*, and *A. altissima;* the major herbaceous species were *P. australis*, *P. japonica*, and *M. sacchariflorus*. At the Hii River site, the major tree species were *S. pierotii* and *J. mandshurica*; the major herbaceous species were *P. australis* and *M. sacchariflorus*. After surveying 30 km of the riparian zone along the river reaches, three healthy mature individuals per tree species and five well-developed plants per herb species across the elevation gradient of the site were selected and marked.

Selected tree species were 3 to 5 m high, and herb species were about 1.5 to 2 m high. The average root depth of the sampled plants was approximately 2 ± 0.5 m for the tree species and 0.3 ± 0.1 m for the herb species (not sampled, data based on the works).

Well-grown leaves exposed to solar radiation were carefully sampled by hand. To measure the difference between the solar radiation exposed and dark-adapted samples, additional leaves of the tree species were shaded for 30 min by covering them with a well-ventilated paper bag before sampling. All sampled leaves were sealed in a plastic bag immediately after sampling and stocked in an icebox filled with dry ice (~ -70 °C) to transport to the laboratory, where samples were frozen at -80 °C until analysis. It took 8 hr at most to transport to the laboratory. The half-life period of $H_2O_2$ is about 1.4 to 58 hr in water (Asaeda et 2011b).

The low temperature in the stocked icebox may be a stress to changing $H_2O_2$ concentration. However, the temperature declined quickly to below -10 °C, which is lower than the freezing point of $H_2O_2$ as the tissue thickness was less than 1 mm. There was no significant difference in the $H_2O_2$ tissue concentrations between the icebox-stocked and immediately analyzed samples ($p$ = 0.76) and between icebox-stocked and non-stocked samples ($p$ = 0.90).

The exact location of all sampled plants was recorded using GPS (Garmin eTrex). Absolute elevations of sampling points and the ordinary water level above the sea level (referenced to Tokyo peil (T.P.)) were obtained from the surveyed sectional map of the Upstream Arakawa River Management Office for the Arakawa river site and the River Management Offices of Izumo for the Hii River site. Soil samples were collected in triplicate from 20 cm below the ground surface from all plant sampling points. The riparian soil was stocked in tightly sealed plastic bags and brought to the laboratory for analysis. Soil samples were collected from the same place where the plants grow, and leaves were sampled from that plant species as well.

### 3.3.3 $H_2O_2$ assays of plant leaves

In the analytical process of $H_2O_2$ concentration, the fresh plant leaves were dry frozen with liquid nitrogen and ground (~500 mg) together with an ice-cold 50 mM phosphate buffer (pH 6.0). Polyvinylpyrrolidone (PVP) was added to this extraction to mask the effect of phenolic compounds in the plant materials. The extraction was centrifuged at 5000 rpm at 4 ºC for 15 min, and the supernatant was separated. An aliquot of 750 µL extracted supernatant was mixed with 2.5 mL of 0.1% titanium sulfate in 20% (v/v) $H_2SO_4$ (Jana and Choudhuri 1982). The mixture was centrifuged at 5,000 × g at 20℃ for 15 min. The intensity of the yellow color developed through the reaction was measured spectrophotometrically (UV mini 1210, Shimadzu, Japan) at a wavelength of 410 nm. The absorption at 410 nm includes the effect of other soluble compounds. Thus, the $H_2O_2$ concentration was calculated from the slope of the standard curve obtained from the known $H_2O_2$ concentration, which was offset, derived by the intercept absorption rate with zero $H_2O_2$ concentration samples. The results were compared with those of the e-FOX method, and a suitable correlation ($r$ = 0.971) was obtained. The results were presented as micromol/gFW (Fresh weight).

### 3.3.4 Soil analyses

The moisture content of the soil samples was determined using the weight loss method. The soil sample was weighed initially and subjected to drying at 105 ºC in an electric oven until a constant weight over time was recorded. Soil moisture content was estimated by the difference between the initial and final weights of the sample. Soil nutrient analyses were conducted only for the fine sediment component (< 128 micro meter). TN and TP were analyzed separately from the soil in different method. TN in the sediment (oven- dried) was analyzed by a CHN elemental analyzer (Yanako CHN coder MT-5 and Autosampler MTA-3, manufactured by Yanako CO., Ltd., Japan). TP was determined by the molybdenum blue colorimetric method[84] after digestion with $H_2SO_4$–$HClO_4$ (APHA, 1998).

### 3.3.5 Distribution analysis of target species

In both river sites, all individual trees taller than 1 m were counted per species for the entire gravel bar for the Arakawa River and the area between the dikes of the Hii River along a 2.7 km long stretch[86]. Compared with the channel morphology obtained by the MLIT River Management Office of each river, the Arakawa Upstream River Management Office, and the Izumo River Management Office, tree densities were obtained for every 0.5 m elevation.

### 3.3.6 Statistical analysis

Data analyses were carried out using R (R 2011) for the dependent variables. Raw data of all variables were checked for normal distribution with the one-sample Kolmogorov-Smirnov test and for homogeneity of the variances with Levene's test. When necessary, arcsine transformation was performed. Results are presented as mean ± SD (n = 3). Data were subjected

to a one-way Analysis of Variance (ANOVA) followed by Dunkan's multiple range test to evaluate the mean difference at a 0.05 significance level. The correlation coefficient ($r$) and the significance levels ($p$) were used to evaluate the strength and significance of the parameters estimated. Non-linear regression analysis was performed to view species to species effect among parameters ($H_2O_2$, soil moisture content, TN, TP, and TN: TP). A general additive model was performed to find out the efficacy of derived Monod equations among parameters (soil moisture, $H_2O_2$, TN, TP, TN: TP, and elevation).

## 3.4 Results

### 3.4.1 Edaphic condition of the riparian zone

The soil moisture content gradually decreased with increased elevation away from the channel at both the Arakawa River and Hii River sites ($r$ = -0.90, $p$ <0.01 for the Arakawa River and $r$ = -0.98, $p$ <0.01 for the Hii River) (Fig. 3.5.1). The distributions were empirically given as follows:

For the Arakawa River (gravelly) site ($r$ = 0.99):

$$\text{Soil moisture } (\%) = 45.0 \exp(-0.53 \text{ elevation}) \quad (1)$$

For the Hii River (sandy) site ($r$ = 0.99):

$$\text{Soil moisture } (\%) = 45.0 \exp(-0.265 \text{ elevation}) \quad (2)$$

where elevation is given in meters above the ordinary water level.

No significant difference was observed in the deviation from these curves between the previously observed results in other studies and the present study (Arakawa River, $r$ = 0.91, $p < 10^{-7}$ for 2006, $r$ = 0.89, $p < 10^{-7}$ for 2008, and $r$ = 0.78, $p < 10^{-7}$ for 2010; Hii River, $r$ = 0.99, $p$ < 0.01 for 2014 and $r$ = 0.98, $p$ < 0.01 for 2015) (Karunaratne et al., 2003; Asaeda et al., 2008). For any given elevation, soil moisture is always higher in the Hii River, owing to the smaller particle size of the riparian soil.

Unlike soil moisture content, the sampled riparian soil TN and TP concentrations did not show a specific trend with respect to surface elevation (Arakawa River, $r$ = 0.4, $p$ = 0.12 for TN and $r$ = 0.35, $p$ = 0.18 for TP; Hii River, $r$ = -0.16, $p$ = 0.6, for TN and $r$ = 0.33, $p$ = 0.2 for TP). The average riparian soil TN concentration at the Arakawa River site was lower than that at the Hii River. At the Arakawa River site, soil TN values ranged between approximately 0.1% and 0.2%, regardless of elevation, whereas the values distributed widely between 0.1% and 0.4% at the Hii River site. These high values can be caused by the smaller particle size of the riparian soil at the Hii River. No significant difference was observed from the previous studies (Karunaratne et al., 2003; Asaeda et al., 2008).

Riparian soil TP was relatively similar in both rivers, ranging between 0.01% to 0.05%. The TN:TP ratio ranged from 1 to 4 at both river sites (Vamsi-Krishna et al., 2021). The elevation ranges, leaf $H_2O_2$ concentration of target species, and leaf $H_2O_2$ concentration compared with the soil moisture distribution are shown in Fig. 3.5.2 and Fig. 3.5.3. *Salix* spp. were distributed at elevations lower than 2.5 m from the ordinary water level at both river sites, while other tree species were distributed from 2.0 m upwards. For herbs, *Phragmite*s spp. were primarily located at elevations lower than 2.5 m from the ordinary water level, while *M. sacchariflorus* thrived from 1.5 m upwards.

### 3.4.2 Foliar $H_2O_2$ concentration with respect to soil moisture content

Fig. 3.5.4(a) displays the relation between soil moisture content and foliar $H_2O_2$ concentration of trees. This figure includes the foliar $H_2O_2$ values of *S. subfragilis* at the Miharu Reservoir and soil moisture values from other reports (Castro-Díez et al., 2009; Berendse et al., 1998) for comparison. Salix species are generally found at sites with more soil moisture. With increasing

soil moisture, all *Salix* species showed a significant decrease in foliar $H_2O_2$ concentrations regardless of river and reservoir sites ($r = –0.89$, $p < 0.01$ for *S. pierotii*; $r = –0.92$, $p < 0.1$ for *S. gilgiana*; $r = –0.5$, $p < 0.1$ for *S. subfragilis*). A near constant value was maintained with higher soil moisture contents until the soil moisture content reached 35%. Thus, the lowest leaf $H_2O_2$ concentration (20 μmol/gFW) was recorded at higher than 35% soil moisture content.
The soil moisture content and $H_2O_2$ concentration of other tree species were positively correlated ($r = 0.66$, $p < 0.01$). The value of $H_2O_2$ concentration (approximately 15 μmol/gFW) was lowest at around 5% soil moisture content (the driest condition), then rose with increasing soil moisture until approximately 20% to 30%, where it attained about 40 μmol/gFW.
Foliar $H_2O_2$ concentration of herbaceous species concerning soil moisture content is presented in Fig. 3.5.4(b). Regardless of sites, similar species-specific trends were observed. *M. sacchariflorus* had a higher $H_2O_2$ concentration at low soil moisture conditions, which decreased with increasing soil moisture until 15% ($r = –0.94$, $p < 0.001$). It slightly increased again with higher moisture content. *Phragmites* spp. showed a uniquely decreasing trend with soil moisture content ($r = –0.84$, $p < 0.001$). In general, herb species were negatively correlated with soil moisture ($r = –0.80$, $p < 10^{-5}$).

### 3.4.3 Foliar $H_2O_2$ concentration with respect to other factors

TN and TP contents of plant biomass have organ-specific differences. Plant's N: P ratios <10 and >20 often (not always) correspond to N- and P-limited biomass production in short term periods respectively, although it can vary in the long term (Güsewell et al., 2003). The TN contents of plant biomass were approximately 0.9%, 0.4%, and 2.1% of roots, stems, and leaves of *Salix* spp., respectively, and 1.3%, 0.6%, 3.0% for *R. pseudoacacia*. TP contents were 0.1%, 0.01%, 0.12% for *Salix* spp. and 0.05%, 0.12%, and 0.07% for *R. pseudoacacia*. For herbs, whole-plant values were 1.9±0.25% for TN and 0.19±0.03% for TP. The nitrogen and phosphorus ratio of plant biomass's range was 10 to 30. This was much larger than the nitrogen and phosphorus ratio of soils, which was 1 to 4. Therefore, compared with phosphorus, nitrogen seems to be more restrictive for plant growth. However, given the correlation between biomass (of *P. australis* or *M. sacchariflorus*) and riparian soil, the TN and TP contents were 0.24±0.05 ($p = 0.37$) for TN and 0.1±0.37 ($p = 0.71$) for TP (data not shown). There was no correlation between biomass and TN or TP contents in the riparian soil.

The $H_2O_2$ concentration with respect to riparian soil TN or TP contents is shown in Fig. 3.5.5. *J. mandshurica* were distributed at a wide range of TN concentrations, compared with other species, while *Salix* species' habitat had relatively low riparian soil TN concentration. However, the $H_2O_2$ concentration of all species scattered largely, and there was no significant correlation with riparian soil TN or TP concentrations, and TN: TP (*S. gilgiana*: $r = -0.16$, $p = 0.73$ for TN and $H_2O_2$, $r = -0.43$, $p = 0.34$ for TP and $H_2O_2$, $r = -0.06$, $p = 0.90$ for TN: TP and $H_2O_2$; *S. pierotii*: $r = -0.31$, $p = 0.55$ for TN and $H_2O_2$, $r = 0.66$, $p = 0.15$ for TP and $H_2O_2$, $r = -0.46$, $p = 0.36$ for TN: TP and $H_2O_2$; *J. mandshurica*: $r = -0.80$, $p = 0.1$ for TN and $H_2O_2$, $r = -0.78$, $p > 0.1$ for TP and $H_2O_2$, and $r = -0.13$, $p = 0.76$ for TN: TP and $H_2O_2$; *R. pseudoacacia*: $r = -0.35$, $p = 0.25$ for TN and $H_2O_2$, $r = -0.56$, $p = 0.08$ for TP and $H_2O_2$, $r = -0.256$, $p = 0.45$ for TN: TP and $H_2O_2$; *A. altissima*: $r = -0.187$, $p = 0.67$ for TN and $H_2O_2$, $r = -0.71$, $p = 0.178$ for TP and $H_2O_2$, $r = 0.25$, $p = 0.690$, for TN: TP and $H_2O_2$).

For herb species, *M. sacchariflorus* were distributed over a relatively wide range of riparian soil TN concentrations (0.15% to 0.26%), and its foliar $H_2O_2$ concentration varied between 16 to 50 μmol/gFW. *P. australis* exhibited 12 to 32 μmol/gFW of $H_2O_2$ concentrations in 0.10% to 0.35% of riparian soil TN concentration. *P. japonica* were distributed near the shoreline of the channel, where TN concentration was relatively low. There was no significant correlation

between $H_2O_2$ and TN or TP concentrations and TN: TP in riparian soil (*P. japonica*: $r = -0.063$, $p = 0.871$ for TN and $H_2O_2$, $r = -0.497$, $p = 0.174$ for TP and $H_2O_2$, $r = 0.041$, $p = 0.917$ for TN: TP and $H_2O_2$; *P. australis*: $r = 0.454$, $p = 0.546$ for TN and $H_2O_2$, $r = -0.754$, $p = 0.246$ for TP and $H_2O_2$, $r = 0.748$, $p = 0.246$ for TN: TP and $H_2O_2$; *M. sacchariflorus*: $r = 0.044$, $p = 0.866$ for TN and $H_2O_2$, $r = -0.241$, $p = 0.352$ for TP and $H_2O_2$, $r = 0.181$, $p = 0.486$ for TN: TP and $H_2O_2$).

The non-linear regression analysis was performed for each species to find out effect of different parameters (soil moisture, $H_2O_2$, TN, TP, TN: TP). *Salix* spp., *R. pseudoacacia, A. altissima*, and *J.mandshurica* individually show significant correlation between soil moisture content and $H_2O_2$ ($p < 0.001$, $p < 0.001$, $p = 0.002$, and $p < 0.05$ respectively), whereas no significant results were observed ($p = 0.459$ for *Salix* species, $p = 0.884$ for *R. pseudoacacia,* $p = 0.186$ for *A.altissima*, and $p = 0.652$ for *J.mandshurica*) among parameters (soil moisture, TN, TP, and TN:TP). Herb species also exhibit a similar type of significant trend in the nonlinear regression analysis. *Phragmites* spp. and *M. sacchariflorus* indicate significant correlation between soil moisture content and $H_2O_2$ ($p < 0.001$ for both species) On the contrary, no significant results were noticed ($p = 0.076$ for *Phragmites* spp., and $p = 0.138$ for *M. sacchariflorus*) among parameters (soil moisture, TN, TP, and TN:TP).
We cannot find significant trends between $H_2O_2$ concentration and riparian soil TN or TP. One of the reasons is because the variation range of TN and TP was too small to affect $H_2O_2$ concentration in the observed sites[64], indicating the insignificant effect of nutrients on $H_2O_2$. However, widely various TN or TP variations may have a possibility to affect $H_2O_2$ concentration.

### 3.4.4 Modeling foliar $H_2O_2$ concentration

From the above discussion we can conclude that due to the insignificant impact of nutrients especially TN or TP concentration, the $H_2O_2$ concentration in plants is solely related to soil moisture content. The simple empirical equations can be formulated based on the Monod equations. The equations show the fundamental structure as easily explicit and broaden the underlying mechanism of the species.
For tree species:
*Salix* spp.

$$H_2O_2(\mu mol/gFW) = 20.0\frac{25^4}{25^4+\text{Soil Moisture}^4} + 25.0 \quad (3)$$

(for the range of soil moisture < 60%, $r = -0.60$, $p < 10^{-4}$)
Other tree spp. (*Juglans mandshurica*, *Robinia pseudoacacia*, *Ailanthus altissima*):

$$H_2O_2(\mu mol/gFW) = 30.0\frac{\text{Soil Moisture}^4}{25^4+\text{Soil Moisture}^4} + 15.0 \quad (4)$$

(for the range of soil moisture < 40%, $r = 0.76$, $p < 10^{-4}$)
For herbaceous species:
*Phragmites* spp.

$$H_2O_2(\mu mol/gFW) = 50.0\frac{15^{1.5}}{15^{1.5}+\text{Soil Moisture}^{1.5}} \quad (5)$$

(for the range of soil moisture < 45%, $r = 0.92$, $p < 10^{-9}$)
*Miscanthus sacchariflorus*:

$$H_2O_2(\mu mol/gFW) = 45.0\frac{8^3}{8^3+\text{Soil Moisture}^3} + \frac{\text{Soil Moisture}^2}{35.0} \quad (6)$$

(for the range of soil moisture < 30%, $r = 0.89$, $p < 10^{-3}$)
where soil moisture is given by %.
Soil moisture distribution depends on the sediment particle size composition. This relationship was similar for either gravelly or sandy rivers (Fig. 3.5.1). For simplicity, soil moisture at the ground surface at the Arakawa River site, equation (1), and the Hii River site, equation (2), are

used as representative of those gravelly rivers and sandy rivers in general. Substituting equations (1) and (2) into (3) to (6), $H_2O_2$ concentration for each type of plant is given as a function of the elevation. The estimated $H_2O_2$ concentration is presented in Fig. 3.5.6 and Fig. 3.5.7. The locations of observed plants in the present study and other reports are displayed by thick lines in the figures.

Both *Salix* spp. and other tree species are restricted to areas with stress levels less than 40 to 45 micromol/gFW. Therefore, *Salix* spp. can only distribute starting from the ordinary water level up to 4 m in gravelly rivers and 6 m in sandy rivers. In contrast, other tree species can distribute from 1 m upwards in gravelly rivers and 3 m upwards in sandy rivers. For herbaceous species, all species areas appear where soil moisture results in an $H_2O_2$ concentration below micromol/gFW. *Phragmites* spp. can, therefore, distribute at elevations up to 3 m in gravelly rivers and more than 5 m in sandy rivers. *M. sacchariflorus* grows at higher elevations and cannot grow below1.5 m above the ordinary water level.

$H_2O_2$ is generated under stressed conditions. Thus, the intensity of stresses is correlated to the $H_2O_2$ concentration. $H_2O_2$ concentrations of light-exposed and dark-adapted samples are shown in Fig. 3.5.8. There was no difference in $H_2O_2$ concentration between the two treated leaves ($p = 0.96$) for all tested species, indicating that dark conditions do not cause significant stress.

To ensure the efficacy of our derived equation, we try to analyze their suitability with the general additive model (GAM). We found that GAM supports our derived equations appropriately among different parameters (elevation, soil moisture content, $H_2O_2$, TN, TP, and TN: TP). The $H_2O_2$ concentration of tree species increased with higher soil moisture content, yet in terms of *Salix* spp., the relation is vise-versa. In the case of herb species, *Phragmites* spp. shows a significant negative correlation with soil moisture content and $H_2O_2$, whereas *M. sacchariflorus* shows a "U" shaped correlation with soil moisture content and $H_2O_2$. Considering these situations and derived equations, tree and herb species were divided into four groups in GAM analysis. Twso groups from tree species (*Salix* spp. and other tree species), and another two groups from herb species (*Phragmites* spp. and *M. sacchariflorus*). *Salix* spp., *Phragmites* spp., *M. sacchariflorus*, exhibit significant correlation with soil moisture content and $H_2O_2$ among parameters (elevation, soil moisture content, $H_2O_2$, TN, TP, and TN: TP) (Tables 4, 5, 6, and 7). In the case of other tree species, significant correlation occurs with $H_2O_2$ and soil moisture content shown in Table 5, and elevation shows a strong correlation with $H_2O_2$ (Table 5).

### 3.5 Identifying oxidative stress in different elevation levels using figures

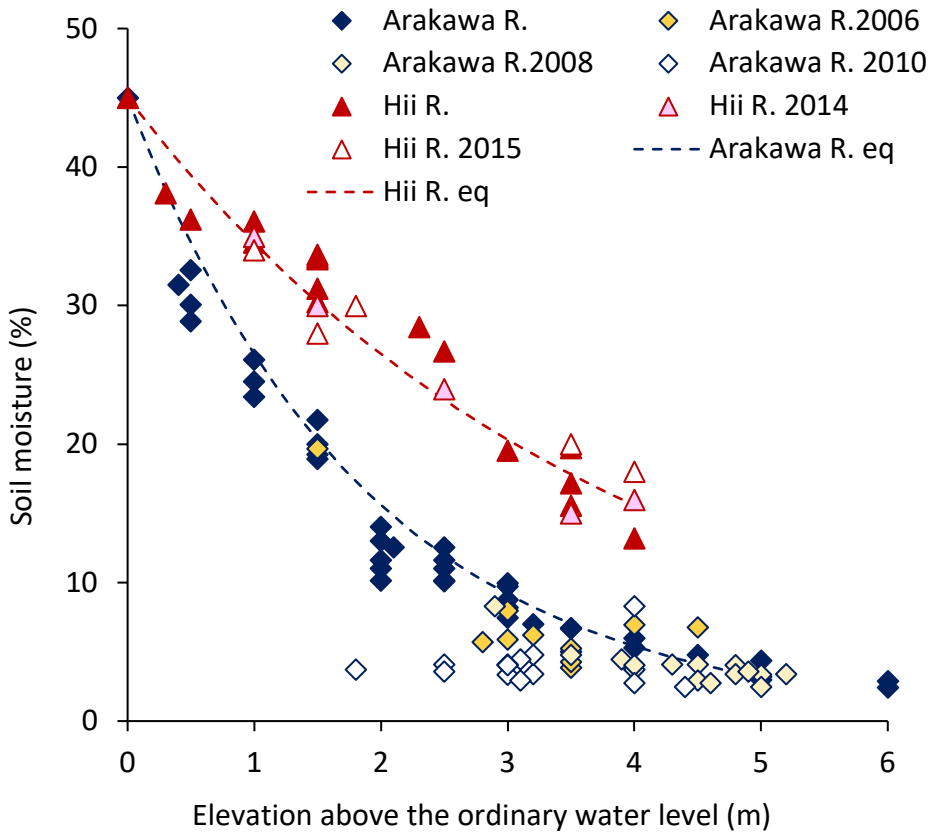
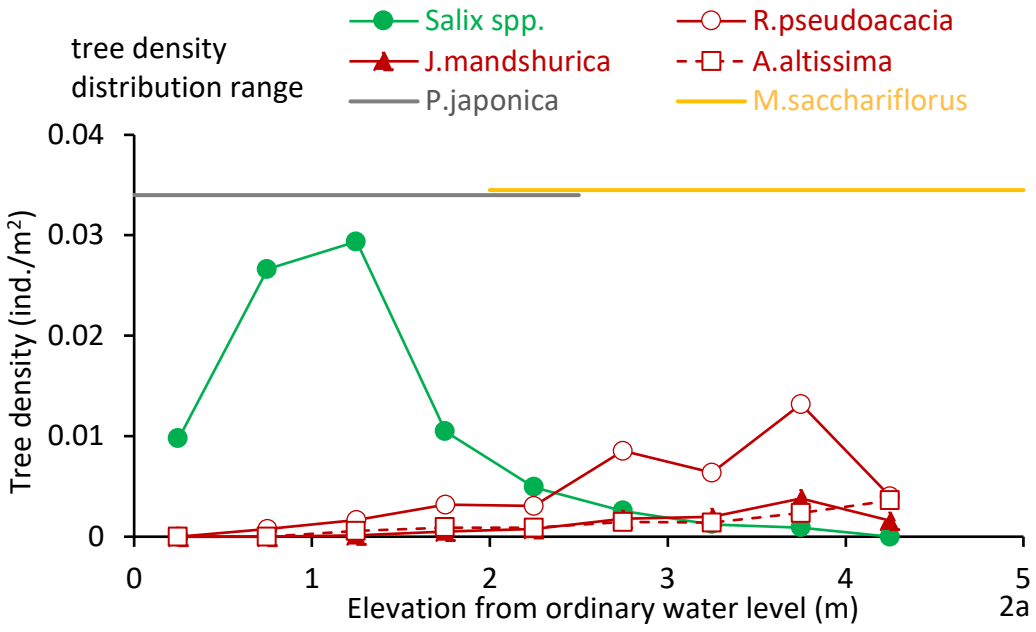



Figure 3.5.1. Relationship between elevation above ordinary water level and soil moisture. Data are from this study and previous studies from 2006 to 2015 (Castro-Diez et al.; Berendse et al.).



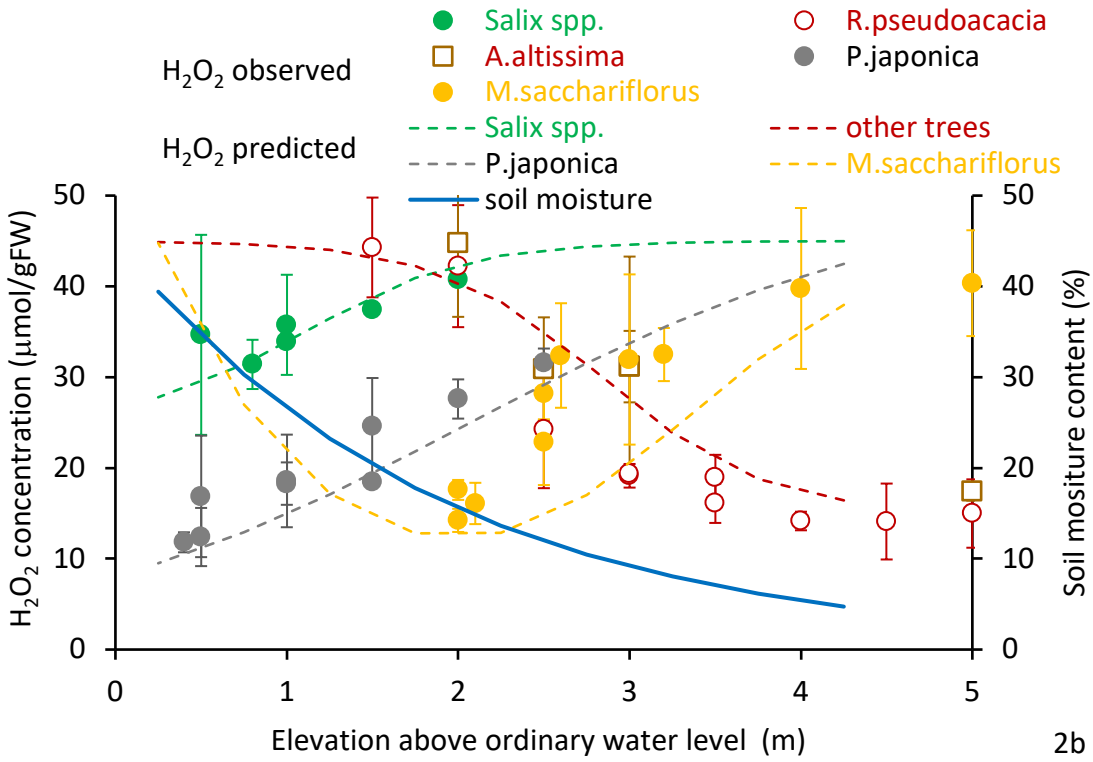
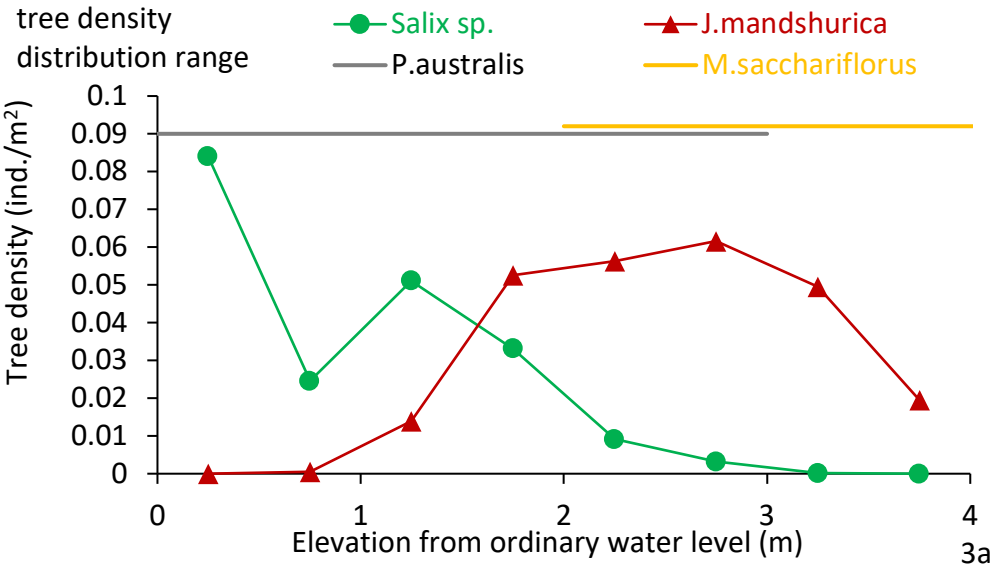



Figure 3.5.2. (a) Tree density and herb colonies at the Arakawa River site elevations of entire observed area. (b) Soil moisture and $H_2O_2$ concentration of samples and simulated results from equations (3) to (6) distributions are also shown. Vertical bars in (b) are standard deviations.



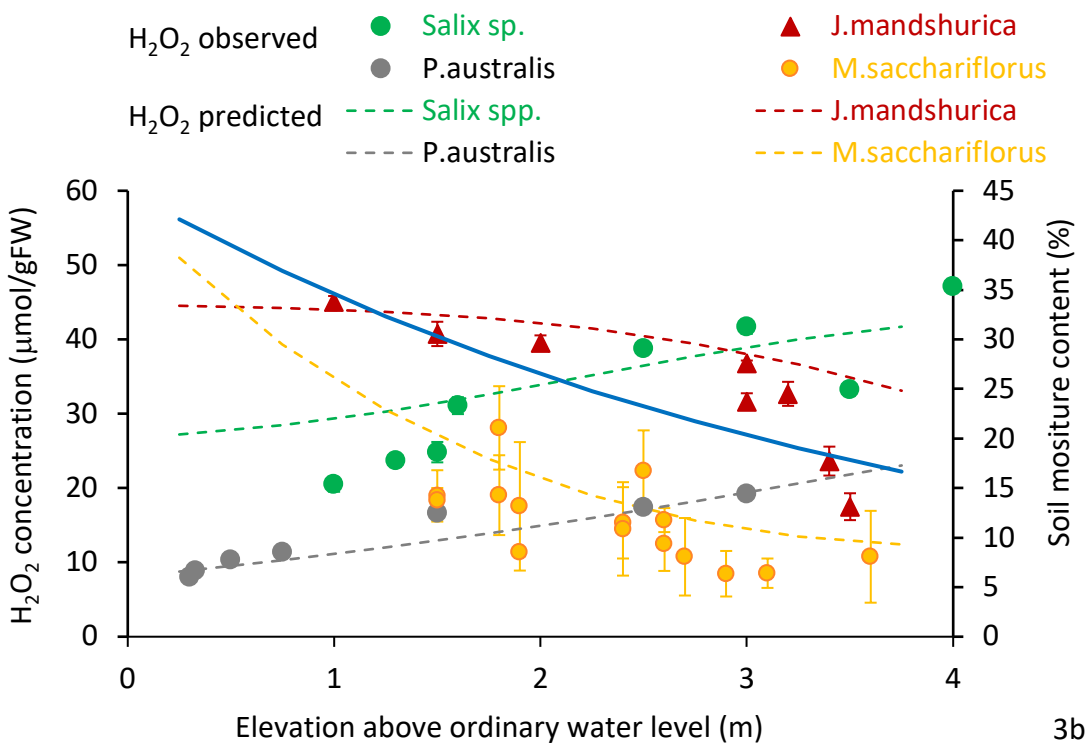



Figure 3.5.3. (a) Tree density and herb colonies at different elevations at the Hii River site of entire observe area. (b) Soil moisture and $H_2O_2$ concentration of samples and simulated results from equation (3) to (6) distribution are also shown. Vertical bars in (b) are standard deviation.

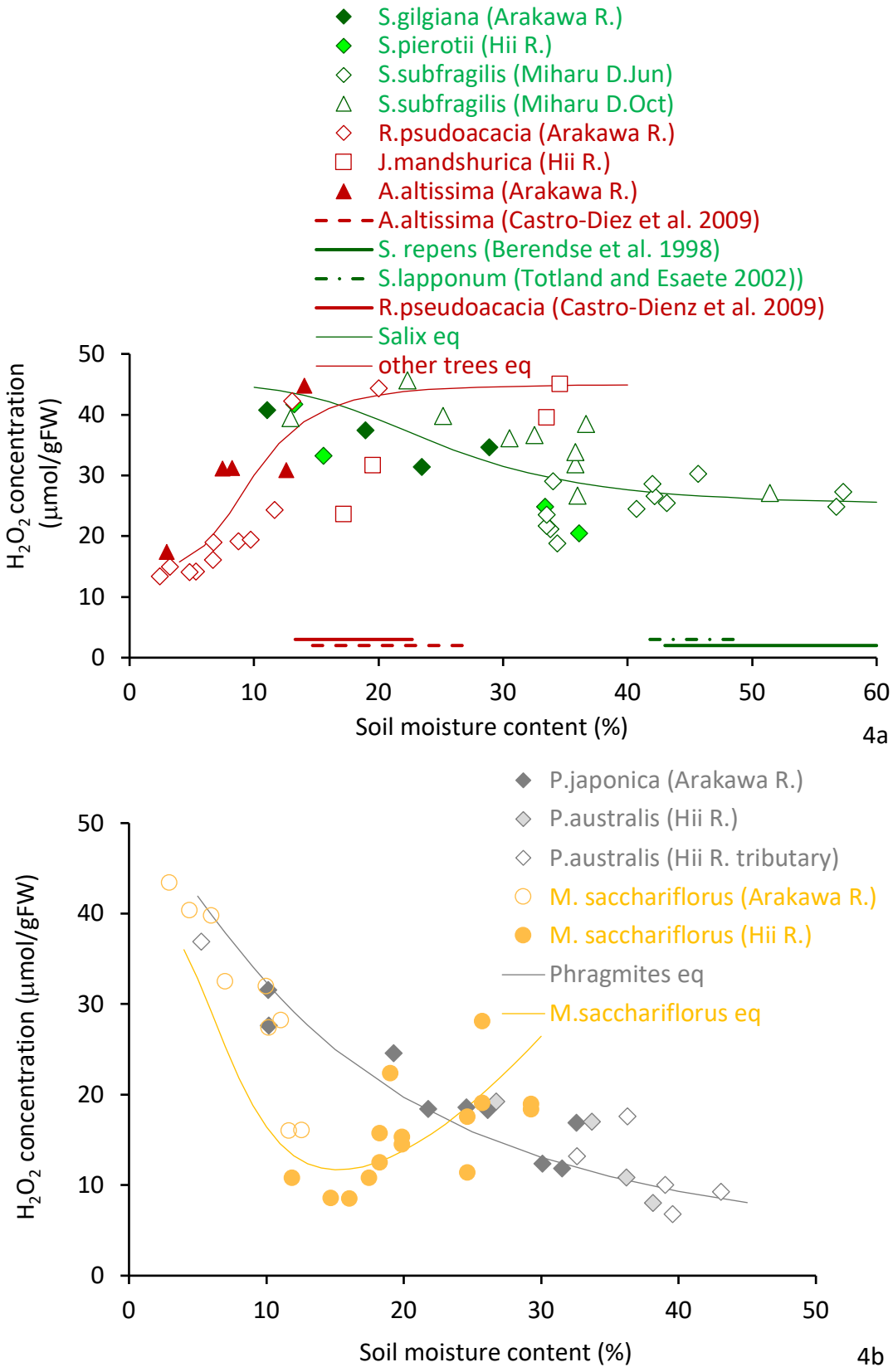



Figure 3.5.4. Relationship between soil moisture and leaf $H_2O_2$ for (a) different tree species and (b) herbaceous species. Reported soil moisture contents are added (Castro-Diez et al.; Berendse et al.; Totland and Esaete).

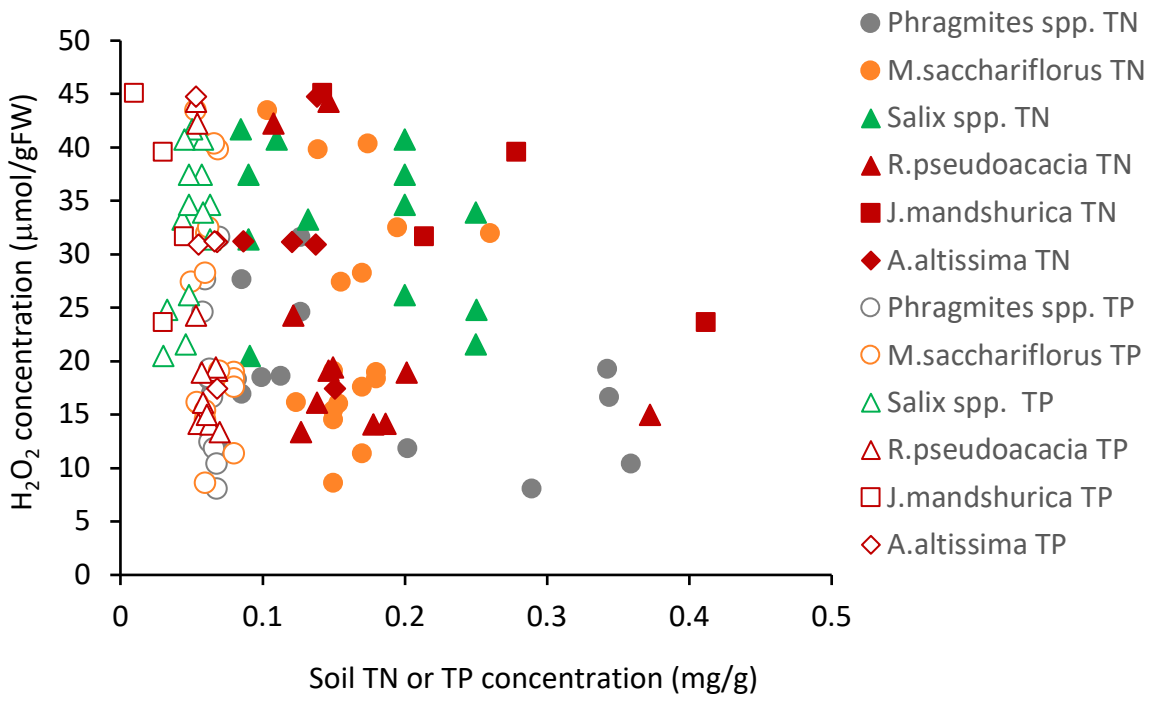



Figure 3.5.5. Relationship between leaf $H_2O_2$ concentration and soil TN or TP contents.

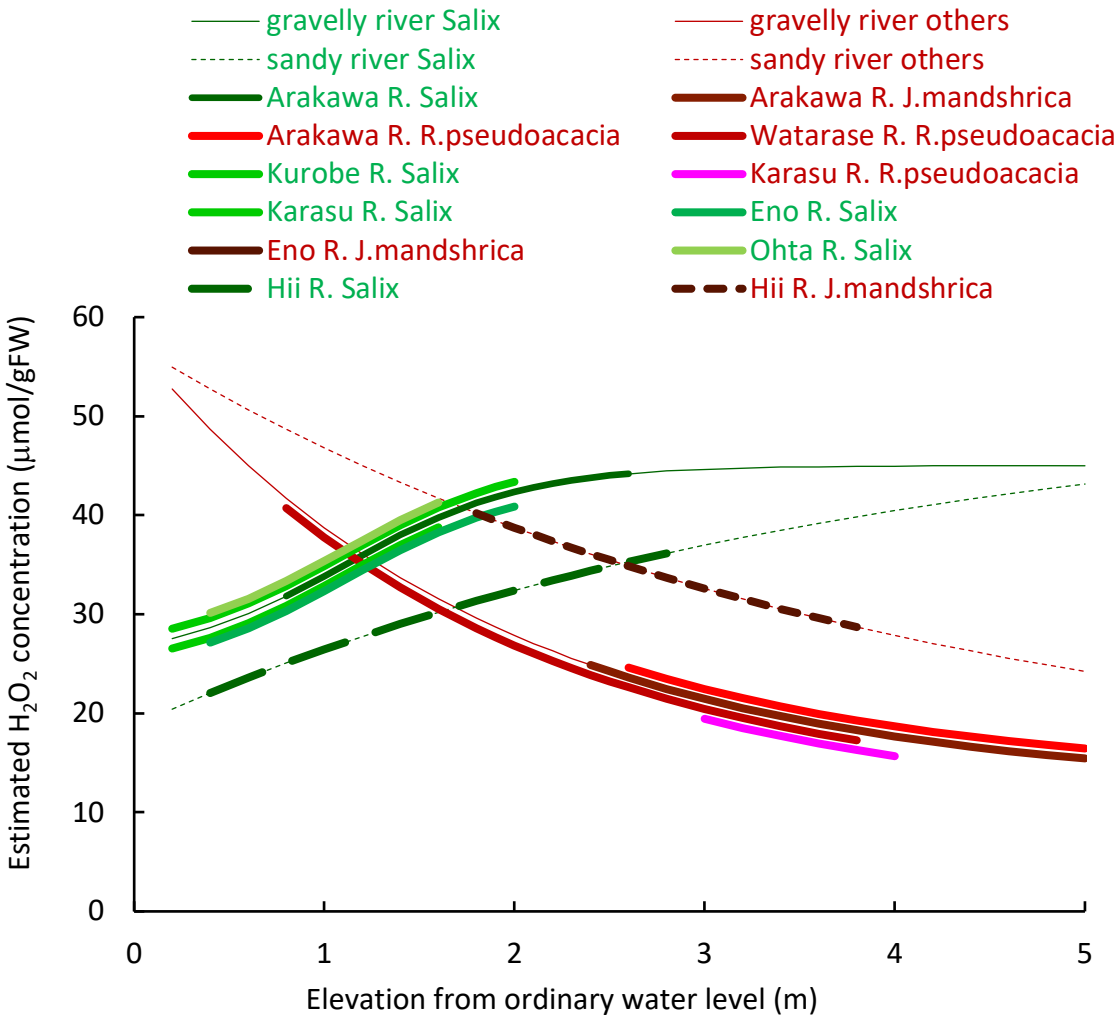



Figure 3.5.6. Estimated $H_2O_2$ concentration of tree species as a function of elevation from ordinary water level, compared with observed distribution (solid lines: gravelly reaches; dashed lines: sandy reaches; thick lines: observed distribution; green lines: *Salix* spp.; red lines: other species; Arakawa R., Hii R., Watarase R., Eno R., Ohta R.: this study; Karas R.; Kurobe R.)

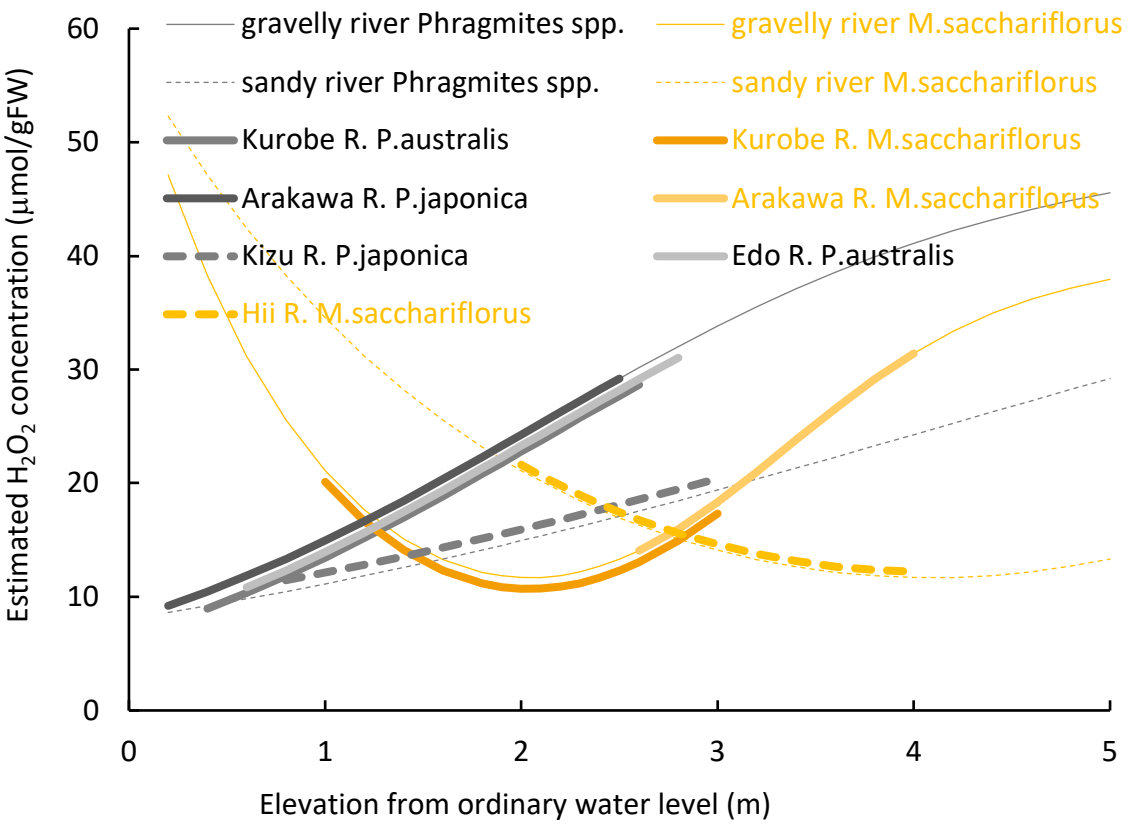



Figure 3.5.7. Estimated $H_2O_2$ concentration of herb species as a function of elevation from ordinary water level, compared with observed distribution (solid lines: gravelly reaches; dashed lines: sandy reaches; thick lines: observed distribution; gray lines: *Phragmites* spp.; orange lines: *M.sacchariflorus*; Arakawa R., Hii R., Watarase R., Edo R.: this study; Karas R.; Kurobe R.)

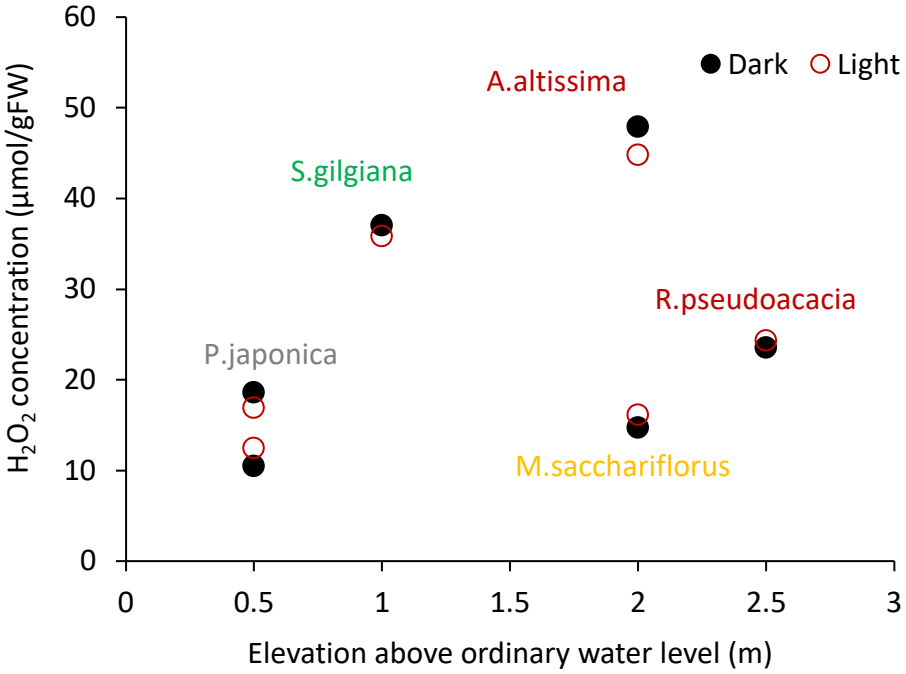



Figure 3.5.8. $H_2O_2$ concentration differences of light-exposed and dark-adapted samples.

Figure 3.5.9. Studied sites at Arakawa River (left) and Hii River (right).

Table 4: General additive model for *Salix spp* to find out correlation among parameters

| Approximate significance of smooth terms | | | | |
|---|---|---|---|---|
| Parameters | edf | Ref.df | F | p-value |
| s(Elevation) | 1 | 1 | 0.41 | 0.53735 |
| s(Soil Moisture) | 1 | 1 | 14.230 | <0.05 |
| s(Soil TN) | 1 | 1 | 0.001 | 0.97290 |
| s(Soil TP) | 1 | 1 | 0.359 | 0.56564 |
| s(Soil TN: TP) | 1 | 1 | 0.005 | 0.94761 |

R-sq.(adj) = 0.766 Deviance explained = 85.6%
GCV = 20.288 Scale est. = 11.593

Table 5: General additive model for other tree species to evaluate interaction among parameters

| Approximate significance of smooth terms | | | | |
|---|---|---|---|---|
| Parameters | edf | Ref.df | F | p-value |
| s(Elevation) | 1.980 | 1.998 | 10.618 | <0.01 |
| s(Soil Moisture) | 1 | 1 | 7.231 | <0.05 |
| s(Soil TN) | 1 | 1 | 0.639 | 0.431164 |
| s(Soil TP) | 1.606 | 1.844 | 2.273 | 0.078004 |
| s(Soil TN: TP) | 1 | 1 | 0.415 | 0.525103 |

R-sq.(adj) = 0.728 Deviance explained = 78.2%
GCV = 45.069 Scale est. = 35.012

Table 6: General additive model for *Phragmites spp.* to observe correlation among parameters

| Approximate significance of smooth terms | | | | |
|---|---|---|---|---|
| Parameters | edf | Ref.df | F | p-value |
| s(Elevation) | 1 | 1 | 0.422 | 0.5368 |
| s(Soil Moisture) | 1 | 1 | 11.343 | <0.05 |
| s(Soil TN) | 1 | 1 | 0.054 | 0.8232 |
| s(Soil TP) | 1 | 1 | 0.309 | 0.5958 |
| s(Soil TN: TP) | 1 | 1 | 0.063 | 0.8090 |

R-sq.(adj) = 0.897 Deviance explained = 94%
GCV = 8.8054 Scale est. = 4.7414

Table 7: General additive model for *Miscanthus sacchariflorus* to find out relationship among parameters

| Approximate significance of smooth terms | | | | |
|---|---|---|---|---|
| Parameters | edf | Ref.df | F | p-value |
| s(Elevation) | 1 | 1 | 0.001 | 0.9762 |
| s(Soil Moisture) | 1.981 | 1.998 | 6.642 | <0.05 |
| s(Soil TN) | 1 | 1 | 1.216 | 0.2988 |
| s(Soil TP) | 1.574 | 1.812 | 1.077 | 0.4555 |
| s(Soil TN: TP) | 1.290 | 1.490 | 0.779 | 0.3683 |

R-sq.(adj) = 0.864 Deviance explained = 92.2%
GCV = 29.554 Scale est. = 15.916

### 3.6 Discussion

In Japanese rivers, the volume of sediments has substantially decreased over the last five decades because of the construction of dams upstream, gravel mining, and the reduction of sediment inflow due to the afforestation of the upstream mountainous areas. Therefore, the riparian morphology and sediment characteristics remain relatively stable, even though it is subjected to recurrent large flooding events (Liebault 2002; Lach et al., 2002). Most of the sampling points were inundated only during a large flooding event, which occurs approximately once every 10 years, with the inundation lasting for a maximum of one week. Therefore, the measured soil moisture content is characteristic of the study site most of the time. The moisture content that the riparian zone reaches is highly affected by sediment particle composition; it is higher at sandy channels than at gravelly channels, as shown in the present study. There were good agreements between the results of previously observed cases (from equations (1) and (2)) and the current study at both river sites.

Several other factors can affect riparian vegetation stress. In the riparian zone of the observed reach, generally nitrogen rather than phosphorus becomes critical for plant growth (Kachi and Hirose 1983), and it may thus influence the growth of vegetation via soil moisture. However, in the present study, there was no correlation between plant tissue $H_2O_2$ concentration and TN along with TP concentration in the riparian soil for the tree and herb species as shown in Fig. 4.5.6. Therefore, nitrogen and phosphorus were considered as neither major factors for limiting growth nor regulating species distribution in both rivers. Also, solar radiation did not affect the $H_2O_2$ concentration as expressed in Fig. 4.5.5. The last major flooding was more than two years ago, and no erosion or deposition trace was observed on the soil surface. Thus, mechanical disturbances on the ground surface during floods likely did not affect the distribution of plants. Therefore, soil moisture seems to be the major component of stress for the plants in this case.

#### 3.6.1 Threshold $H_2O_2$ concentration

When a plant is exposed to environmental stress, it generates $H_2O_2$ in different organelles. This occurs partly in the scavenging process of various reactive oxygen species and partly directly. However, some $H_2O_2$ is scavenged by the antioxidant activities and finally converted to water and oxygen. Under high-stress conditions, plant tissues become damaged and die off due to the high concentration of $H_2O_2$. Therefore, there is likely a threshold concentration below which plants do not deteriorate or die. In the present study, no cases with $H_2O_2$ concentrations higher than 40 to 50 µmol/gFW were observed for tree species. Concentrations above 40 to 50 µmol/gFW of $H_2O_2$ seem to be too high to maintain a healthy condition for a tree species. For

example, *Salix subfragilis* in the Miharu Dam highly deteriorated at $H_2O_2$ concentration levels ~40 to 50 μmol/gFW, which was caused by at least 5 months of low soil moisture. The low soil moisture condition seems to be a high stressor for *Salix* spp. At the same time, each species distributed only the elevation where the $H_2O_2$ concentration becomes lower than these values. In the present study, *R. pseudoacacia* and *A. altissima* growing on low elevation sites along the Arakawa River had a high $H_2O_2$ concentration of 40 to 50 μmol/gFW. These plants may experience greater stress with high soil moisture. All *R. pseudoacacia* and *A. altissima* plants intrude and grow at the lower elevation sites (below 1 m, 30% moisture; Fig. 4.5.1), had died off over the course of several years (Asaeda et al 2011) without being subjected to flood disturbance. This situation indicates that more than 40 to 50 micromol/gFW of $H_2O_2$ concentration reflects harsh conditions for *R. pseudoacacia* and *A. altissima* (Fig. 4.5.2(a)). In contrast, all *S. gilgiana* growing at elevations above 3 m had disappeared after several years.[26,52,54] *S. gilgiana* cannot spread at this elevation, probably because of very low soil moisture content. For herbaceous species, no *Phragmites* spp., were found at elevations with less than 10% soil moisture in the gravelly channel and 20% in the sandy channel. This corresponds to an $H_2O_2$ concentration of 35 □mol/gFW and above. *M. sacchariflorus* distributed at relatively low soil moisture, with the lowest being about 5%, corresponding to higher than 40 micromol/gFW of $H_2O_2$ concentration. Although the water potential was not measured in this study, the sediment's logarithmic scale of suction (pF) was approximately 4 at 10% moisture content. The pF ~3-4 corresponds to the wilting point and is nearly the critical condition for the continued growth of many herbaceous plants. The 40 to 50 micromol/gFW of $H_2O_2$ concentration obtained for the existing plants seems to be the critical value for survival. The lowest elevation where *M. sacchariflorus* was found was approximately 1.5 m above the ordinary water level, where the plant had about 30 micromol/gFW of $H_2O_2$ concentration. The location seemed close to the lowest elevation for the distribution, although this was not absolutely critical. Thus, the $H_2O_2$ concentration of *M. sacchariflorus* is U-shaped with respect to soil moisture. For other species, *Salix* spp. and *Phragmites* spp. were not found in water, and the $H_2O_2$ concentration is unknown, and other tree species growing at very low soil moisture conditions were not obtained. However, there is a possibility the $H_2O_2$ concentration also has a U-shaped trend.

The threshold condition of $H_2O_2$ concentration spans a relatively wide range, from 35 to 50 micromol/gFW species specifically. However, plants begin to die off with $H_2O_2$ concentrations higher than the threshold value, as was observed in this study. Thus, the threshold condition delineates the habitat conditions where plants can form.

### 3.6.2 Characteristics of tree species

*Salix* species usually are dominant at low-elevation zones (Hernandez-Leal et al., 2019). In the present study, *Salix* species always developed above the ordinary water level, and no *Salix* was found in the constantly inundated zone. *Salix* spp. make use of hydrochory: dispersal of seeds through water, which encroaches onto the shoreline in spring. Therefore, the distribution of *Salix* spp. seems to reflect the physiological survival of occupied seeds.

For *Salix* spp., $H_2O_2$ concentration decreases in accordance with soil moisture content till 40%. This situation indicates low stress in high soil moisture content. Then, it slightly increased with more than 40% soil moisture. The preferable soil moisture of these *Salix* species appears to be around 40%, which was near saturation. Several experiments indicated that even steady inundation does not lead to high stress as long as oxygen concentration is sufficiently present in the substrate. Other tree species in the present study provided a relatively unique increasing trend of $H_2O_2$ concentration from 5% of soil moisture to higher levels. The soil moisture thus

is the major component determining the potential locations for spatial distribution in the riparian zone.
For *R. pseudoacacia*, the highest photosynthesis rate was reported at 17% soil moisture. It was substantially lower at 8% and 24%, and the highest reported leaf water potential was at 13% soil moisture, compared to lower soil moisture contents. These results suggest the species' preferred specific soil moisture conditions, which is confirmed in the present results (Fig. 4.5.2(a) and Fig. 4.5.3(a)). The soil moisture content at the root zone of trees was not measured in the present study. However, it can be derived from the soil moisture distribution in Fig. 4.5.1. This species does not seem to prefer a high moisture level, although it is typically found in riparian zones. Specifically, this species can thrive at higher elevations in the riparian zone. Since this species uses hydrochory like the *Salix* spp., these results also imply that seeds can reach the preferred elevations only during very high water levels. *A. altissima* showed a similar trend of $H_2O_2$ concentration in response to soil moisture content as found in *R. pseudoacacia*. The $H_2O_2$ concentration level of *J. mandshurica* was slightly lower than *R. pseudoacacia* and *A. altissima*, indicating that this species prefers higher moisture content. Its high buoyancy can be more easily dispersed downstream and captured ashore at the riverside, where the soil moisture content is more congenial to its growth.

### 3.6.3 Characteristics of herbaceous species

$H_2O_2$ concentration in herbaceous species decreased with increasing soil moisture, and this relationship was consistent regardless of species. *M. sacchariflorus* is one of the most common species of the riparian zone and is distributed at higher elevations than *Phragmites* spp. However, the $H_2O_2$ concentration of *M. sacchariflorus* was as high as 40 micromol/gFW when it grew on soil with approximately 5% moisture content. No distribution was found at higher elevations where the riparian soil has less than the soil moisture content. *Phragmites* spp., on the other hand, is typically distributed in the lower elevation zone where the soil moisture content is >10%. The tissue $H_2O_2$ concentration of *Phragmites* spp. in response to soil moisture content also indicates its preference for higher moisture conditions.

### 3.6.4 $H_2O_2$ concentration as the monitoring system

Riparian plants are subject to various stress factors, and they establish successfully only in areas where stress levels are sufficiently low. The riparian zone is often affected by flood disturbance, which changes the channel topography. However, although the reduction of sediment flux erodes the channel bed, it provides a stable habitat for riparian vegetation. Groundwater level variation takes a longer period than surface water and does not change much during a short period. If the soil moisture distribution is properly maintained with an appropriate period of low flow, regardless of short flood occurrences, the elevations where these species distribute or disappear can be predicted. Measurement of $H_2O_2$ can be a method to evaluate the threshold values of certain determining abiotic variables beyond which plants cannot survive. Therefore, their chance of disappearance under these conditions can be forecasted. This method is also more precise than most previous methods based on plant traits and empirical monitoring of their spatial distribution only. It is difficult to understand their preferred habitats by the general condition of their spatial distribution, owing to various types of stresses combined with different intensities and frequencies, and exposure periods. Also, it is highly dependent on confounding factors like timing and unusual events. The physiological condition of each species determines their unique distribution, excluding physical mortality.

## 3.7 Conclusion

Riparian vegetation encounters different environmental stresses. The findings of this study demonstrate that the spatial distribution of different species in a riparian zone occurs in the

context of elevation and soil moisture content. The nutrient level especially TN and TP cannot play significant role due to a small variation of TN and TP concentration. The $H_2O_2$ concentration and soil moisture content solely significant in the observed sight. The species-specific distribution zones can be explained by the $H_2O_2$ concentration in the plant, which could be a rapid, efficient, and reliable monitoring indicator for vegetation distribution. This study suggests that foliar $H_2O_2$ concentration represents the sum of all abiotic stress, and the plant population decreases or cannot survive when it is beyond a critical value.

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# Chapter 4
# Identification of environmental stressors for riparian plants using hydrogen peroxide as an oxidative marker

## 4.1 Background of the study

The effect of environmental stress on plants has recently been intensely studied, resulting in reports of several plant body features being subjected to stresses (Asaeda et et 2021, 2022). The available soil moisture along a riparian zone is a critical factor for plant occurrence (Ozinga et al. 2005), as water acts as a different type of support agent by initiating germination and determining plant establishment and growth. Due to frequent flood disturbances the sediment accumulations cannot develop properly. As a result, its nutrient level may decrease. The sediment nutrient level may also determine which species can distribute at specific zones. The sediment nutrient level in riparian soil, especially TN or TP concentration, can filter and alter its biogeochemistry. For instance, wetland plants take up 16% to 75% of TN. An overabundance of solar energy generates ROS, including superoxide radicals, due to the underutilization of energy absorbed by the PSII antennae complex in the PSII reaction center (Hanna and Jan 2016)), and damages cellular components, such as D1 protein, which otherwise recovers the damaged photosynthesis apparatus.

## 4.2 Objective of this study

The objective of the current study is to find the environmental stressors for riparian plants considering soil moisture, soil nutrient, and high solar radiation by quantifying hydrogen peroxide concentrations.

## 4.3 Methodology

### 4.3.1 Study site and species

Leaves of riparian plants, such as *Artemisia princeps Sicyos angulatus*, and *Solidago altissima*, were collected for this study. Sampling was conducted at the Arakawa- Taroemon reserver (350 56' 52.2"N, 139032' 13.1"E to 35° 59' 7.3" N, 139° 30' 58.2"E) on a sunny day on June 1$^{st}$, August 2$^{nd}$, and September 9$^{th}$, 2022. The collected samples were transported to the laboratory after finishing the sampling. Fully expanded leaves from the middle part of plants in each replicate were collected. The collected samples were prepared for analysis soon after arriving in the laboratory, which was described later. Soil samples were collected near collected trees leave samples to analyze soil moisture and macronutrients such as total Nitrogen (TN) or total phosphorus (TP).

### 4.3.2 Preparation of collected samples

Approximately 40 to 50 mg of the plant leaf was weighed and placed into a 15 ml centrifuge tube with a combination of beads (3mm and 10mm) (BMS Inc.). For each leaf, triplicate was performed. The centrifuge tube was frozen with liquid nitrogen and ground to a powder using Shake Master (BMS Inc., Japan). A volume of 5ml potassium phosphate buffer (pH 6, 50mM) was added, and a small amount of polyvinylpyrrolidone (PVP) was used to prevent the effect of the phenolic compound. The mixture was centrifuged (Kokusan H60-R, Japan) twice at 5500 rpm for 10 min, and the supernatant was collected as an extract to analyze $H_2O_2$ in eFOX and $Ti(SO_4)_2$ methods. The extract was transferred to a -80$^{o}$C refrigerator until analysis.

### 4.3.3 Determination of $H_2O_2$ Content with eFOX and $Ti(SO_4)_2$ assay

The modified ferrous-xylenol orange assay was used to measure $H_2O_2$ (Queval et al., 2008; Cheeseman 2006). The supernatant (100 µL) was added to 1 mL of the assay solution containing 250 µM ferrous ammonium sulfate, 100 µM sorbitol, 100 µM xylenol orange, 25 mM $H_2SO_4$ (sulfuric acid), and adding 1% ethanol to the reagent increases its sensitivity to $H_2O_2$ by 50% (Fujifilm wako pure chemical corporation, Japan), which has been deoxygenated with gaseous nitrogen to prevent artifacts. Reaction mixtures were incubated at room temperature for 15 min. The absorbance was measured at 560 nm by spectrophotometry (UVmini-1240, Shimadzu, Japan). $H_2O_2$ content was calculated by a standard curve using a series of diluted solutions of commercial high-grade 9.8 M $H_2O_2$ (30%) (w/w) (Dautania et al. 2014; Asaeda et al. 2022b). Brennan and Frenkel (1977) method was used to measure $H_2O_2$. The titanium (II) metal ion in the acidic solution forms a peroxide complex with $H_2O_2$, a yellow-colored compound. In a 10 ml round centrifuge tube, 750 µL of extraction was taken. 2.5 mL of 0.1% of $TiSO_4$ in 20% $H_2SO_4$ solution was added. The mixture was centrifuged at 10000rpm for 15 min at room temperature. 1 mL of the supernatant was transferred into a 1 mL spectrophotometer cell. The absorbance was measured at 410 nm. For blank, a mixture of 750 µL of 0.05 M phosphate buffer (pH 6.0) and 2.5 mL of 0.1 $TiSO_4$ in 20% $H_2SO_4$ was used.

### 4.3.4 Measurement of soil moisture

The moisture content of the soil samples was determined using the weight loss method. The soil sample was weighed initially and subjected to drying at 105 ºC in an electric oven until a constant weight over time was recorded. Soil moisture content was estimated by the difference between the initial and final weights of the sample.

### 4.3.5 Quantification of soil TN and TP

Soil nutrient analyses were conducted only for the fine sediment component (< 128 micro meter). TN and TP were analyzed separately from the soil combinedly. TN and TN were analyzed with a soil analyzer (EWTHA1J, Japan). Approximately 1 gram of soil was mixed with 30 mL of the supplied buffer. The mixture was shaken vigorously for 3 min. This mixture was filtered with Mili-Q pore (0.22 µm) filter paper, so the solution remained clear. An equal volume of the filtered mixture and buffer was inserted in the respective chamber. Soil cartilage was kept in the proper position. The reading of TN and TP was used using a standard curve. A standard solution of TN and TP (1000mg/L) was used to make the standard curves.

### 4.3.6 Statistical analysis

Raw data were checked for normal distribution with the one-sample Kolmogorov–Smirnov test as well as for homo- geneity of the variances with the Levene's test. All data were subjected to one-way analysis of variance (one-way ANOVA), followed by Turkey's multiple comparison test to evaluate mean differences at a 0.05 significance level ($p < 0.05$). Pearson's correlations were evaluated for the chlorophyll *a*/*b* ratio, antioxidative enzyme activity, soil nutrient concentrations, and total biomass (aboveground and belowground). All statistical analyses were performed using IBM SPSS version 28.

## 4.4 Results

The $H_2O_2$ concentration gradually decreased with increasing soil moisture content for *Artemisia princeps* ($r=-0.637$, $p<0.001$)and *Sicyos angulatus* ($r=-0.796$, $p<0.001$), whereas $H_2O_2$ concentration increased with increasing soil moisture for *Solidago altissima* ($r=0.658$, $p<0.001$) (Figure 4.5.1). The $H_2O_2$ concentration with respect to PAR, as well as riparian soil

TN or TP contents, is shown in Figure 4.5.2. The $H_2O_2$ concentration of all species scattered largely, and there was no significant correlation with $H_2O_2$ among PAR, riparian soil TN or TP concentrations, and TN: TP (*Artemisia princeps*: $r$ = -0.10, $p$ = 0.73 for TN and $H_2O_2$, $r$ = -0.23, $p$ = 0.34 for TP and $H_2O_2$, $r$ = 0.33, $p$ = 0.90 for TN: TP and $H_2O_2$; r=0.47, p=0.31 for PAR and H2O2; *Sicyos angulatus* $r$ = -0.07, $p$ = 0.55 for TN and $H_2O_2$, $r$ = -0.02, $p$ = 0.15 for TP and $H_2O_2$, $r$ = -0.15, $p$ = 0.36 for TN: TP and $H_2O_2$, r=0.46, p=0.27 for PAR and H2O2; *Solidago altissima*: $r$ = -0.01, $p$ = 0.1 for TN and $H_2O_2$, $r$ = -0.41, $p$ > 0.1 for TP and $H_2O_2$, and $r$ = 0.45, $p$ =0.76 for TN: TP and $H_2O_2$, r=-0.09, p=0.46 for PAR and H2O2). GAM is performed to evaluate the correlations with H2O2 among parameters (TN, TP, TN:TP, and PAR). Tables 8, 9, and 10 show that $H_2O_2$ only significantly correlated with soil moisture content. Deviance explained lies between 67 to 80% which implies high accuracy of the findings. There is no significant correlation observed between soil moisture and TN or TP concentration (Figure 4.5.3).

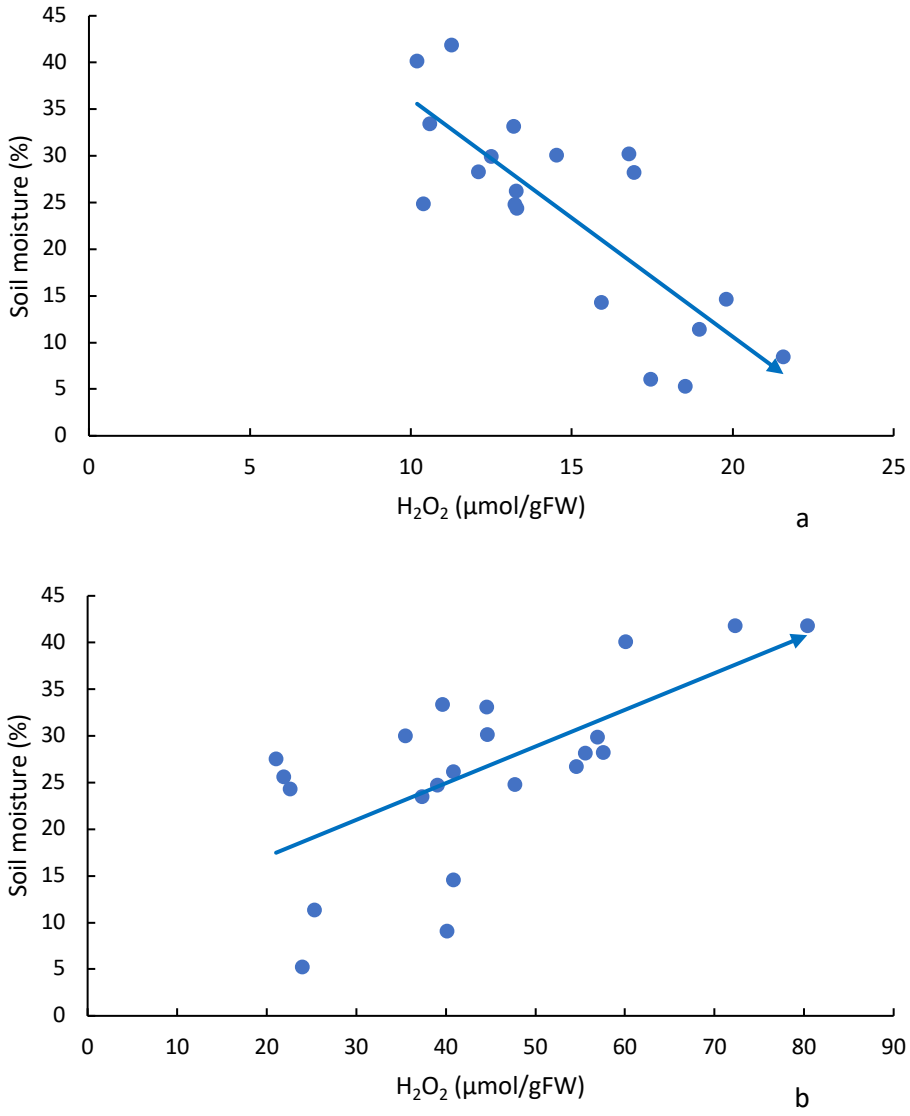


Figure 4.5.1: Relationship between soil moisture and $H_2O_2$ in Sicyos angulatus (a), and Solidago altissima (b).

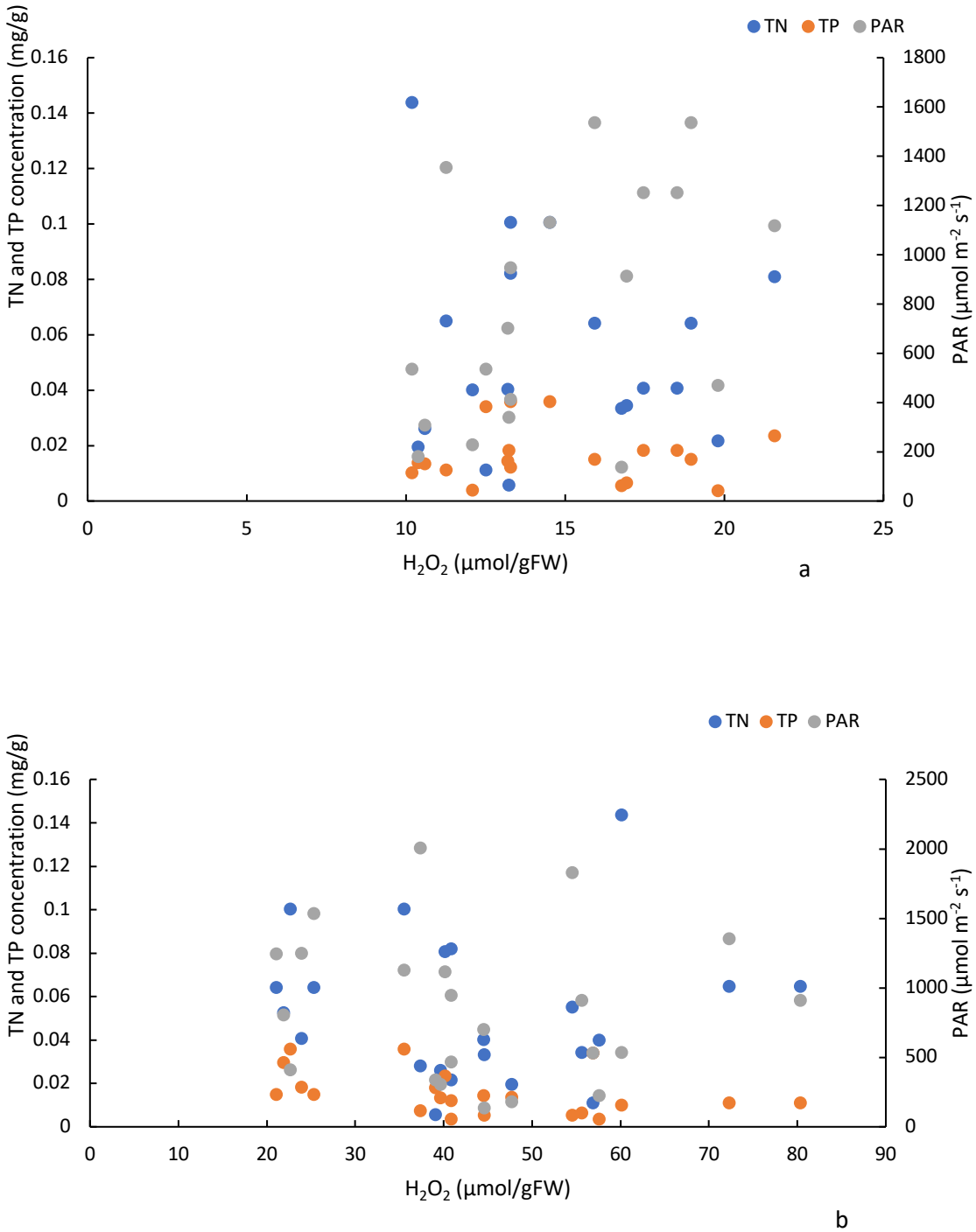


Figure 4.5.2: Relationship between $H_2O_2$ and TN or TP concentration, and PAR *Sicyos angulatus* (a), and *Solidago altissima* (b).

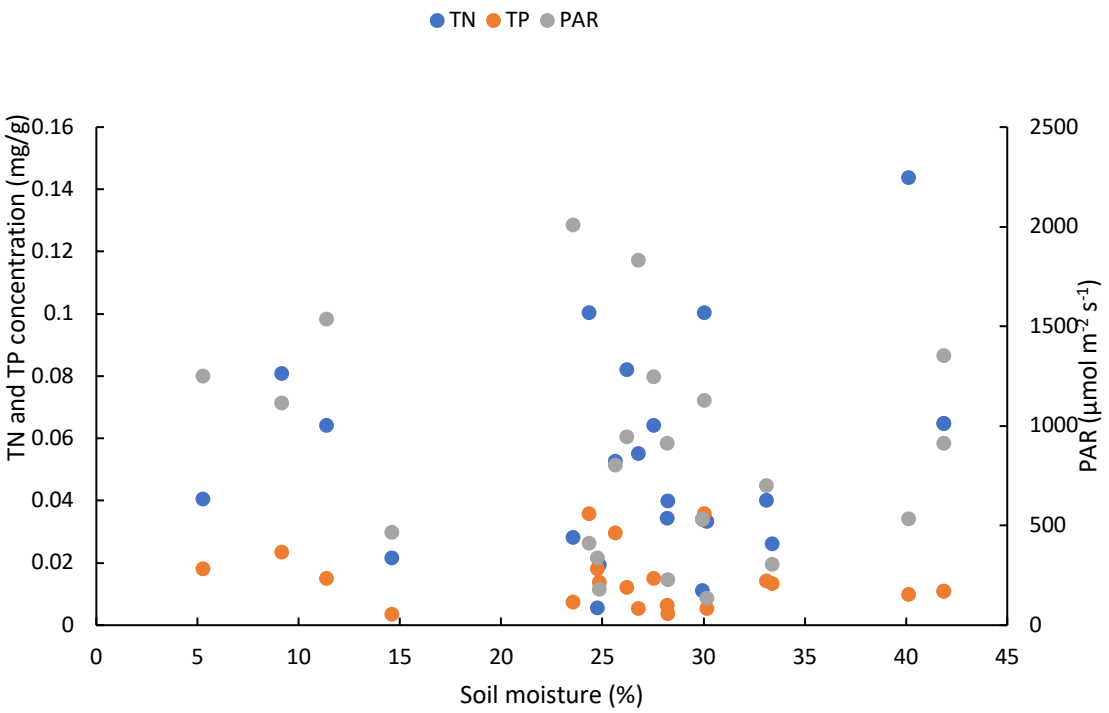


Figure 4.5.3: Relationship between soil moisture and TN, TP, and PAR

Table 8: General additive model for *Sicyos angulatus* to find out correlation among parameters with $H_2O_2$

| Approximate significance of smooth terms | | | | |
|---|---|---|---|---|
| Parameters | edf | Ref.df | F | p-value |
| s(Soil moisture) | 1 | 1 | 9.412 | <0.01 |
| s(Soil TN) | 1 | 1 | 3.340 | 0.087 |
| s(Soil TP) | 1.845 | 2.198 | 1.081 | 0.377 |
| s(Soil TN: TP) | 1 | 1 | 3.377 | 0.086 |

R-sq.(adj) = 0.474;  Deviance explained = 67.1%

Table 9: General additive model for *Solidago altissima* to observe correlation among parameters

| Approximate significance of smooth terms | | | | |
|---|---|---|---|---|
| Parameters | edf | Ref.df | F | p-value |
| s(Soil Moisture) | 2.129 | 2.477 | 9.112 | <0.01 |
| s(Soil TN) | 1 | 1 | 2.851 | 0.11515 |
| s(Soil TP) | 1 | 1 | 0.454 | 0.51215 |
| s(Soil TN: TP) | 1 | 1 | 1.524 | 0.23888 |

R-sq.(adj) = 0.623 Deviance explained = 74.5%

### 4.6 Discussion

Riparian vegetation plays a vital role in protecting banks from erosion, controlling flow velocity during heavy floods, increasing riparian infiltration, trapping, and stabilizing sediment (Gurnell 2014). Thus, the distribution of species-spe- cific elevations in the riparian zone is due to the effect of the soil moisture content (Takahashi and Nakamura 2010; Ye et al. 2019). This study indicates that soil moisture content is species-specific. Many studies have reported that reed communities always inhabit a river bank close to the water (Hudon et al. 2016; Volesky et al. 2017), whereas trees occupy higher elevations (Gurnell et al. 2016). Stomata close in response to reduced soil moisture, which then decreases photosynthetic activity during exposure to high solar radiation, which accelerates accumulation of $H_2O_2$ (Chaves and Oliveira 2004). In Japanese rivers, the volume of sediments has substantially decreased over the last five decades because of the construction of dams upstream, gravel mining, and the reduction of sediment inflow due to the afforestation of the upstream mountainous areas. Therefore, the riparian morphology and sediment characteristics remain relatively stable, even though it is subjected to recurrent large flooding events (Liebault and Piegay 2002; Lach and Wyzga 2002; Asaeda et al., 2015; Asaeda and Sanjaya 2017). Therefore, the measured soil moisture content is characteristic of the study site most of the time. The moisture content that the riparian zone reaches is highly affected by sediment particle composition.

#### 4.6.1 Soil nutrient and plant stress

Higher soil nutrient content leads to reduced abiotic stress, and thus plant diversity and coexistence may increase due to less competition (Kepfer-Rojas et al. 2019). However, the opposite scenarios have also been reported. Increasing nutrient content can favors competitive species that are capable of efficiently capturing resources (Dingaan et al. 2017). Plant growth in riparian ecosystem depends on nutrient-rich organic matter and on the dissolved nutrients in the water (Asaeda et al. 2011b). In the riparian zone of the observed reach, generally nitrogen rather than phosphorus becomes critical for plant growth (Kachi and Hirose 1983; Asaeda et al., 2011c; Asaeda and Rashid 2012; Asaeda and Sanjaya 2017), and it may thus influence the growth of vegetation via soil moisture. However, in the present study, there was no correlation between plant tissue $H_2O_2$ concentration and TN along with TP concentration in the riparian soil for the tree and herb species as shown in Fig. 6. Therefore, nitrogen and phosphorus were considered as neither major factors for limiting growth nor regulating species distribution in both rivers. Also, solar radiation did not affect the $H_2O_2$ concentration as expressed in Fig. 2. The last major flooding was more than two years ago, and no erosion or deposition trace was observed on the soil surface (Asaeda et al., 2011). Thus, mechanical disturbances on the ground surface during floods likely did not affect the distribution of plants. Therefore, soil moisture seems to be the major component of stress for the plants in this case.

In the past two decades, invasive species began to form colonies in many rivers (Ministry of Lands Infrastructure Transportation and Tourism in Japan [MLIT], 2016). It often covers extensive areas of the channel bed and completely changes the ecosystem there (Collier et al., 1999; Yarrow et al., 2009). It affected stream ecosystems extremely, retarding flow velocity, increasing sedimentation (Collier et al., 1999), and exile of native species (Santos et al., 2011; Gillard et al., 2017). Therefore, though the effects were particularly eminent in Japanese gravel rivers, the invasive species is a worldwide problem.

Vegetation management requires monitoring of plants. Common approaches are adaptive management to understand the condition of plants, where the growth and prosperity or shrinkage of stands are monitored for a long period. It is extremely time-consuming and costly. The current approach used in the study quantifies the environmental stress in rela- tively no time, which involves a very simple methodology of analysis there by helping to quantify the environmental stress associated with different species and to understand the level of risk associated with each species and manage them accordingly (Asaeda et al. 2020, 2021). This method also helps to restore the species that are on a verge of extinction by examining the favorable location of colonization quanti- fying the environmental stress in a short period.

When a plant is exposed to environmental stress, it generates $H_2O_2$ in different organelles. This occurs partly in the scavenging process of various reactive oxygen species and partly directly (Mittler 2002; Sharma et al., 2012). However, some $H_2O_2$ is scavenged by the antioxidant activities and finally converted to water and oxygen. Under high-stress conditions, plant tissues become damaged and die off due to the high concentration of $H_2O_2$. The low soil moisture condition seems to be a high stressor for each species.

## 4.7 Conclusion

Riparian vegetation encounters different environmental stresses. The findings of this study demonstrate that the observed species in a riparian zone occurs in the context of soil moisture content. The nutrient level especially TN and TP cannot play significant role due to a small variation of TN and TP concentration. The $H_2O_2$ concentration and soil moisture content solely significant in the observed sight. This study suggests that foliar $H_2O_2$ concentration represents the sum of all abiotic stress, and the plant population decreases or cannot survive when it is beyond a critical value.

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# Chapter 5 Exploring nonchemical approach to eradicate cyanobacteria using $H_2O_2$ as an indicator

## 5.1 Background of the study

Cyanobacteria are photoautotrophs in nature. The presence of light directly affects the growth of cyanobacteria, even moderate light changes are stressful (Welkie et al. 2019). The ability of cyanobacteria to respond to varying light intensities provides insights into photosynthesis and biotechnology applications (Flombaum 2013). The physiological metabolism of cyanobacteria depends on the light intensity, so different light intensities need to be investigated (Asaeda et al., 2022a; Barnuevo and Asaeda, 2018). Due to extreme light conditions (very low or very high), photoinhibition can occur [Walsh et al., 1997]. In photoinhibition, electron transport in photosystem II is reversibly reduced to protect the photosynthesis apparatus (Hanelt 1996) as well as other photoprotective mechanisms, macroalgae, and phytoplankton (Hortonn and Ruban 2005; Kirilovsky 2007; Zhang et al. 2008). Chlorophyll fluorescence characteristics in response to light change result from photoinhibition (Jones and Hoegh-Guldberg 2001).
During cyanobacteria growth, higher temperatures promote metabolic acceleration. At the same time, at the ecosystem level, they contribute to the formation of thermal stratification, which promotes biomass accumulation at the water's surface (O'Neil et al., 2012). *P. ambiguum* and *P. foetida* grows best at 25°C to 33°C (Bryanskaya et al., 2008; Chamizo et al., 2020). Subtropical and tropical regions can experience cyanobacterial blooms all year round due to their ability to proliferate at high temperatures (Rangel et al., 2016; De la Escalera et al., 2017). Despite their preferential growth conditions at higher temperatures, little is known about how fluctuating temperatures affect their growth.

The shortage of nutrient conditions, including phosphorous (P) and nitrogen (N), is identified as a dominant stressor that suppresses the growth of cyanobacteria. Phosphorus is an important macronutrient to plankton growth in many ways. Phosphorus makes rigid structures in cell walls, membranes, and nucleic acids by making covalent links between monomers. Phosphorus is also involved in cell metabolism directly by storing energy as polyphosphate bodies in plankton cells. The absolute concentrations of P and N and the stoichiometric ratio of these elements often play an important role in plankton growth in lakes. N:P mass ratio varies between 240 and 0.5, depending on the variation of P concentration in lakes. When the mass ratio of N:P exceeds 10, P is considered as the limiting factor. On the other hand, when N:P less than 10, N becomes the limiting factor on phytoplankton growth, including cyanobacteria in freshwater bodies. Hence, both surplus and deficiency of nutrients could cause significant alternations in cyanobacteria biomass and cellular stress. The combined effect of various abiotic stresses on the production rate is often reported (Asaeda et al., 2022). Some combinations inhibit growth due to the contradicting impacts of stressors; however, a significant reduction of biomass is also reported as caused by simultaneous exposure to multiple stressors compared to a single stress source. Hence, excessive radiation stress combined with a shortage of P and N nutrients could generate huge cellular stress and cyanobacterial growth inhibition.

## 5.2 Objective of the study

There is a huge possibility of using abiotic stresses like light intensities and temperatures instead of chemical methods (Asaeda et al., 2020, 2021, 2022). Therefore, this study aims to (1) find the threshold level of oxidative stress of cyanobacteria using $H_2O_2$ as a biomarker, (2) develop a nonchemical approach using abiotic stress, such as different light intensities and temperatures to eradicate cyanobacteria, (3) effects of the PAR regime and phosphorus

concentrations on cyanobacteria stress, particularly endogenous $H_2O_2$ concentrations, under the condition of naturally produced $H_2O_2$ from organic matter, (4) combined effects of the PAR regime and P concentrations on $H_2O_2$ concentrations, and (5) relationship between $H_2O_2$ concentrations and antioxidant enzyme activities of cyanobacteria, aiming at the possibility of applying $H_2O_2$ concentrations as a proxy to detect stress intensity in algal management and the contribution rate of the biological $H_2O_2$ production rate in the treatment.

## 5.3 Material and methods for Quantifying oxidative stress of cyanobacteria

### 5.3.1 Short-term exposure

Two cyanobacterial species *Microcystis aeruginosa* [NIES-111) and *Phormidium ambiguum* (NIES-2119), were obtained from the National Institute for Environmental Studies (NIES), Japan. The Autoclaved 100% BG 11 (Rippka et al. 1979) medium was used for both cultures throughout the experiment. The strains were started culturing at 30°C [± 0.3°C). The strains were cultured with white fluorescent light, containing a flux of 10–20 µmol $m^{-2}$ $s^{-1}$ in a 12 h:12 h light: dark cycle inside an incubator (Cool Incubator KMH-259 from AS ONE Corporation). During the light phase of the incubation period, each culture was manually shaken twice daily. Subcultures were conducted (Abenayaka et al. 2017, 2018) until a sufficient amount of *M. aeruginosa* and *P. ambiguum* were obtained.

### 5.3.2 Experimental procedure

A sufficient amount of *M. aeruginosa* and *P. ambiguum* were transferred into three incubators at 30°C (± 0.3°C), 20°C (± 0.3°C), and 10°C (± 0.3°C) at 10-20 µmol $m^{-2}$ $s^{-1}$ PAR for 23 days to acclimatize. The diurnal variations of PAR were performed after the 23 days of incubation, starting at 6:00 and continuing until 21:00. The PAR was 0 µmol $m^{-2}$ $s^{-1}$ in the initial stage at 6:00. The PAR increased hourly by 100 µmol $m^{-2}$ $s^{-1}$ from 6:00 and reached maximum 600 µmol $m^{-2}$ $s^{-1}$ during the period of 11:00–13:00. After that, light intensity decreased parallelly. It came to 0 µmol $m^{-2}$ $s^{-1}$ at 18:00. The samples were collected every three-hour intervals at 6:00, 9:00, 12:00, 15:00, 18:00, and 21:00. Collected samples were allocated in 1.5 mL tubes taking 1 mL of *M. aeruginosa* and *P. ambiguum* cells which was centrifuge at 10,000 rotation per minute (rpm) for 10 minutes in 4°C. The supernatant was removed and kept at -80°C for bioassays described below.

### 5.1.3 Long-term exposure

#### 5.3.3. 1 *M. aeruginosa P. ambiguum*, and *P. foetida* cell culture

The strains of *M. aeruginosa*, *P. ambiguum* (NIES-2119), and *P. foetida* (NIES-512) were provided by the National Institute for Environmental Studies, Japan. Throughout the experiment, autoclaved BG 11 medium (Rippka 1979) was provided to each species. The strain was cultured at 30°C (±0.3°C) with a white fluorescent lamp that provided a 12 h:12 h cycle of light and darkness in an incubator (KMH-259, Japan). Light intensity in the incubator ranges between 10 and 20 µmol $m^{-2}$ $s^{-1}$ for *M. aeruginosa* and *P. ambiguum,* and 20 to 30 µmol $m^{-2}$ $s^{-1}$ for *P. foetida*. When the cultures were incubated under light, they were manually shaken for a day thrice. Until enough *P. ambiguum,* and *P. foetida* were produced, it was subcultured.

#### 5.3.3.2 Experimental procedure

We transferred sufficient *M. aeruginosa*, *P. ambiguum* and *P. foetida* into three incubators at 30°C (± 0.3°C), 20°C (± 0.3°C), and 10°C (± 0.3°C) for 24 days at 10-20 µmol $m^{-2}$ $s^{-1}$ for *M. aeruginosa* and *P. ambiguum* and 20-30 µmol $m^{-2}$ $s^{-1}$ light intensity for *P. foetida*. Unfortunately

after 24 days of acclimatization at 10°C *M. aeruginosa* dies. In the case of *M. aeruginosa* the results were described for 30°C and 20°C. The effects of light exposure on *P. ambiguum* and *P. foetida* at each temperature were examined by introducing white fluorescent light at 10, 30, 50, 200, 100, and 600 µmol $m^{-2}$ $s^{-1}$. In order to maintain 12 hours of light and 12 hours of darkness, an automatic timer was set (REVEX PT7, Japan). A quantum sensor (Apogee, MQ-200) was used to measure light intensities, which were then uniformly adjusted in the medium. Culture flasks were gently shaken three times per day for homogeneous exposure to light. Samples were taken twice for analysis. The first samples were taken at 12:00 noon on the 24 days of acclimatization, considered starting day. Following the first sampling, a second sampling was conducted at seven days intervals at the same time. The starting day collection sample is identical this is because preliminary light intensities were changed during the collection time. Collected samples were allocated in 1.5 mL tubes, taking 1 mL of *P. ambiguum* and *P. foetida* cells in different tubes, and centrifuged at 10,000 rotations per minute (rpm) for 10 minutes at 4°C. The supernatant was removed and kept at -80°C for bioassays described below. Each culture group was replicated three times to conduct the experiment.

### 5.3.4 Nutrient depletion

#### 5.3.4.1 Culture and incubation

*P. ambiguum*, an odor-forming benthic cyanobacterial species, was obtained from the National Institute of Studies (NIES), Japan. The strain was cultured and acclimatized for 30 days in an autoclaved BG 11 medium, maintained at 20 °C under controlled PAR conditions with white fluorescent light, having flux of 20 µmol$m^{-2}$$s^{-1}$ in a light-and-dark cycle of 12 h:12 h. The cultures were manually shaken twice a day. Cells were subcultured by diluting with new BG 11 medium as needed.

#### 5.3.4.2 Experimental procedure

After 30 days, well-grown cyanobacterial cells were collected by centrifugation, washed once with distilled water, and then re-suspended in modified BG 11 media. All experiments were conducted by using incubators (MIR-254, Sanyo, Tokyo, Japan) with a nutrient level of BG-11 medium consisting of $NaNO_3$ 17.6 mM, $K_2HPO_4$ 0.2296 mM, $MgSO_4.7H_2O$ 0.0146µM, Na2CO3 0.0189µM, Citric acid 0.0031µM, Ferric ammonium citrate 0.0023µM, EDTA ($Na_2$ salt) 0.0297µM, $H_3BO_3$ 4.6253µM, $MnCl_2.4H_2O$ 0.9145µM, $ZnSO_4.7H2O$ 0.0765µM, $Na_2MoO_4.2H_2O$ 0.1611µM, $CuSO_4.5H_2O$ 0.0316µM, and $Co(NO_3)_2.6H_2O$ 0.0023µM in 1 L of deionized water, adjusted for N and P concentrations, respectively, at 3000 µg$L^{-1}$ of nitrogen and 0.1, 1.0, 10, 100, and 1000 µg$L^{-1}$ of phosphorus. Six different PAR intensities - namely, 10, 20, 30, 50, 100 and 200 µmol$m^{-2}$$s^{-1}$ by white fluorescent light (Toshiba, Japan) and VBP-L24-C2 PAR source (Valore, Kyoto, Japan) were used with 12 h:12 h PAR and dark cycle. The PAR intensities were measured using a quantum sensor (EKO Instruments Co., Ltd., Japan) and adjusted uniformly in the media. The temperature was kept constant (20 °C) throughout the experiment. At 12:00, after 7 days, samples were taken for the subsequent stress response analysis. Collected samples were subjected to bioassays that are described later.

### 5.3.5 Biochemical parameters used for analysis.

#### 5.3.5.1 Estimation of total soluble protein content

Total soluble protein concentration was measured following [Abeynayaka et al., 2017]'s method with minor modifications. Cyanobacterial cells were unfrozen and 1 mL of 500mM NaOH solution was added for extraction. The supernatant was collected after centrifugation at 4°C for 15 minutes at 10,000 rpm. 51µL supernatant extract was added in 969µL coomassie

Bradford protein assay for each sample. The mixture was kept at room temperature for 10 min, and the absorbance was recorded at 595 nm in UVmini-1240. A series of known albumin concentration standard curves was used to measure the known protein concentration.

**5.3.5.2 Chlorophyll-a concentration measurement**

Chl-a concentrations were quantified using the (ISO 10260:1992; Gregor and Mars$alek, 2004)'s method. 1.5 mL of 90% ethanol was added to the unfrozen cyanobacteria cells. The mixture was homogenized by vortexing and kept at room temperature (25± 2°C) for 24h in darkness by wrapping it with aluminum foil. Each sample was centrifuged at 12,000 rpm, and the supernatant was measured with UVmini-1240 at 665nm and 649nm absorption wavelengths. The formula for calculating Chl-a content is as follows::

Chl-a (μg/mL) = $(13.95*A_{665} - 6.88*A_{649}) *1.5$

**5.3.5.3 Identification of cell growth and calculating growth rate**

$OD_{730}$ measurements were performed using a UV-Vis spectrophotometer (UVmini-1240, Shimadzu, Japan) to determine the cyanobacteria growth rate. $OD_{730}$ was measured using a previously proposed methodology (Axler et al., 1994) with a minor modification. 1mL *P. ambiguum* cells were homogenized with 3mm beads (Bio Medical Science Inc.) and measured at a wavelength of 730 nm. The reading was taken twice., The first sample was collected on the 24$^{th}$ day of acclimatization, known as starting day, while the second sample was collected seven days later.

**5.3.5.4 $H_2O_2$ concentration measurement assay**

A modified version of the xylenol orange (eFOX) method was used to measure the $H_2O_2$ content of *P. ambiguum* following the methods of (Queval et al., 2008; Cheeseman 2006). Unfrozen 1 mL cell pellets were homogenized in 1 mL of 100 mM pH 7.0 phosphate buffer and centrifuged at 12,000 rpm for 10 minutes at 4°C to extract the cellular $H_2O_2$. The supernatant (100 μL) was added to 1 mL of the assay solution containing 0.250 mM ferrous ammonium sulphate, 0.1 mM sorbitol, 0.1 mM xylenol orange, 1% ethanol, and 25 mM $H_2SO_4$, which had been deoxygenated with gaseous nitrogen to prevent artifact production in hydrogen peroxide during the reaction. After 15 minutes of reaction, the absorbance at 560 nm was measured by spectrophotometry. $H_2O_2$ content was calculated by a standard curve using a series of diluted solutions of commercial high-grade 9.8 M $H_2O_2$ (Dautania et al. 2014; Asaeda et al. 2022a).

**5.3.5.4 CAT assay**

CAT activity was measured spectrophotometrically at room temperature by monitoring the decrease in absorbance at 240 nm resulting from the decomposition of $H_2O_2$. The activity of CAT was measured by the method of Aebi (1984). The enzyme was extracted with 1 mL potassium phosphate buffer (0.05 M, pH 7.0) containing 0.1 mM EDTA. The reaction mixture contains 15 μL of 0.75M $H_2O_2$, 920 μL potassium phosphate buffer, and 65 μL of enzyme extract. Measurements were taken every 10 seconds for 3 minutes, and the 39.4 mM/cm extinction coefficient was used to calculate the CAT activity.

**5.3.5.5 Statistical analyses**

The collected data were tested for normality with the Shapiro–Wilk's test before the statistical analyses. All results were presented as the mean ± SD (n = 3). The data were subjected to a two-way analysis of variance (ANOVA) to evaluate the combined effect of temperature and

light intensities on Cyanobacteria in the laboratory experiment, and the bivariate analysis was used and followed by Pearson's correlation method to evaluate the relationship among the parameters. The statistical analyses were performed with IBM SPSS V25.

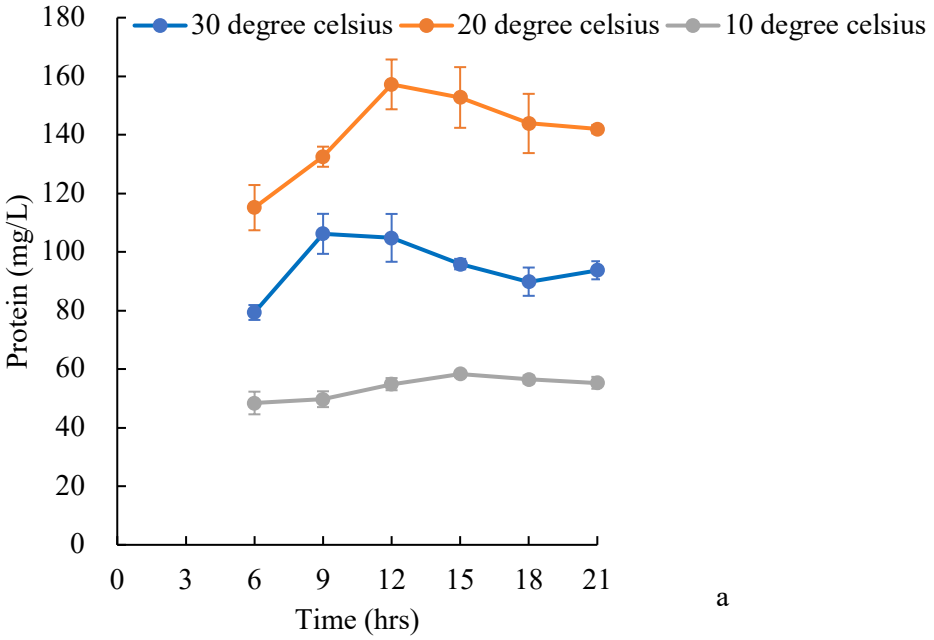
### 5.3.6 Short-term exposure treatment of *M.aeruginosa* and *P. ambiguum*

The protein content of *M.aeruginosa* culture was highest with 20°C, followed by the case of 30°C. After starting light exposure, protein content slightly increased until 12:00 with 20°C, and until 9:00 with 30°C; however, they were maintained with nearly constant values, 140mg/L with 20°C, and 90mg/L with 30°C, despite the slight reduction with time. With a 10°C case, a nearly constant value of about 50mg/L was kept throughout the experiment (Fig. 5.3.6.1 (a)). The protein content of *P. ambiguum* reached its highest, containing almost similar amounts with 30°C and 20°C until 15:00, and maintained the nearly constant value of 145mg/L. At 10°C, protein content was nearly endless at 80mg/L and increased until 15:00, then slightly decreased with time progress (Fig. 5.3.6.1 (b)).

*M. aeruginosa* shows higher $H_2O_2$ concentration at 20°C until 15:00, following 30°C and 10°C, then decreases with decreasing PAR. In the case of *P. ambiguum,* following 20°C and 10°C, the $H_2O_2$ concentration is higher at 30°C at 15:00 and then decreases afterward with decreasing PAR. However, the reduction rate of $H_2O_2$ concentration was less than the increasing rate before and could not reach the initial values of 6:00, even at 21:00, for all temperatures in both species (Fig. 5.3.6.2 (a, b)). The production of $H_2O_2$ of *P. ambiguum* was nearly fourfold higher than *M. aeruginosa* at 30°C.

Chl-a concentration in *M.aeruginosa*, as shown in Fig. 5.3.6.3 (a), is slightly increased between 12:00 and 15:00 at 30°C and 20°C respectively, then was kept nearly the same values afterward with light intensities. . Chl-a concentration was maximum at 20°C approximately 8 mg/L. However, less than 1mg/L, with 10°C. Chl-a concentration of *P. ambiguum* increased until 15:00 in each temperature; after that slightly decreased. Chl-a concentration was maximum at 30°C approximately 17 mg/L. In the meantime, 5mg/L at 10°C (Fig. 5.3.6.3 (b)). At 30°C and 20°C, *P. ambiguum* produces almost twice as much Chl-a as *M. aeruginosa*, while at 10°C, the production increases fivefold.

In the case of *M.aeruginosa* and *P. ambiguum,* CAT activity generally increased until 15:00 for all temperature cases, then decreased with the PAR (Fig. 5.3.6.4(a, b)). *P. ambiguum* showed a higher CAT activity trend than *M.aeruginosa* at 30°C.



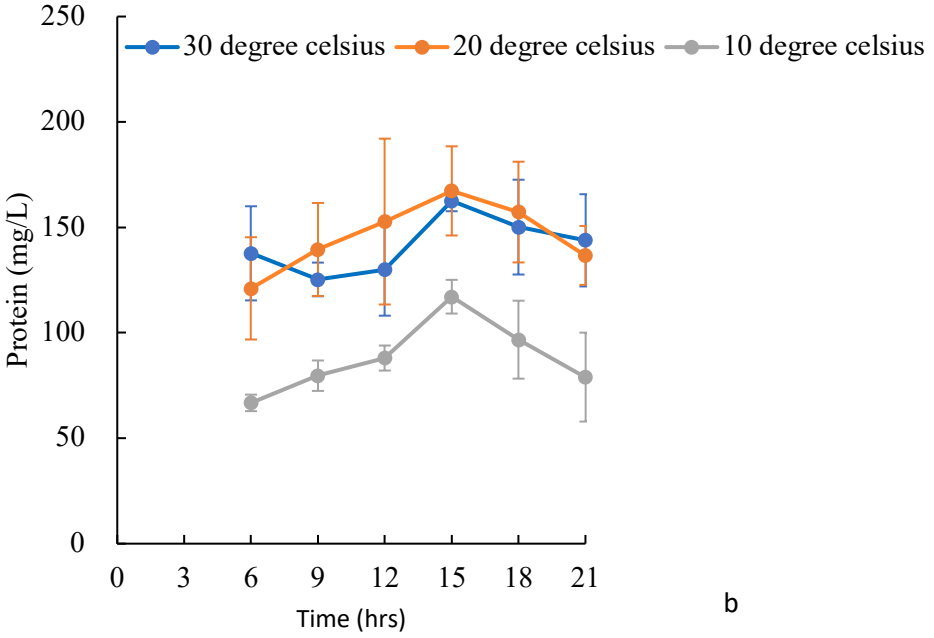
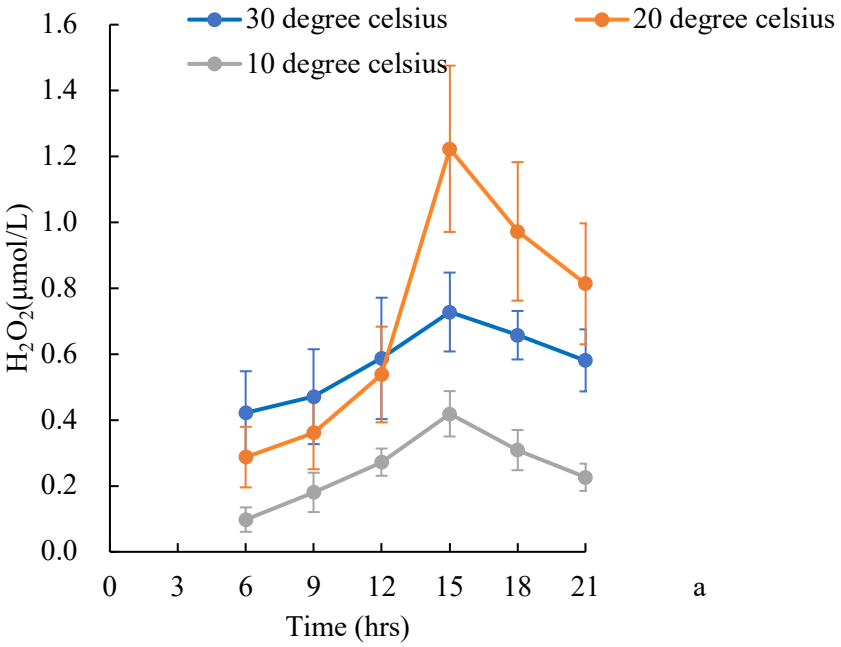




Figure 5.3.6.1 Diurnal variations of light intensities in the protein contents of *M. aeruginosa* (a) and *P. ambiguum* (b) at different temperatures. Three different colours indicate the incubated temperatures. The error bars indicate standard deviations.



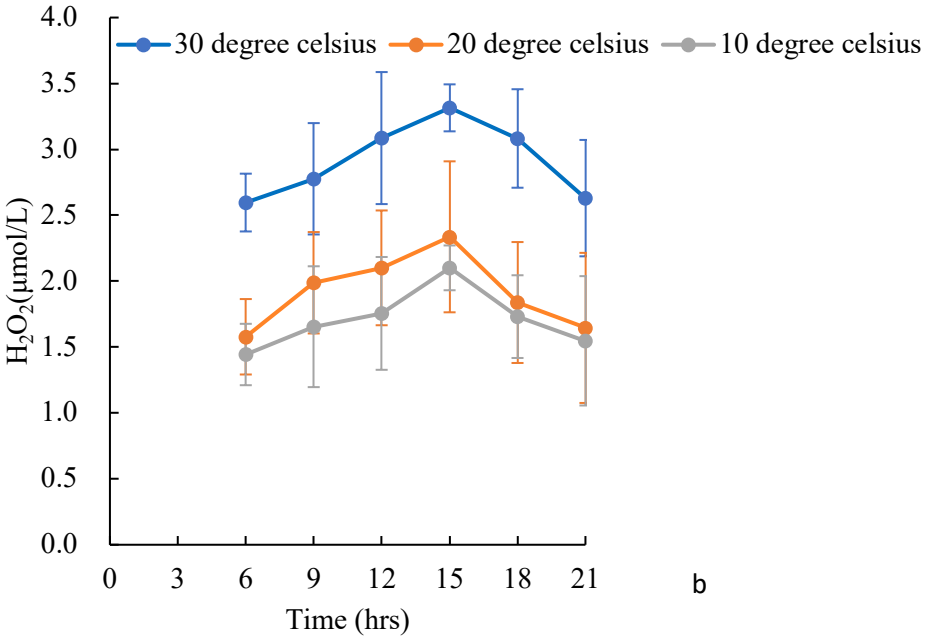
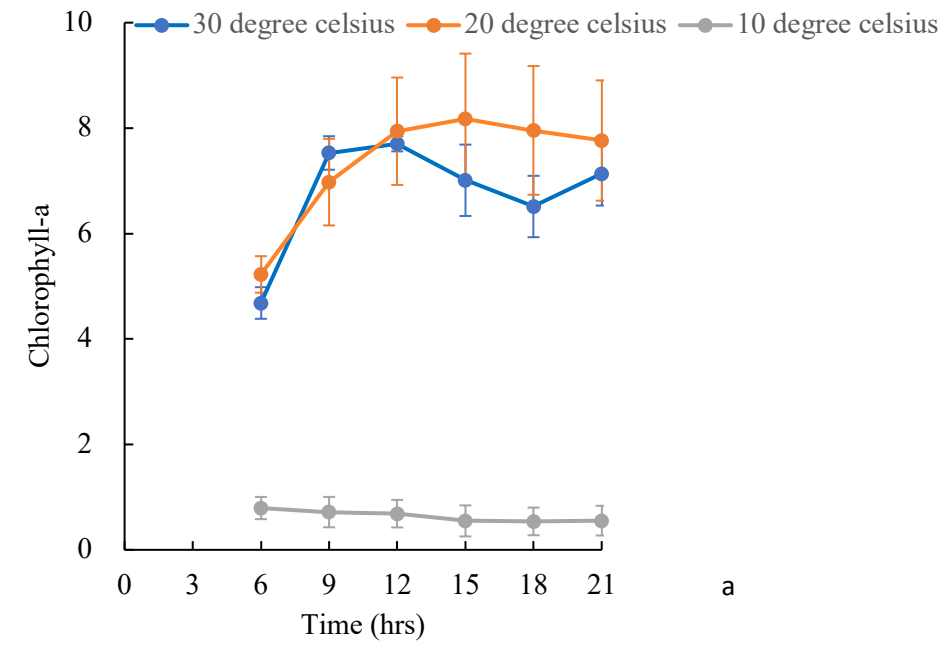




Figure 5.3.6.2 Diurnal variations of light intensities in the $H_2O_2$ contents of *M. aeruginosa* (a) and *P. ambiguum* (b) at different temperatures. Three different colours indicate the incubated temperatures. The error bars indicate standard deviations.



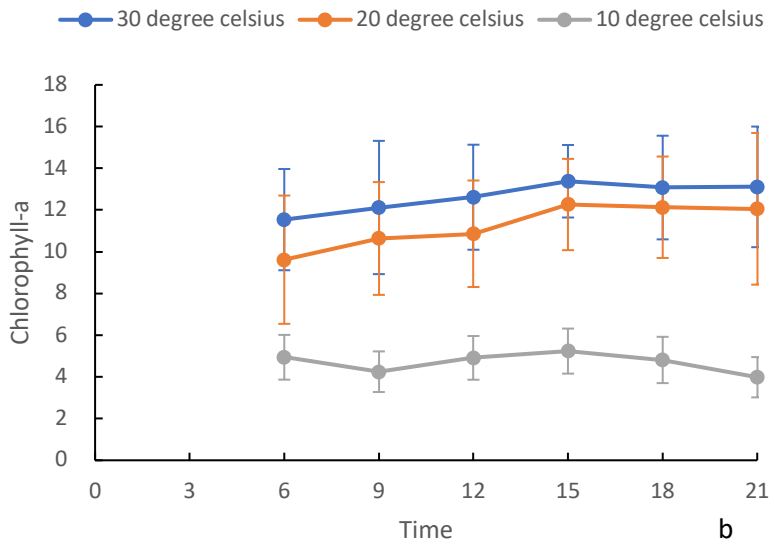
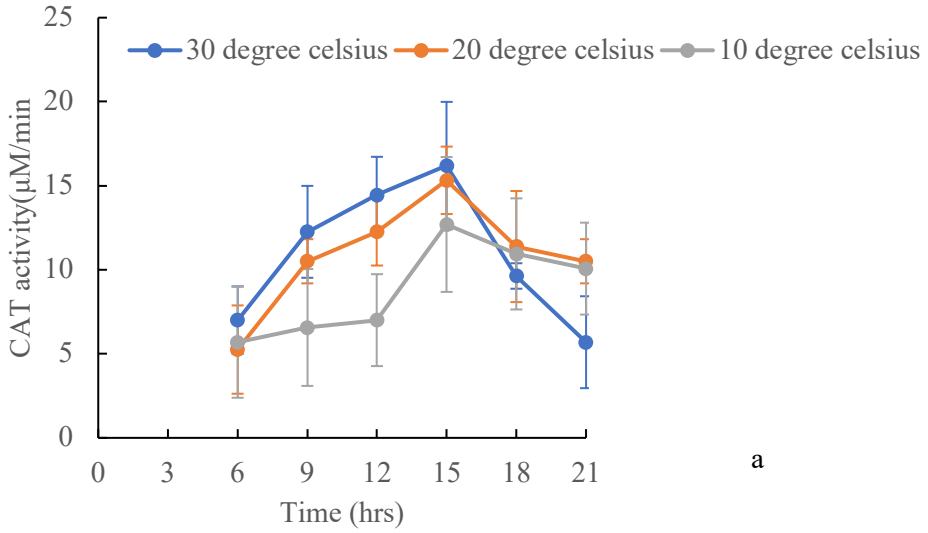
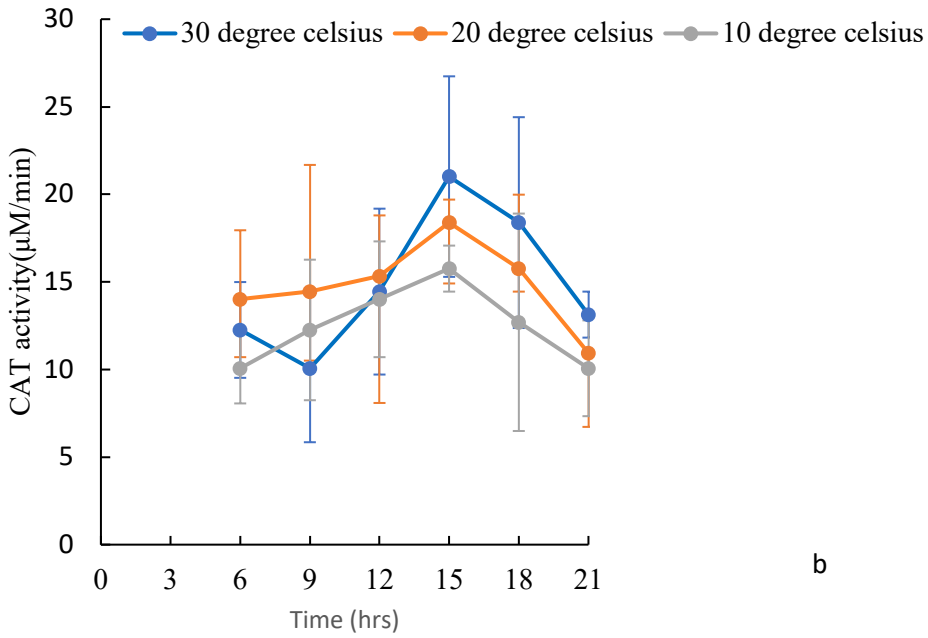




Figure. 5.3.6.3. Diurnal variations in the Chl-a of *M. aeruginosa* (a) and *P. ambiguum* (b) at different temperatures. Three different colours indicate the incubated temperatures. The error bars indicate standard deviations.

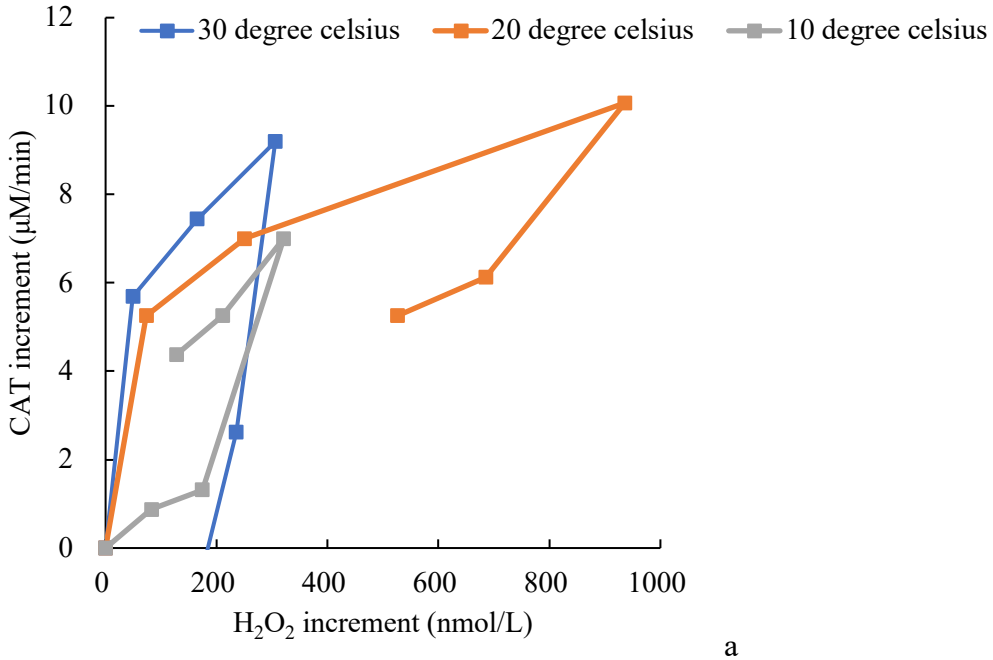



a

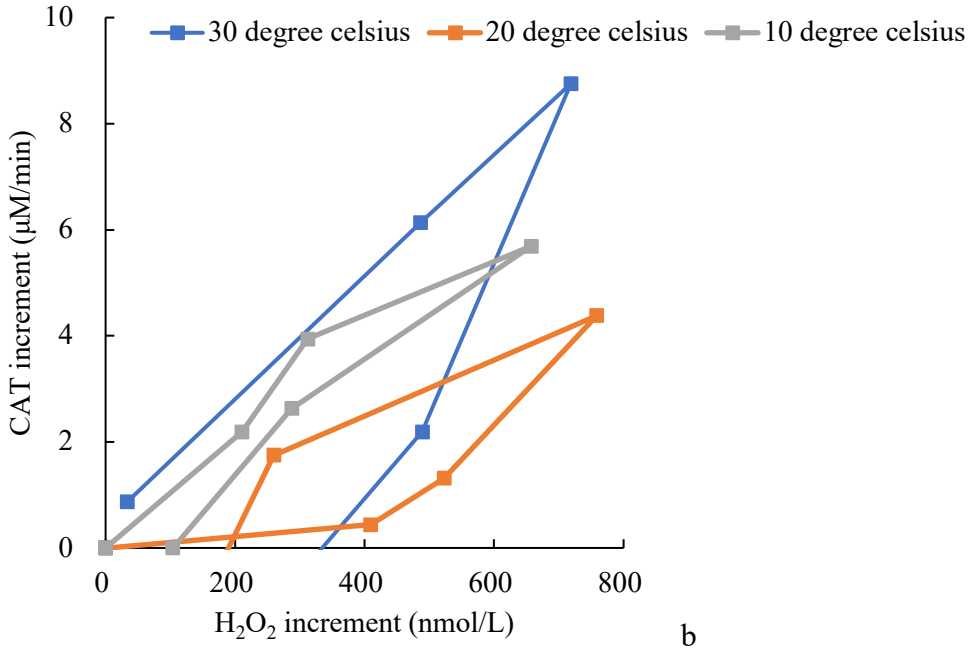



b

Figure. 5.3.6.4. CAT activity of *M. aeruginosa* (a) and *P. ambiguum* (b) in different temperatures in diurnal light intensities. Three different colours indicate the incubated temperatures. The error bars indicate standard deviations.




a




b

Figure. 5.3.6.5. Diurnal variational increment of CAT activity in response to $H_2O_2$ concentration of *M. aeruginosa* (a) and *P. ambiguum* (b) in different temperatures. Three different colours indicate the incubated temperatures. The error bars indicate standard deviations.

### 5.3.7 Discussion

#### 5.3.7.1 The steadiness of cyanobacterial biomass

The protein content was used as a reference for cyanobacteria biomass, corresponding to one-third to one-half of the cell biomass [Lopez et al., 2010). After 23 days of acclimatization at

the same temperature as the experiment, the experiment was started and the protein contents were 80mg/L at 30°C, 115mg/L at 20°C, and 50mg/L at 10°C in *M. aeruginosa*. Although there was a slight increase with 20°C and 30°C cultures after starting the light exposure, nearly constant protein contents were maintained afterward.
The protein content of *P. ambiguum* was 137mg/L at 30°C, 121mg/L at 20°C, and 66mg/L at 10°C in the starting point. Although there was a slightly increased till 15:00, nearly constant protein contents were maintained afterward. Thus, a roughly steady condition was produced in the experiment. The protein content of *P. ambiguum* has higher values than *M. aeruginosa* in each temperature.

### 5.3.7.2 The effect of temperature on diurnal light intensities

The growth of cyanobacteria is reduced in low temperatures, especially at 10°C (You et al. 2018). In the present study, as the temperatures shifted to 10°C or 20°C from 30°C, $H_2O_2$ concentration, protein concentration, and Chl-a concentration became highest at 20°C particularly in the afternoon for *M. aeruginosa*, On the other hand, the highest values are taken at 30°C with *P. ambiguum* when temperatures shifted to 10°C or 20°C from 30°C. A high decrease in $H_2O_2$ concentration, protein concentration, and Chl-a concentration was observed at 10°C in *M.aeruginosa* compared to *P.ambiguum*.

After 23 days of incubation of sufficient *M. aeruginosa* and *P.ambiguum,* $OD_{730}$ at 30°C was (0.995 to 1.122 and 1.753 to 1.891, respectively), 20°C (0.935 to 1.265 and 1.713 to 1.801 respectively), and 10°C (0.522 to 0.591 and 1.239 to 1.387 respectively). *M. aeruginosa* has lower $OD_{730}$ values at every temperature than *P.ambiguum*. Both species showed a significant correlation between protein concentration and $OD_{730}$ with lowering temperatures (30 to 10°C, $p<0.001$ for both species) (data were not shown). A significant negative correlation was observed from 30°C to 10°C ($r= -0.472$, $p<0.05$) in Chl-a/protein with $OD_{730}$ of *M. aeruginosa*, while in 30°C to10°C ($r=-0.333$, $p>0.05$) for *P.ambiguum* indicating that lower temperatures are unsuitable for cyanobacterial growth.

### 5.3.7.3 The trend of antioxidant activity

The $H_2O_2$ contents and the antioxidant activity, such as CAT of *M. aeruginosa* and *P. ambiguum,* were highly responsive to the diurnal variations in light intensity. This study used diurnal light intensities in 30°C, 20°C, and 10°C temperatures, where $H_2O_2$ levels increased during high PAR and decreased at lower PAR. If cellular $H_2O_2$ level increases, the antioxidant activities will also increase parallelly to prevent damage caused by oxidative stress [Pruchniak et a. 2015; Lin et al., 2012). As observed with the $H_2O_2$ levels, CAT also varied during the same time frame and followed the $H_2O_2$ levels, which increased at higher light intensities and vice-versa. CAT reaches a maximum at 15:00, which is 3h after the maximum light intensity (Fig. 5.3.6.4 (a, b)).

As a nearly steady condition was maintained before starting the light exposure; thus, the CAT activity before the light exposure was balanced with $H_2O_2$ to keep the homeostasis. The increments of $H_2O_2$ and CAT after starting the light exposure are shown in Fig. 5.1.5 (a, b). Similar trends were taken by these three temperature cultures, such as CAT activity is proportional to $H_2O_2$ concentration until 200 nmol/L of $H_2O_2$, which corresponds to the peak of PAR. The increment of CAT was substantially suppressed with higher $H_2O_2$ concentration, although the light intensity was already decreasing. The increment of CAT activity with $H_2O_2$ increment was highest at 20° for *M. aeruginosa* meanwhile 30°C for *P. ambiguum*. This shows the preference for the summer season in the case of *M. aeruginosa.* It can be predicted that the

experiment groups, which receive higher PAR, go through an enhanced rate of photosynthesis. This is evidenced by the increased $H_2O_2$ formed after exposure to higher light intensity (Exposito-Rodriguez et al., 2017; Pagey et al. 2011). As a result, at higher PAR, where the photon energy exceeds endurable levels for the photosystem, photoinhibition occurs to prevent photodamage (Virtanen et al., 2019; Nishiyama et al. 2006), during which $H_2O_2$ production, as well as CAT activity, is reduced with higher light exposure (Vanderauwera et al. 2005; Asaeda et al. 2022b), The $H_2O_2$ contents is directly correlated with light intensity. CAT almost decreased to the initial conditions by 21:00 in different temperatures. Therefore, cells may undergo oxidative stress balanced with antioxidant activities during dark conditions despite the low-level antioxidant activities. Protein synthesis of photosystems will be suppressed as long as $H_2O_2$ remains in cells (Latifi et al., 2009). If $H_2O_2$ continues for a long duration, the cell cannot function properly, which can eventually lead to cell mortality (Brutemark et al., 2015].

### 5.3.7.4 The mechanism of the preference of *M.aeruginosa* in summer time

Surface water temperature in the summer season is ~ 30°C. Compared with the highest values of *P. ambguum* at 30°C, *M. aeruginosa* has a lower $H_2O_2$ concentration at 30°C than at 20°C, indicating a lower stress level at high temperatures. This indicates that *M. aeruginosa* can efficiently proliferate at high water temperatures, especially 30°C. The present study results correspond to those (Imai et al., 2009). The natural ponds generally experienced increased temperatures from spring to summer but decreased temperatures from summer to autumn. Thus, *M. aeruginosa* may dominate early in the year because it responds to a seasonal rise in water temperature.
On the contrary, *P. ambguum* cells become bleached at 30°C when incubated for around a month (Stam et al., 1979). It has been found by other researchers that *P. ambguum* is abundant during autumn (Borah et al., 2022). Our present study reveals similar findings for *P. ambguum* in accordance with the temperatures.

However, this study only involved a single-day diurnal variation which shows the effect of different temperatures and light intensities on *M. aeruginosa* and *P.ambiguum*. Therefore, this PAR variation requires an extended exposure period to understand better the ultimate distribution of the remaining $H_2O_2$ and adaptation responses.

### 5.3.7.5 Conclusion

*M. aeruginosa* and *P. ambiguum* showed an increasing trend of $H_2O$, Chl-a, and CAT activity in different temperatures at 15:00. After that, it decreases parallel with decreasing light intensity in diurnal PAR variations. $H_2O_2$ concentration and protein content was highest at 20 °C with *M.aeruginosa*, although values were similar between 20°C and 30°C with P. *ambiguum*. The temperature effect was more significant in *M. aeruginosa* than *P. ambiguum* on $H_2O_2$ concentration, protein concentration, and Chl-a content. The Chl-a/ protein concentration substantially correlates with $OD_{730}$ in decreasing temperatures for both species. CAT activity was proportionate to the $H_2O_2$ increment, which implies the hysteresis effect of daytime PAR and antioxidant activity in the different temperatures. It appears that *M. aeruginosa* has a higher competitive advantage in summer than *P. ambiguum*. These results indicate that lowering the temperatures can be an effective tool to suppress cyanobacteria.

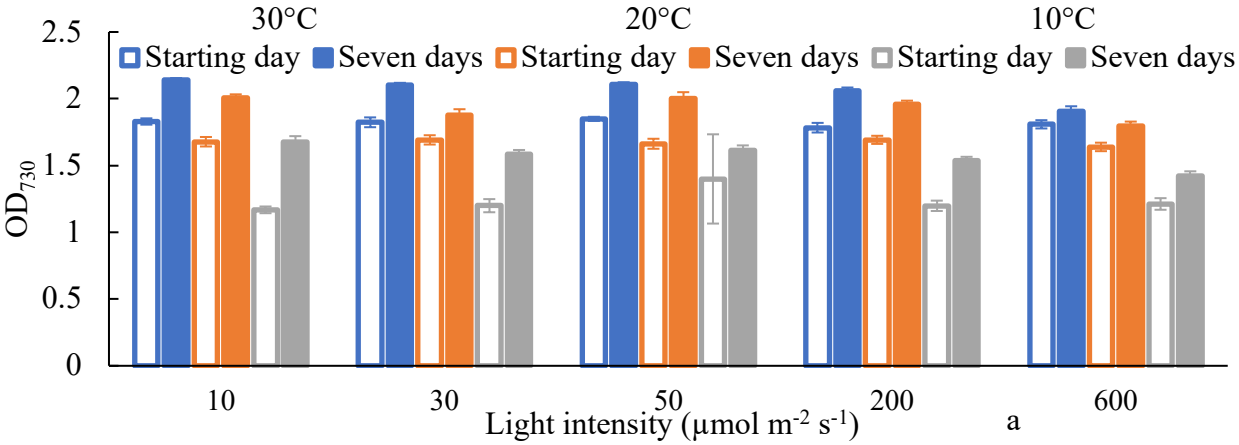
## 5.4 Long term exposure treatment of *P. ambiguum* and *P. foetida*

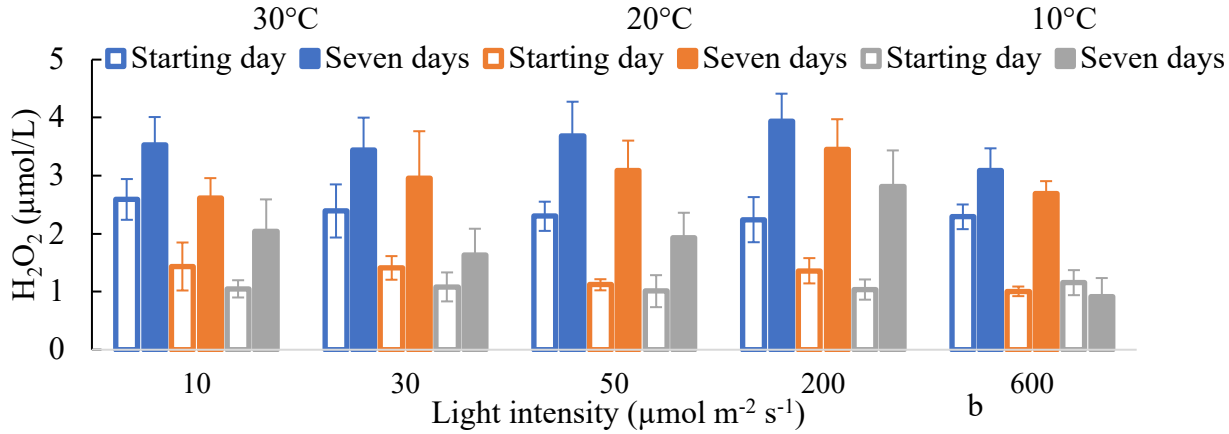
### 5.4.1 Results

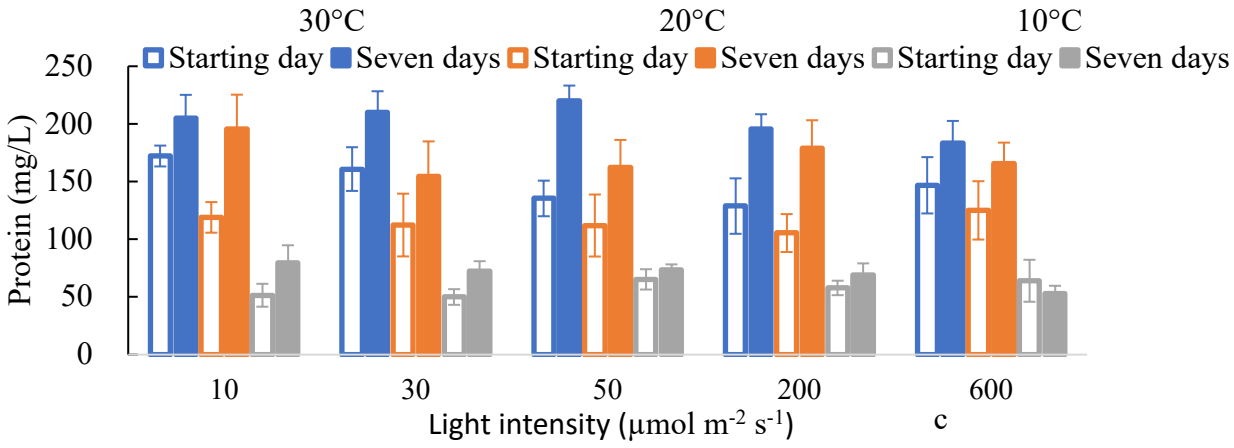
$OD_{730}$, $H_2O_2$ concentration, protein concentration, Chl-a content, and CAT activity were monitored in *P. ambiguum* and *P. foetida* at 30°C, 20°C, and 10°C based on each light intensity for the starting day and the seven days period. Due to preliminary light intensities changing during collection, the starting day collection in each temperature did not show too much fluctuation. In the seven days of exposure, $H_2O_2$ steadily increased with increasing light intensity until 200 μmol $m^{-2}$ $s^{-1}$, while declined at 600 μmol $m^{-2}$ $s^{-1}$ in each temperature (Figure 5.4.1.1b, 2b). In both species, $OD_{730}$ and protein concentration, which had been lower at the starting day with 20°C, increased up to the 30°C level in seven days (Figure 1a, 1c, 2a, 2c). Chl-a substantially decreased from 50 μmol $m^{-2}$ $s^{-1}$ to 200 μmol $m^{-2}$ $s^{-1}$ ($p$<0.001 for both species) and thereafter at 30°C, 20°C, and 10°C in both species in seven days of light exposure (Figure 1d, Figure 2d). The antioxidant activity of CAT was increased until 200 μmol $m^{-2}$ $s^{-1}$ in response to oxidative damage of $H_2O_2$ concentration and decreased with higher light intensity at 30°C, 20°C, and 10°C in both species (Figure 5.4.1.1e, 2e). In Figure 5.4.1.3a and Figure 5.4.1.3b, $OD_{730}$ is presented with respect to protein and Chl-a content, respectively. There is a significantly high proportionate correlation observed between $OD_{730}$ and protein for all temperatures (at 30°C for *P. ambiguum* r=0.991, $p$<0.001; for *P. foetida* r=0.952, $p$<0.001), (at 20°C for *P. ambiguum* r=0.978, $p$<0.001; for *P. foetida* r=0.990, $p$<0.001), (at 10°C for *P. ambiguum* r=0.829, $p$<0.001; for *P. foetida* r=0.715, $p$<0.001) in the seven days of exposure. On the starting day, both Chl-a and $OD_{730}$ were significantly proportionate with protein concentration, whereas, after seven days, Chl-a was significantly small, compared with the starting level ($p$<0.001 for both species). For both species, the $H_2O_2$ per biomass was expressed by the change of $H_2O_2/OD_{730}$ in seven days, which is presented in Figure 5.4.1.4. In every case, $H_2O_2/OD_{730}$ increased following the light intensity until 200 μmol $m^{-2}$ $s^{-1}$, while declined at 600 μmol $m^{-2}$ $s^{-1}$. There was significant negative correlation was found from 30°C to 10°C between $H_2O_2/OD_{730}$ and light intensities (for *P. ambiguum* r=-0.579, $p$<0.01; for *P. foetida* r=-0.853, $p$<0.001).

In the case of *M.aeruginosa*, Due to preliminary light intensities changing during collection, the zero days collection in each temperature did not show too much fluctuation. Figure 5.4.1.5 shows the differences in $OD_{730}$ between 30°C and 20°C regarding different light intensities. $OD_{730}$ values significantly increased from 0 to 7 days at 20°C (p<0.001). In the 7 days at 30°C, $OD_{730}$ decreased with increasing PAR. In the meantime, $OD_{730}$ increased till 30 PAR at 20°C and took nearly the same value thereafter. A positive correlation was found between $OD_{730}$ and different light intensities in 7 days between 30°C and 20° (r=0.417). Protein concentration substantially increased from 0 days to 7 days at 20 °C (p<0.001), whereas at 30°C, it slightly increased until 200 μmol $m^{-2}$ $s^{-1}$, declined with 600 μmol $m^{-2}$ $s^{-1}$ (Figure 5.4.1.6). With respect to light intensity, after 7 days of light exposure, the protein concentration increased until 30 μmol $m^{-2}$ $s^{-1}$, then constantly decreased with increasing light intensities, similarly in 30 °C and 20°C experiments. After 7 days of treatment, $H_2O_2$ concentration and $H_2O_2$/protein or algal biomass significantly increased in both temperatures (p<0.001 for each temperature) (Figure 5.4.1.7 and Figure 5.4.1.8). At 7 days, $H_2O_2$ and $H_2O_2$/protein concentration increased to 200 PAR at each temperature, and then it decreased with higher PAR. A significant positive correlation was observed from 30°C to 20°C between PAR with $H_2O_2$ (r=0.859 p<0.001)and PAR with $H_2O_2$/protein (r=0.914 p<0.001). In both temperatures, Chl-a increased until 50 PAR and continued to decrease with higher PAR (Figure 5.4.1.9). The Chl-a concentration is shown as a function of $H_2O_2$ of *M. aeruginosa* under different light intensities in Figure 5.4.1.10 in

both temperatures. With 20°C, Chl-a concentration increased with less than 0.5 mmol/L of $H_2O_2$. However, with a higher concentration of $H_2O_2$, it decreased. With 30°C, Chl-a concentration slightly increased until 1 mmol/l of $H_2O_2$, then decreased with a higher concentration of $H_2O_2$. CAT activity significantly increased from 0 days to 7 days at 30°C ($p<0.01$) and 20ºC ($p<0.01$) to decrease oxidative stress. CAT activity increased to 200 µmol $m^{-2}$ $s^{-1}$ at each temperature. CAT activity and temperatures showed a positive correlation ( 30°C and 20°C, r=0.415).



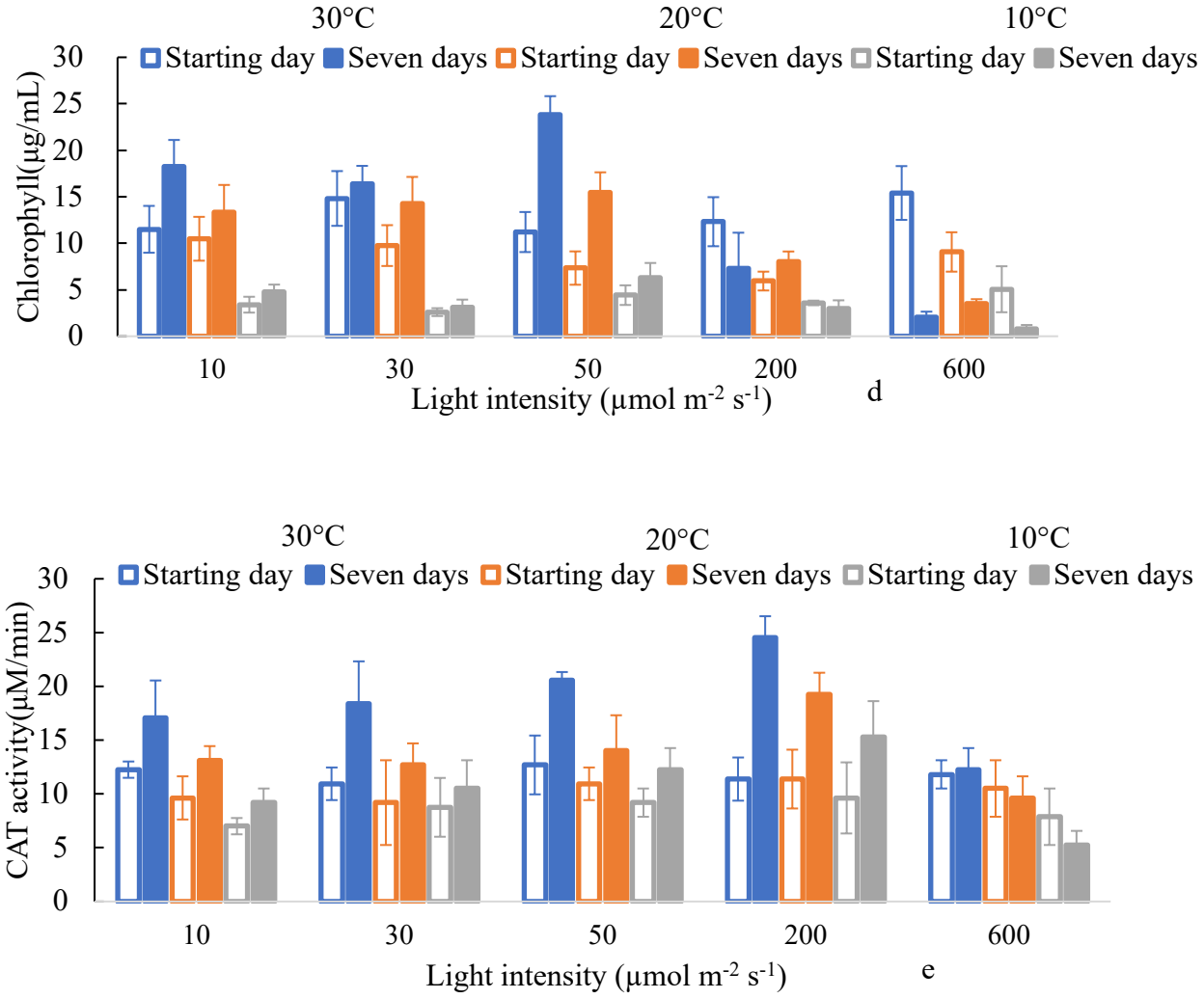
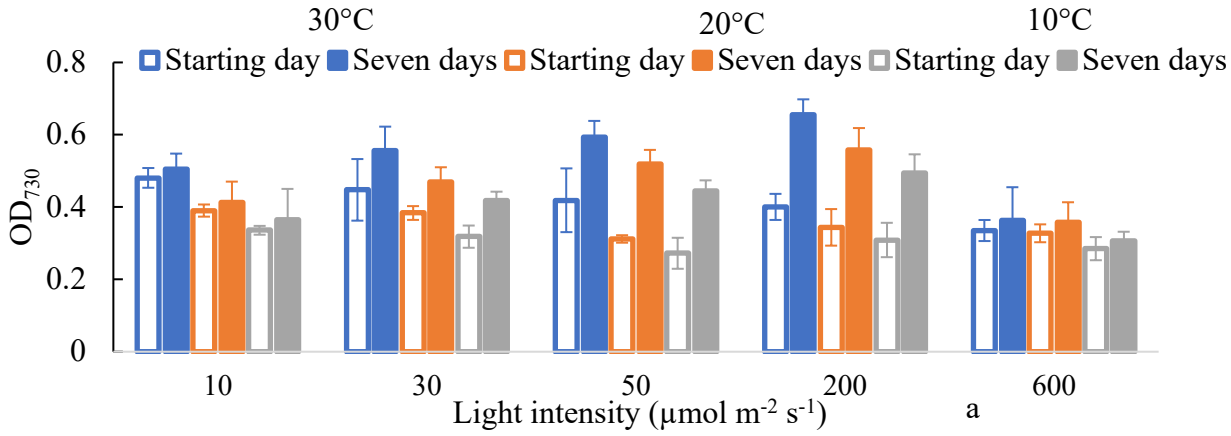
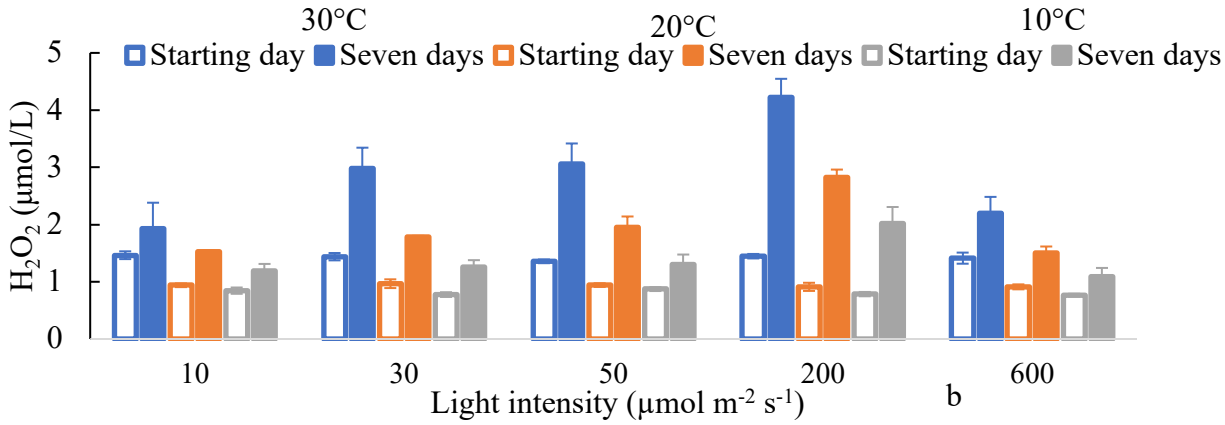
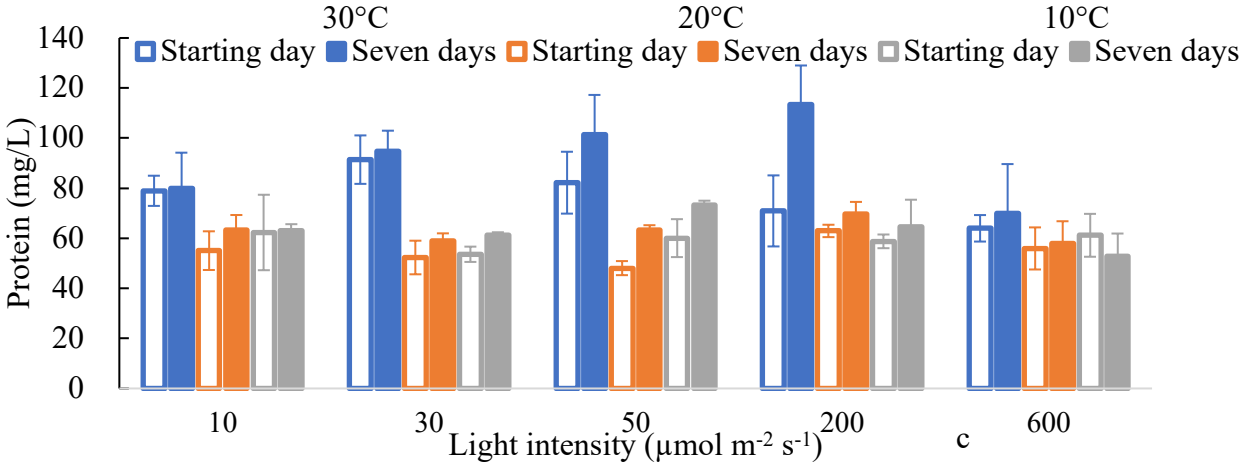
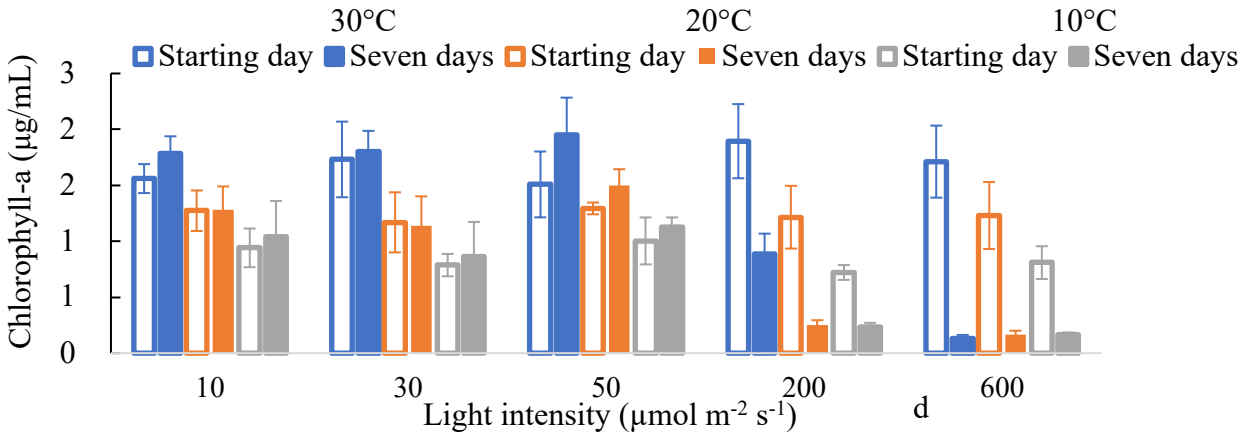



Figure 5.4.1.1: Light intensities at 30°C, 20°C, and 10°C affected $OD_{730}$, $H_2O_2$ concentration, protein concentration, Chl-a content, and CAT activity. Solid quadrate indicates seven-day treatment, whereas blank quadrate indicates starting day. The error bars indicate standard deviations.


30°C
20°C
10°C
Starting day
Seven days
Starting day
Seven days
Starting day
Seven days
0.8
0.6
0.4
0.2
0
OD730
10
30
50
200
600
Light intensity (µmol m-2 s-1)
a


30°C
20°C
10°C
Starting day
Seven days
Starting day
Seven days
Starting day
Seven days
5
4
3
2
1
0
H2O2 (µmol/L)
10
30
50
200
600
Light intensity (µmol m-2 s-1)
b


30°C
20°C
10°C
Starting day
Seven days
Starting day
Seven days
Starting day
Seven days
140
120
100
80
60
40
20
0
Protein (mg/L)
10
30
50
200
600
Light intensity (µmol m-2 s-1)
c



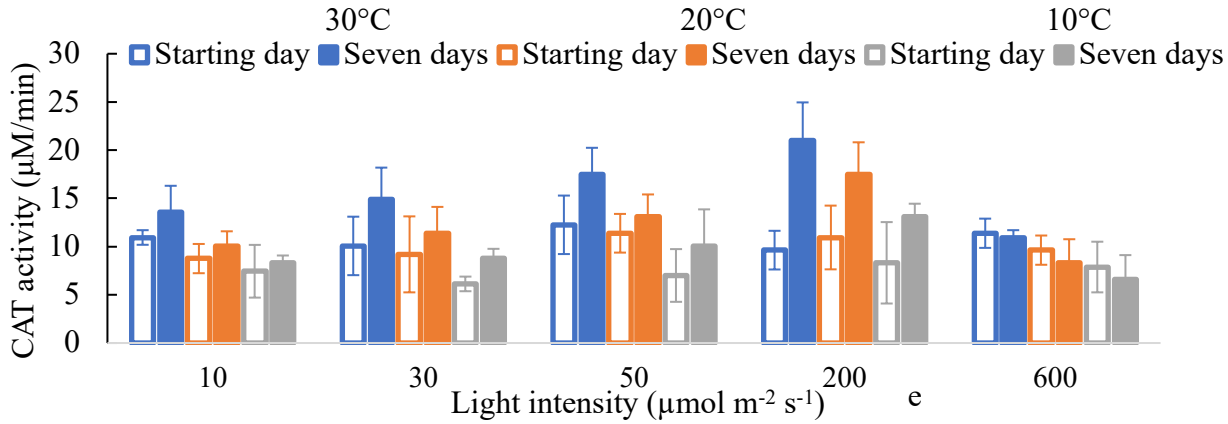
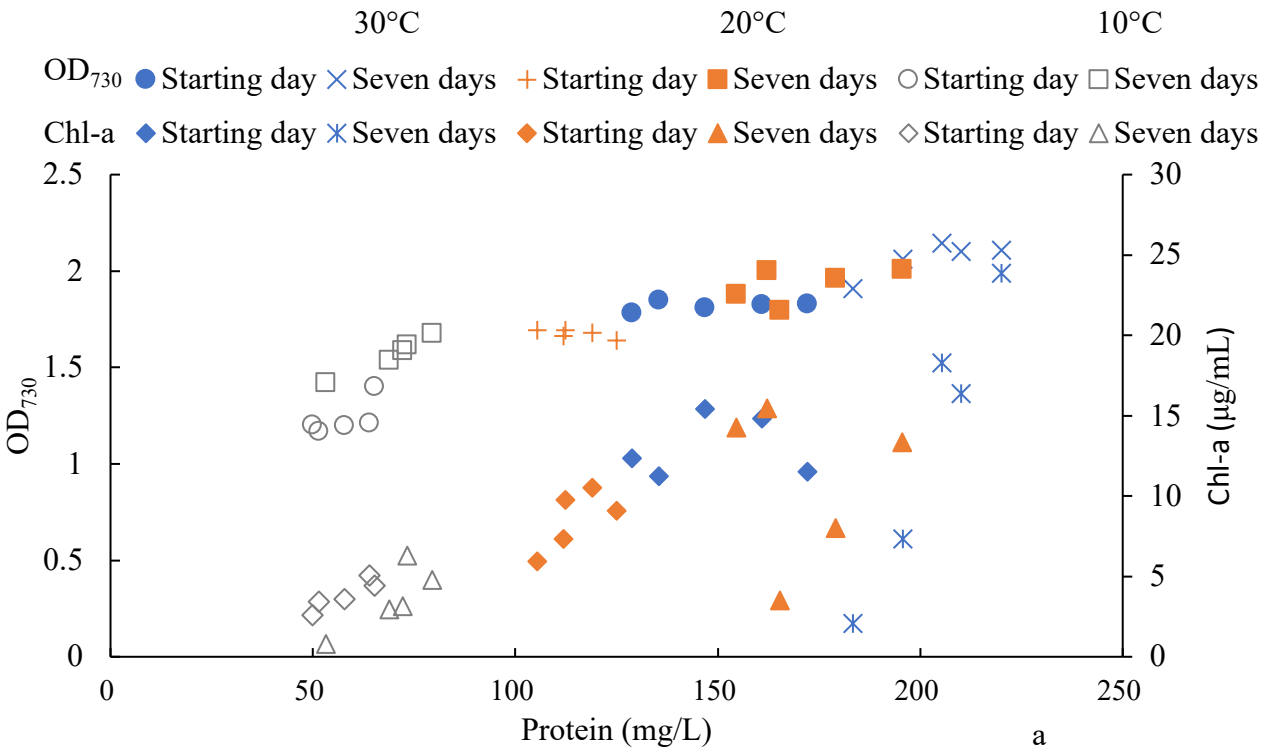




Figure 5.4.1.2: Changes in in $OD_{730}$, $H_2O_2$ concentration, protein concentration, Chl-a content, and CAT activity concerning light intensities at starting and seven days of *P. foetida* at 30°C, 20°C, and 10°C. Solid quadrate indicates seven-day treatment, whereas blank quadrate indicates starting day. The error bars indicate standard deviation.



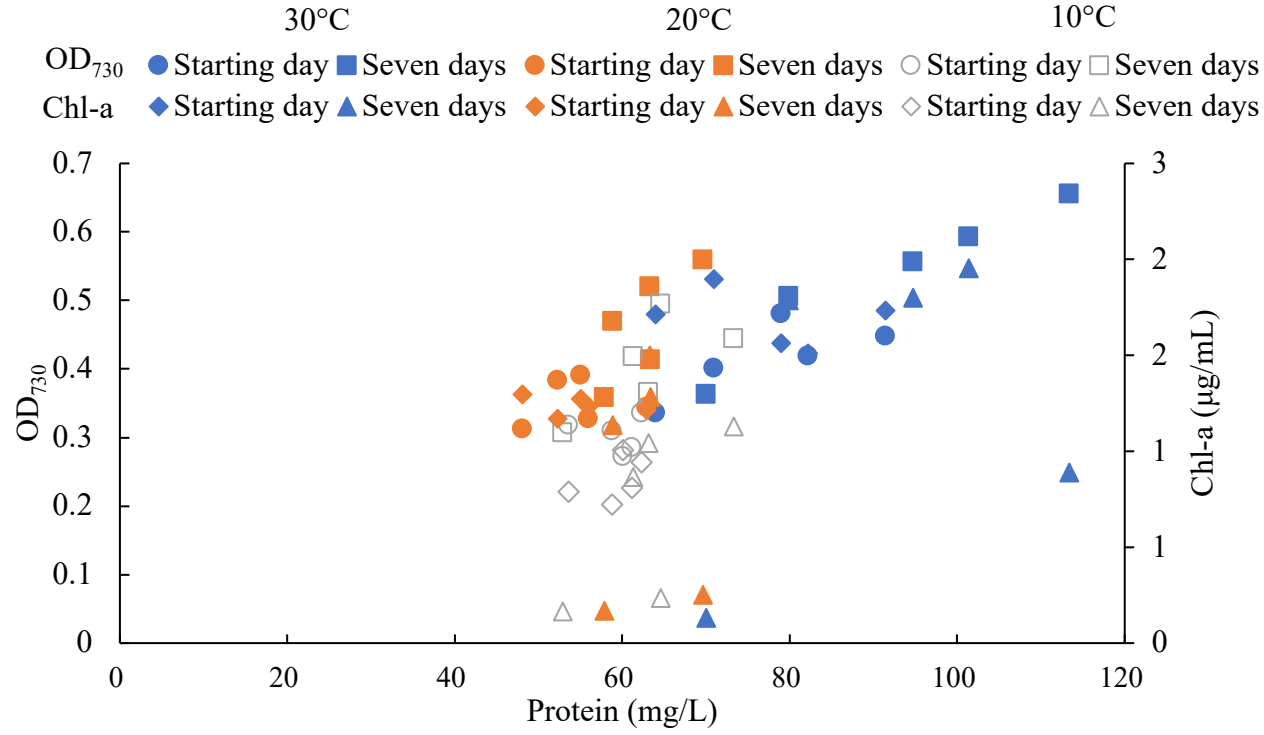




Figure 5.4.1.3: The relationship between protein and $OD_{730}$ and protein as well as protein and Chl-a in *P. ambiguum* (a) and *P. foetida* (b) at 30°C, 20°C, and 10°C.

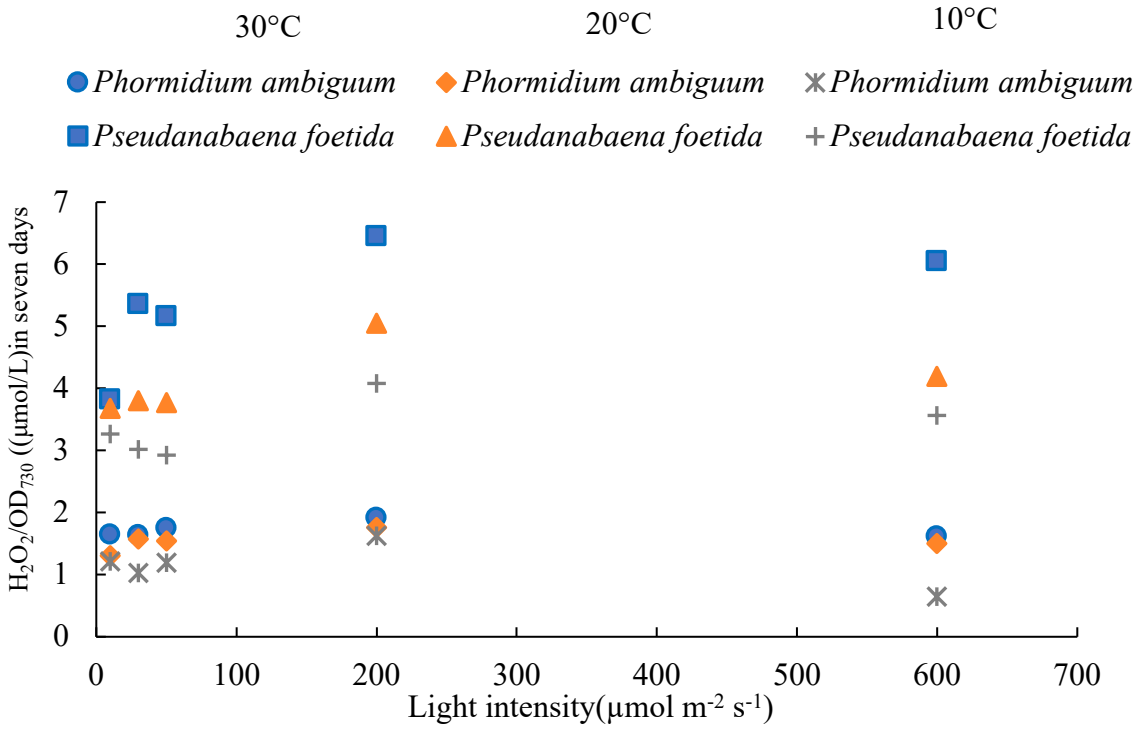



Figure 5.4.1.4. Changes in $H_2O_2/OD_{730}$ in seven days of exposure to different light intensities in *P. ambiguum* and *P. foetida* at 30°C, 20°C, and 10°C.

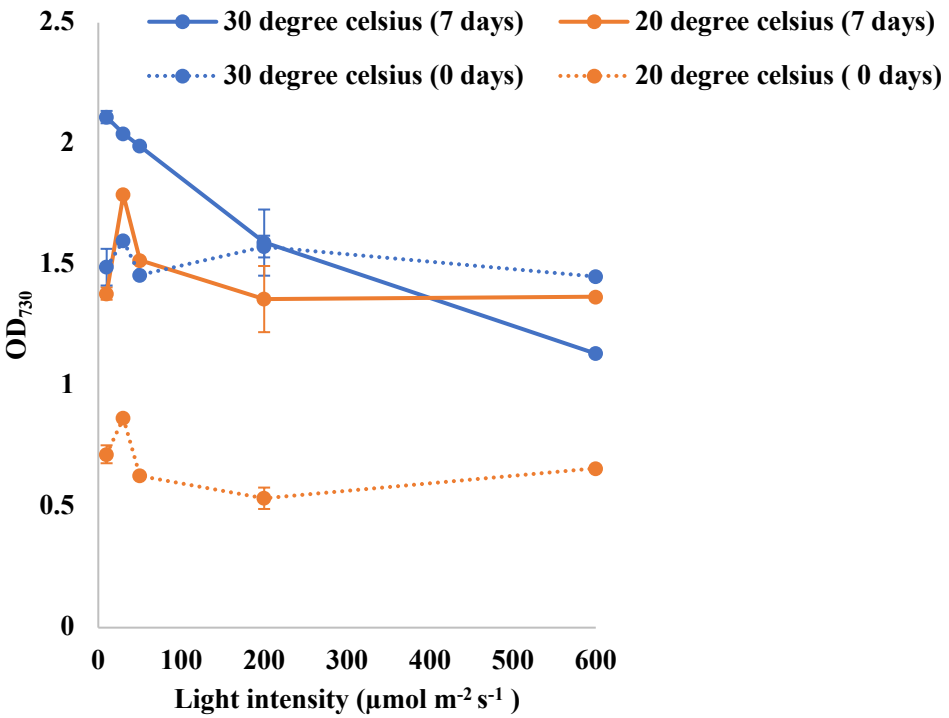



Figure 5.4.1.5 *M. aeruginosa* $OD_{730}$ at 0 and 7 days at 30°C and 20°C. Solid lines indicate seven-day treatment, whereas dashed lines indicate 0 days. The error bars indicate standard deviations.

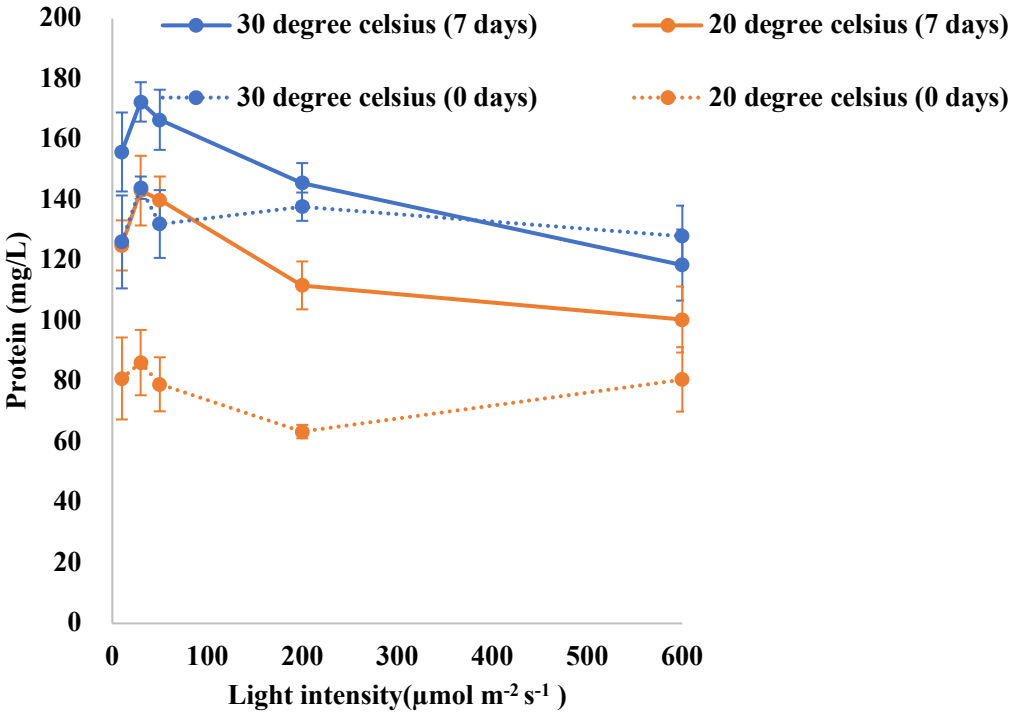



Figure 5.4.1.6 Changes in protein concentration concerning light intensity at 0 and 7 days of observing *M. aeruginosa*. Solid lines indicate seven-day treatment, whereas dashed lines indicate 0 days. The error bars indicate standard deviations.

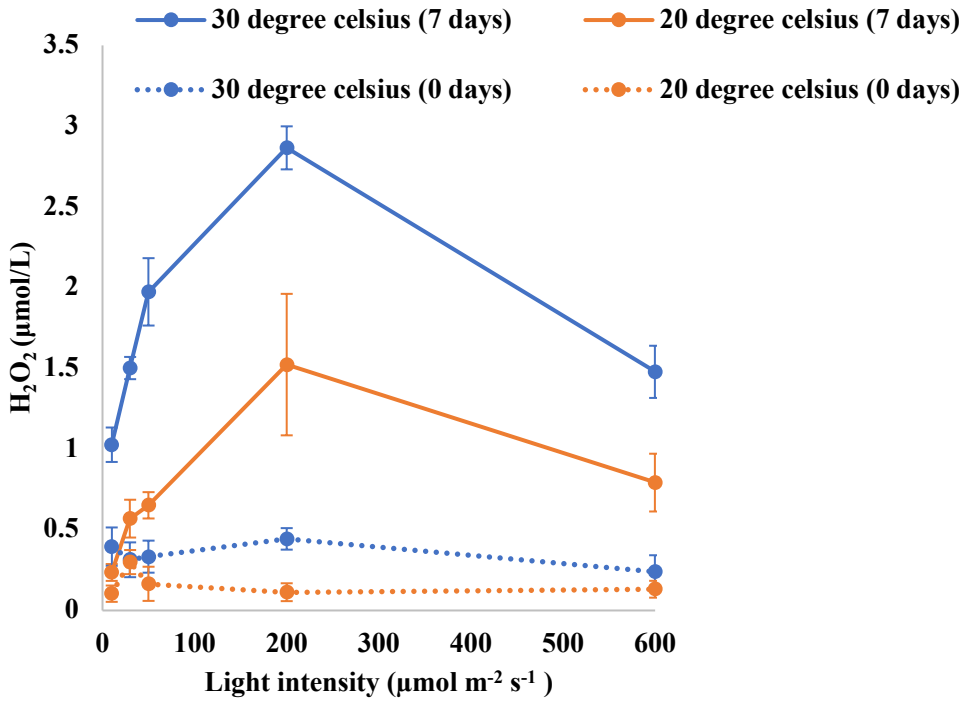



Figure 5.4.1.7. Changes in $H_2O_2$ concentration concerning light intensity in zero and seven days of observing *M. aeruginosa*. Solid lines indicate seven-day treatment, whereas dashed lines indicate 0 days. The error bars indicate standard deviations.

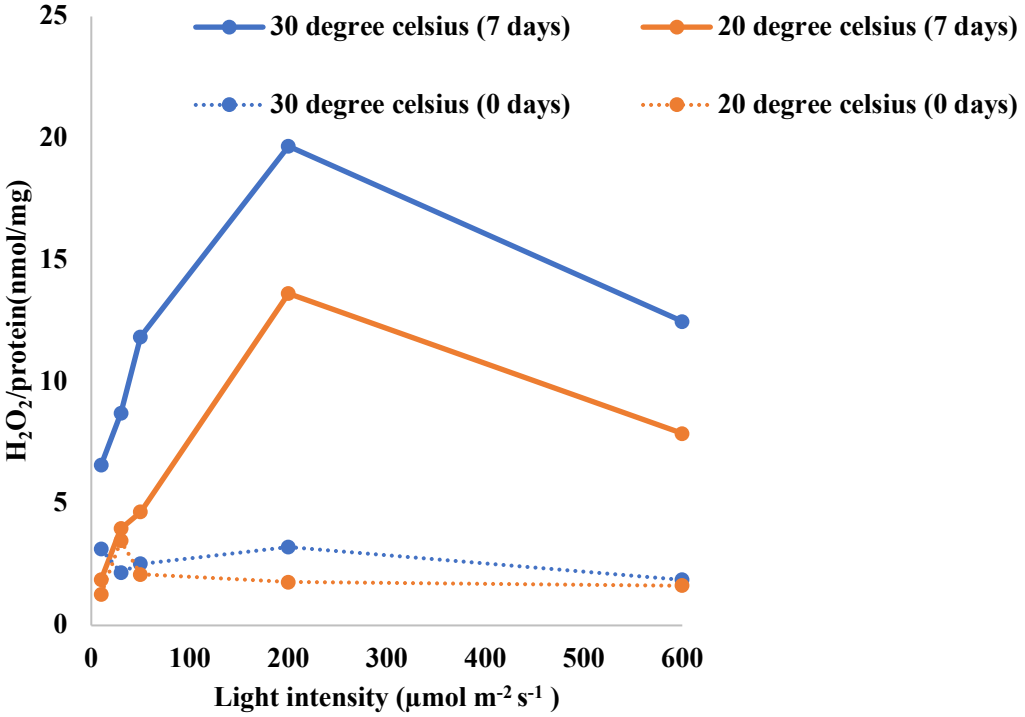



Figure 5.4.1.8. Changes in $H_2O_2$/protein concentration concerning light intensity at zero and seven days of observing *M. aeruginosa*. Solid lines indicate seven-day treatment, whereas dashed lines indicate 0 days. The error bars indicate standard deviations.

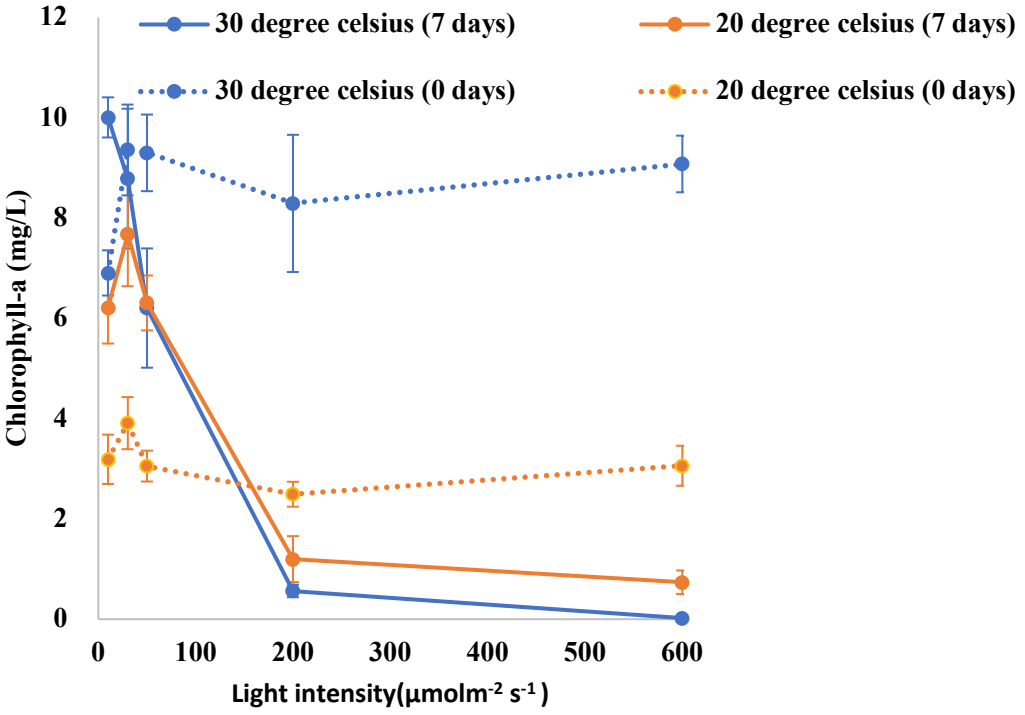



Figure 5.4.1.9 Changes in chlorophyll-a content concerning light intensity in zero and seven days of *M. aeruginosa* observation. Solid lines indicate seven-day treatment, whereas dashed lines indicate 0 days. The error bars indicate standard deviations.

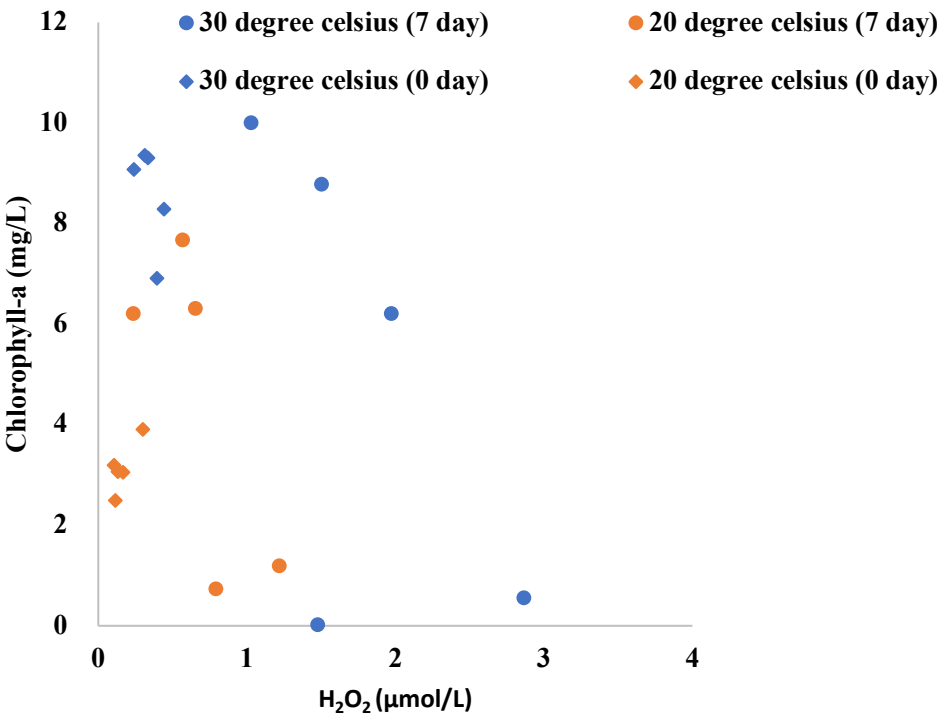



Figure 5.4.1.10 Changes in chlorophyll-a content concerning light intensity at zero and seven days of *M. aeruginosa* observation. Different symbols are used to separate 0 and 7 days of exposure. The error bars indicate standard deviations.

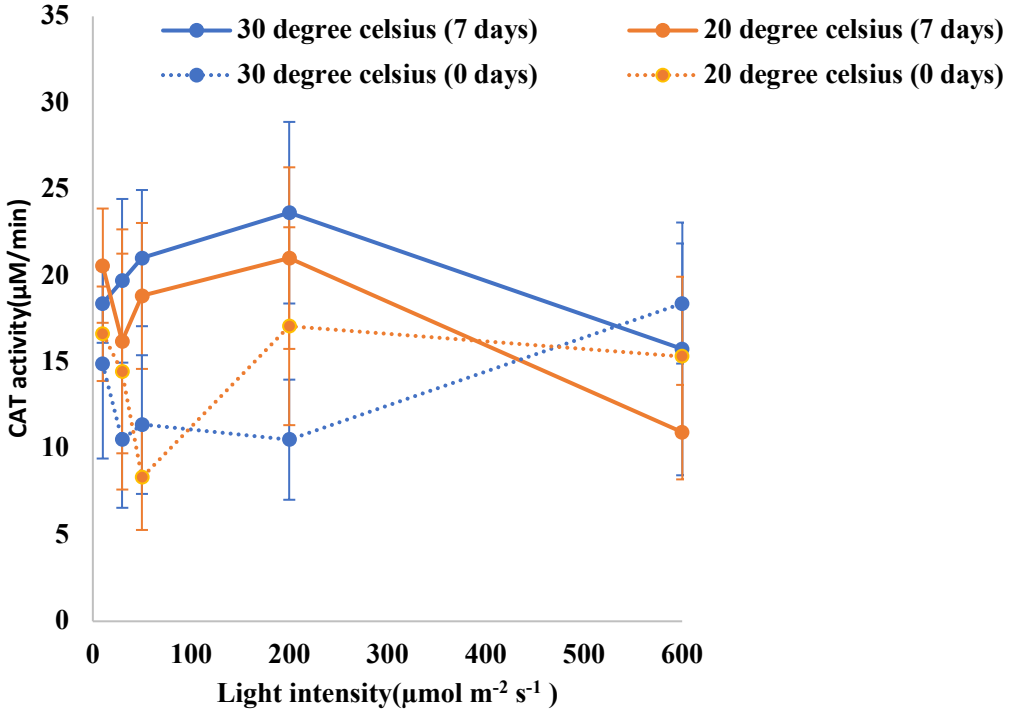



Figure 5.4.1.11 Changes in CAT activity concerning light intensity at zero and seven days of *M. aeruginosa* observation. Solid lines indicate seven-day treatment, whereas dashed lines indicate zero days. The error bars indicate standard deviations.

### 5.4.2 Discussion

#### 5.4.2.1 Effect of light intensities on the response of $H_2O_2$

The excess level of ROS can be generated through abiotic stress (Vesterkvist et al., 2012; Preece et al., 2017; Pham et al., 2018). ROS accumulation in each cyanobacteria species under high light (~ 200 μmol $m^{-2}$ $s^{-1}$) and alterations of temperatures may result in oxidative damage, which may be one reason for cell growth inhibition (Figure 1a and Figure 2a). Cells exposed to high levels of environmental stress produce and accumulate $H_2O_2$. In natural water, cyanobacteria are exposed to various abiotic stresses that enhance oxidative stress, producing $H_2O_2$, which may deteriorate cyanobacterial biomass by producing hydroxyl radicals. The production of $H_2O_2$ may not necessarily be cumulative in abiotic stresses (Mittler et al., 2006; Saints et al., 2010]. The $H_2O_2$ concentration was enhanced until 200 μmol $m^{-2}$ $s^{-1}$ and decreased afterward at each temperature (Figure 1b and Figure 2b). The trend was more prominent at 30°C in the seven days of exposure. In the present study, photoinhibition is seen even with 200 μmol $m^{-2}$ $s^{-1}$ of light intensity, whereas 1000 μmol $m^{-2}$ $s^{-1}$ of light intensity is reported in field observations (Machová et al., 2008; Harel et al., 2004]. In field monitoring, light intensity in water decreases relatively quickly, despite surface colonies receiving a high intensity of solar radiation. As a result, the cyanobacteria colony does not receive strong solar radiation directly, especially *M. aeruginosa*, *P. ambiguum*, and *P. foetida* since it stays in relatively deep water (~ 2m) [Visser et al., 2016]. In addition, they avoid the highest solar radiation in a day, by migrating to deeper zones (Athukorala et al., 2010; Cañedo-Argüelles et al., 2013; McLellan et al., 2017), likely to avoid the high solar radiation and oxidative stress before the recovery of homeostasis via the increasing antioxidant activities (Athukorala et al., 2010).

Laboratory incubations under different $H_2O_2$ concentrations provided the lethal $H_2O_2$ dosage for cyanobacteria in the past (Leunert et al., 2014; Derakhshan et al., 2018; Foo et al., 2020), which implies that cyanobacterial biomass is degraded with higher $H_2O_2$ concentrations. The lethal $H_2O_2$ dosage for cyanobacteria suppression by invitro under different $H_2O_2$ concentrations ranges from 1 to 1000 micromol $L^{-1}$ (Leunert et al., 2014; Bouchard and Purdie 2011; Drábková et al., 2007). This study suggests that if the $H_2O_2$ concentration exceeded ~ 2 to 6 micromol/L (Figure 4) after seven days of treatment, it significantly declined afterward, even against the higher temperature or light intensities, then affects the algal biomass. The high $H_2O_2$ concentration deteriorates the physiological condition without generating further $H_2O_2$ (de Souza et al., 2013; Asaeda et al., 2022). In contrast, Chl-a concentration is affected by even lower levels of light intensities ~50 μmol $m^{-2}$ $s^{-1}$. Low $H_2O_2$ concentration is generated with sufficiently low PAR (~50 μmol $m^{-2}$ $s^{-1}$), and the Chl-a concentration fluctuates positively with the PAR (data are not shown). It may be due to an adaptation to increase productivity. Chl- a concentration increases due to the breakdown of photosynthesis components, such as photosystems I and II (Kura-Hotta et al., 1987) and phycobilisomes (Walsh et al., 1997). In contrast, high Chl-a concentration increases the photosynthesis rate, generating more $H_2O_2$ and deteriorating the photosynthesis apparatus. Thus, increased $H_2O_2$ concentration negatively alters the Chl-a concentration in highly stressed conditions (Asaeda et al., 2022). The $H_2O_2$ concentration is also indicate the environmental stress intensity on plants and productivity (Asaeda et al., 2021).

In the present study, we used protein content as an indicator of biomass rather than the Chl-a content of both cyanobacterial species (Marsac et al. 1977). One-third to one-half of the cyanobacteria cells biomass is protein [López et al., 2010]. There was a high correlation between $OD_{730}$ and protein content (Figure 3a, 3b), indicating that the protein content is a proper biomass reference. Compared with $OD_{730}$, and protein contents, Chl-a is affected by

lower light intensities. The light intensity is the major component to increase the $H_2O_2$ concentration, which may increase the endorsement of $H_2O_2$ in the field treatment. The identification of the required endorsement amount of $H_2O_2$ should be considered, following the suitable factor such as $H_2O_2/OD_{730}$, for the algal blooming suppression.

The growth of *P. ambiguum* is significantly decreased by decreasing temperature from 30°C to 10°C ($p<0.001$ for both species), similar to other studies (Chu et al. 2007; Imai et al. 2009; You et al. 2018] (Fig. 1). Decreasing temperatures (30°C to 10°C) also significantly affect $H_2O_2$, Chl-a, and protein concentration. Algal biomass ($H_2O_2/OD_{730}$) is also significantly lower at 10°C compared to 30°C (Figure 4). These results indicate that *P. ambiguum* and *P. foetida* maintain higher metabolism with higher temperatures, bringing about a high growth rate with high temperatures.

#### 5.4.2.2 Antioxidant activity in response to oxidative stress

The tissue $H_2O_2$ concentration at a particular time is determined as the balance between the generation rate and the scavenging by antioxidant activities. This concentration works as a signal to activate antioxidant behavior. Antioxidant activity increases in response to oxidative stress to prevent cell damage (Liu et al., 2017). Oxidative stress and antioxidative enzymes are triggered by abiotic stress (De Silva and Asaeda, 2017; Rastogi et al., 2010). In the present study, high light intensities (200 to 600 µmol $m^{-2}$ $s^{-1}$) decrease $H_2O_2$ concentration, and low light intensities (10 to 200 µmol $m^{-2}$ $s^{-1}$) increase it. CAT activity proportionally increased with $H_2O_2$ concentration until 200 µmol $m^{-2}$ $s^{-1}$ in each temperature and decreased afterward following the reduction of $H_2O_2$ (Figure 1e and Figure 2e).

#### 5.4.2.3 $H_2O_2$ can be considered a biomarker

$H_2O_2$ is a well-known compound with strong oxidizing capability and is suggested as a promising selective agent against the growth of toxic cyanobacterial species (Drábková et al., 2007). We observed that over 200 µmol $m^{-2}$ $s^{-1}$ light intensity $H_2O_2$ concentration and $H_2O_2/OD_{730}$ (Figure 4) decreased at each temperature. Excessive photosynthetically active radiation (PAR) energy harvesting increases $H_2O_2/OD_{730}$ over 200 µmol $m^{-2}s^{-1}$ light intensity (Welkie et al., 2019; Latifi et al., 2009). Photosystem II (PSII) in the thylakoid membrane produces electrons to fix with solar energy. Higher levels of solar radiation generate more electrons. Therefore, PS-II photoinhibition occurs, resulting in oxidative damage via the production of ROS, mostly $H_2O_2$. It damages cellular components leading to cell death (Latifi et al., 2009). $H_2O_2$ and $H_2O_2/OD_{730}$ negatively affect the Chl-a content (data were not shown). The antioxidant activity of CAT showed a parallelly increasing rate with $H_2O_2$ concentration and algal biomass to prevent cell damage. Therefore, higher $H_2O_2$ levels suppress cyanobacteria growth.

#### 5.4.2.4 Management of cyanobacterial blooms without chemicals

Long-term exposure to light intensities in different temperatures is highly appreciated as non-chemical approach to controlling *P. ambiguum* and *P. foetida*. This result opposes the hypothesis that low-light exposure suppresses cyanobacteria growth. According to Visser et al. (Visser et al., 2016), artificial water mixing in lakes and reservoirs is only sometimes efficient. During artificial mixing, oxygen levels increase in the water, the temperature of the deep layers is raised, and the temperature on the surfaces is lowered. For instance, Destratification (the development of vertical mixing) decreases surface temperature from 28.9°C to 26.4°C, whereas Deep-water aeration increases temperature from 8°C to 23.7°C (Heo and Kim 2004). The

mixing is deep enough to limit light availability and the mixing devices are well distributed horizontally over the lake. Reduced light exposure will lead to decreased growth of cyanobacteria and algae. Among the drawbacks of artificial mixing is the cost of installation, operation, and energy required during the entire growing season. Using intermittent mixing might be beneficial in reducing energy costs and reducing the flotation velocity of cyanobacteria. Light availability under water can influence cyanobacterial growth composition (Havens et al., 1998). When the wind is mixed along with unsustainable vertical heterogeneity of algal biomass, light cannot enter the deeper zone of water. On the other hand, in the calm conditions (stable water column, vertical migration, sustained surface biomass maximum) of a Lake, light may enter the deeper zone (Havens et al., 1998). For example, in comparison with the euphotic depth, Secchi depth is roughly half. Water transparency is measured by Secchi depth, where Secchi depth increases with increasing transparency. A lake's bottom is illuminated when the Secchi depth is roughly half its euphotic depth (Havens et al., 1998). Phytoplankton may experience light limitation when the Secchi depth/total euphotic depth ratio is below 0.5. It is important to consider both species' low-light and high-light vulnerabilities when introducing the system. A destratification process must be designed beforehand based on the intensity and distribution of light and temperature (Asaeda and Imberger 1993; Imteaz and Asaeda 2000). Other types of practical methods can be introduced to control cyanobacterial species, such as exposing deep water to high lights without raising the temperature. There should be a variety of methods developed to illuminate the water column with more light intensity than is tolerated by these species.

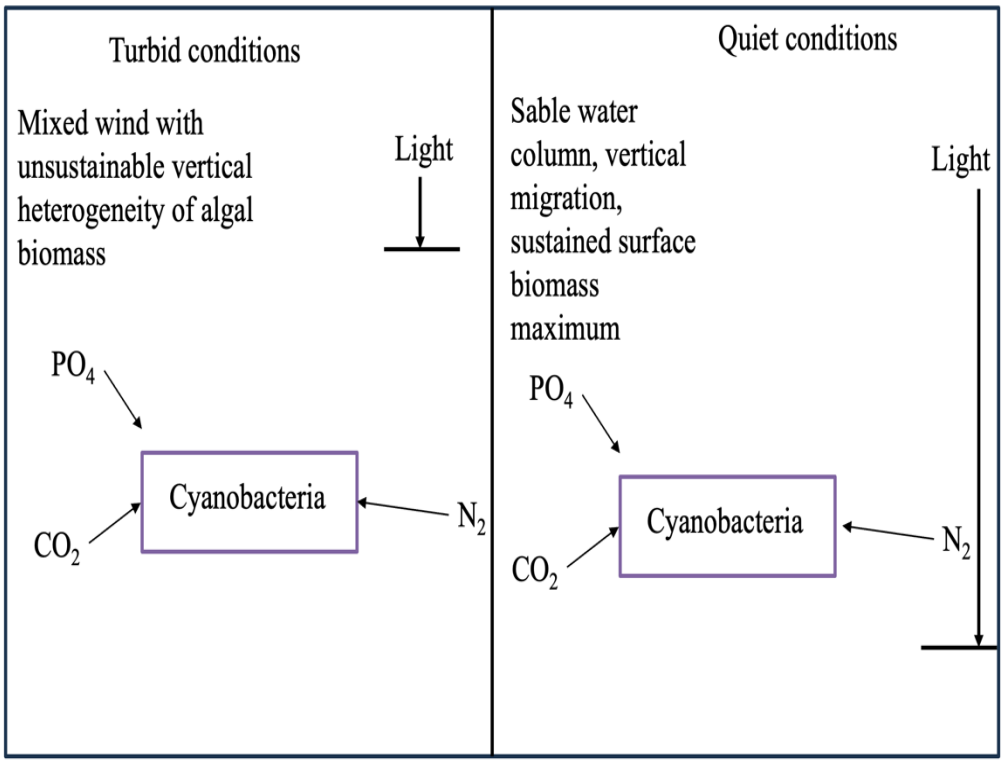



**Figure 5.4.2.4: Management of cyanobacterial blooms with high light intensities.**

### 6.4.2.5 Conclusion

Cyanobacterial species' optimum growth light intensity was 50 μmol$m^{-2}s^{-1}$and showed photoinhibition even after 200 μmol$m^{-2}s^{-1}$ in seven days of the exposure period. Light intensities and temperatures significantly affect $H_2O_2$ concentration, protein concentration, and Chl-a content. Cyanobacterial biomass significantly decreased from 30°C to 10°C in seven days of light exposure time. Lowering the temperature (20°C and 10°C) and high light intensities (~200 μmol $m^{-2}$ $s^{-1}$) can be introduced as a non-chemical approach for long-term treatment. $H_2O_2$ is the most common ROS and can easily be quantified. It could be possible to develop control mechanisms based on high-light intensities, which might lead to lower growth rates, or to improve the existing methods that rely on low-light intensities. Water bodies could be effectively controlled by this method.

## 5.5 Hydrogen peroxide can be a plausible biomarker in cyanobacterial bloom treatment

### 5.5.1 The effect of PAR intensity and phosphorous concentration on $H_2O_2$ concentration

Variations in protein contents of cyanobacterial cultures, grown under different PAR intensity levels with different P concentrations ($mgL^{-1}$), are shown in Fig. 5.5.1.1. Fig. 5.5.1.2 indicates the $H_2O_2$ concentration variations for different PAR intensity levels and each P concentration level ($mgL^{-1}$). Vertical bars indicate standard deviation. Higher protein content was obtained when PAR exposure was lower than 50 $\mu molm^{-2}s^{-1}$. In a PAR intensity range between 0 and 30 $\mu molm^{-2}s^{-1}$, the $H_2O_2$ concentration declined from 50-150 nmol $L^{-1}$ at dark conditions, with increasing PAR. With a PAR intensity between a 30-200 $\mu molm^{-2}s^{-1}$ range, the protein content was slightly reduced ($R^2$=-0.06, $p>0.1$), while the $H_2O_2$ concentration significantly increased ($R^2$ = 0.73, $p<0.001$, and 0.910, 0.720, 0.92, 0.92 and 0.16 for 1000, 100, 10, 1.0, $0.1mgL^{-1}$ of P concentration, respectively), regardless of the P concentration. The variational trend of $H_2O_2$ concentration per protein is shown in Fig. 5.5.1.3 with respect to the PAR intensity. Regardless of the P concentration, it declined with low PAR intensities to 30 $\mu molm^{-2}s^{-1}$ of PAR intensity and then increased with a decreasing enhancement rate. At values higher than 50 $\mu molm^{-2}s^{-1}$ PAR, no significant difference was obtained in the variational trend of the $H_2O_2$ per protein with respect to PAR. The increasing trend of $H_2O_2$ per protein with PAR intensity is mainly attributed to the increasing trend of $H_2O_2$ concentration rather than the reduction of protein content. $H_2O_2$ per protein was generally lower with a higher P concentration ($p<0.03$). $H_2O_2$ per protein, measured as high as 0.5 up to 2.0 $nmolmg^{-1}$ with 100 to 200 $\mu molm^{-2}s^{-1}$of PAR intensities, declined with a higher P concentration (Fig. 5.5.1.4).

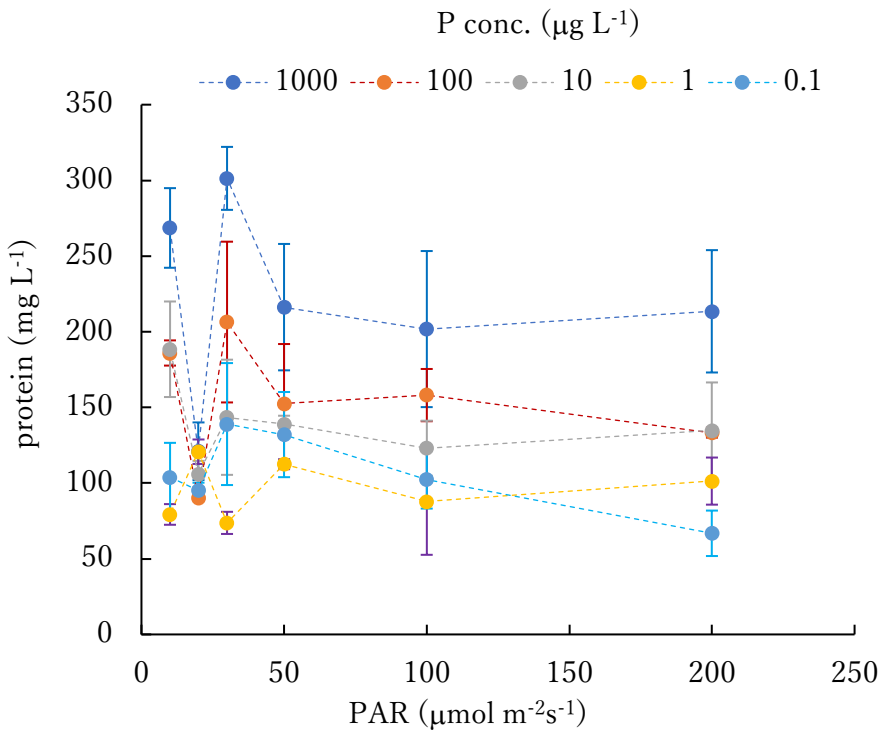



Figure 5.5.1.1. Protein content for different PAR intensity levels and for each phosphorus concentration level ($\mu gL^{-1}$). Vertical bars indicate standard deviation.

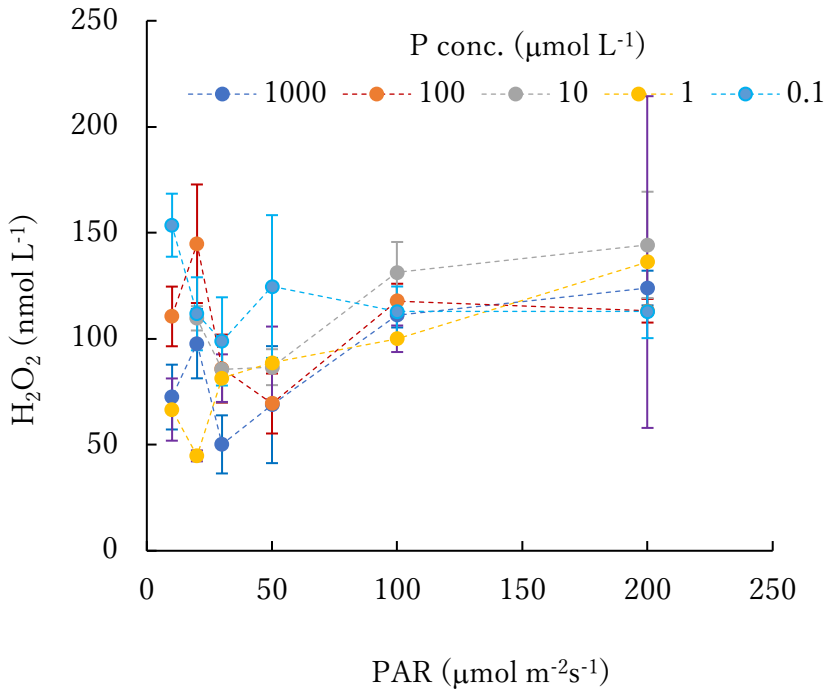



Figure 5.5.1.2. $H_2O_2$ concentration for different PAR intensity levels and for each phosphorus concentration level ($\mu gL^{-1}$). Vertical bars indicate standard deviation.

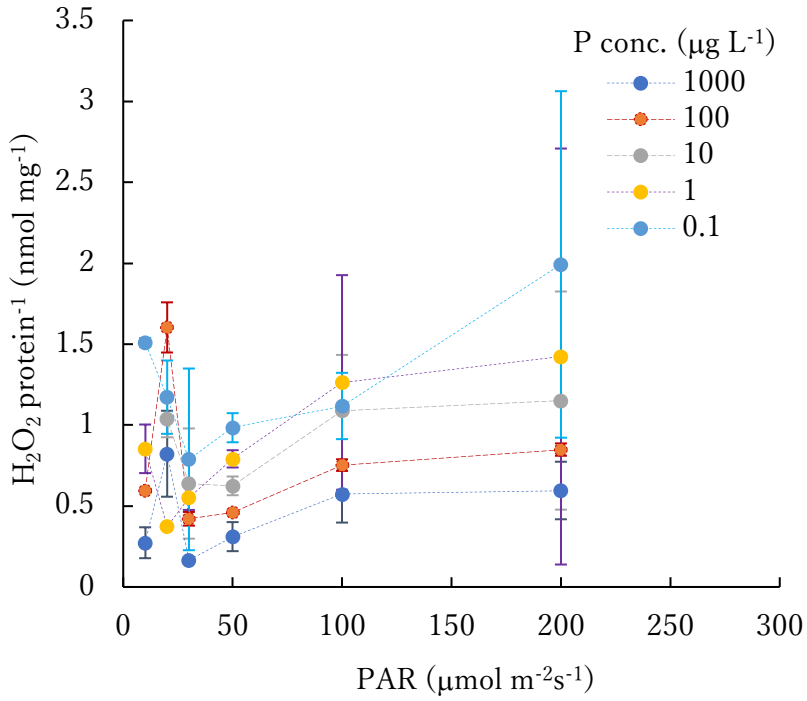



Figure 5.5.1.3. $H_2O_2$ content per protein for different PAR intensity levels and for each P concentration level ($\mu gL^{-1}$). Vertical bars indicate standard deviation.

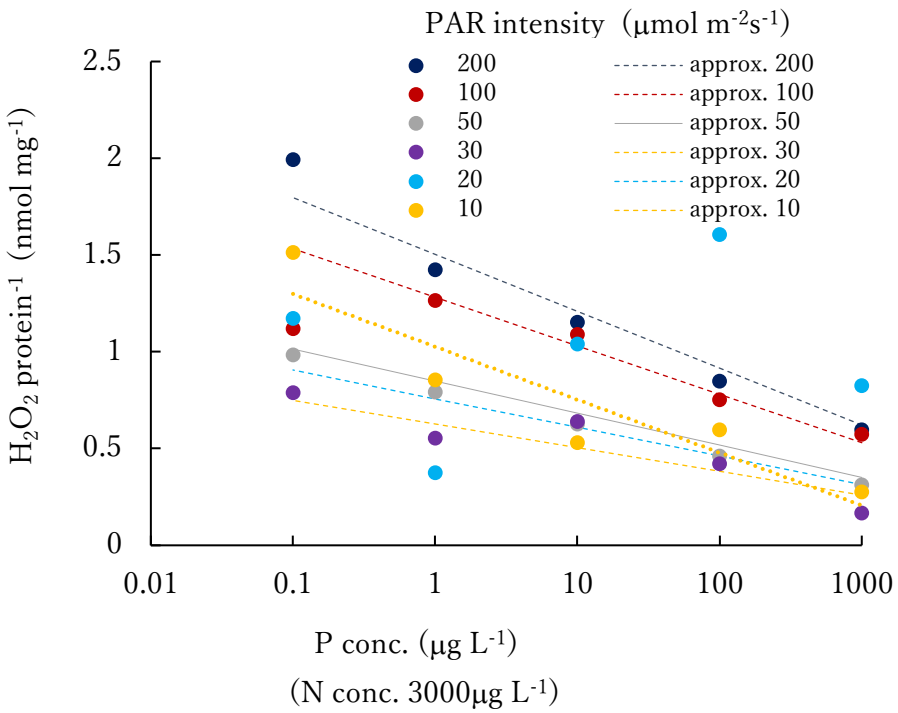



Figure 5.5.1.4. $H_2O_2$ content per protein for different P concentration level ($mgL^{-1}$) and for each PAR intensity level ($\mu molm^{-2}s^{-1}$). Vertical bars indicate standard deviation. Dotted lines show the approximate relation for each light intensity.

### 5.5.2 Antioxidant activities with respect to $H_2O_2$ concentration per protein

SOD activity was uniquely proportionate to $H_2O_2$ per protein (Fig. 5.5.2.1). The approximate relation is shown by the diagonal line, where the $H_2O_2$/protein ($nmolmg^{-1}$) = 0.176(min)*SOD ($nmolmg^{-1}$ $min^{-1}$), ($R^2$ = -0.805, $p<0.01$).
CAT activity is shown as a function of $H_2O_2$ concentration per protein, separately shown by each P concentration in Fig. 5.5.2.2 For each P concentration level, CAT activity per protein linearly increased with the $H_2O_2$/protein. The increasing rate was higher based on PAR intensity (18.73CAT/$H_2O_2$, $R^2$ = 0.573 for 1000μgPL$^{-1}$, 13.82CAT/$H_2O_2$, $R^2$ = 0.977 for 100 μgPL$^{-1}$; 12.89 CAT/ $H_2O_2$, $R^2$ = 0.793 for 10 μgPL$^{-1}$; 14.53CAT/ $H_2O_2$, $R^2$ = 0.949 for 1μgPL$^{-1}$; and 9.22CAT/$H_2O_2$, $R^2$ = 0.766, for 0.1μgPL$^{-1}$), and the proportional coefficient was found to have a significant positive correlation with the logarithmic scale of the P concentration ($p<0.01$).

On the other hand, for each PAR intensity level, CAT activity did not have significant positive correlation with the P concentration level (Barrington et al., 2013)

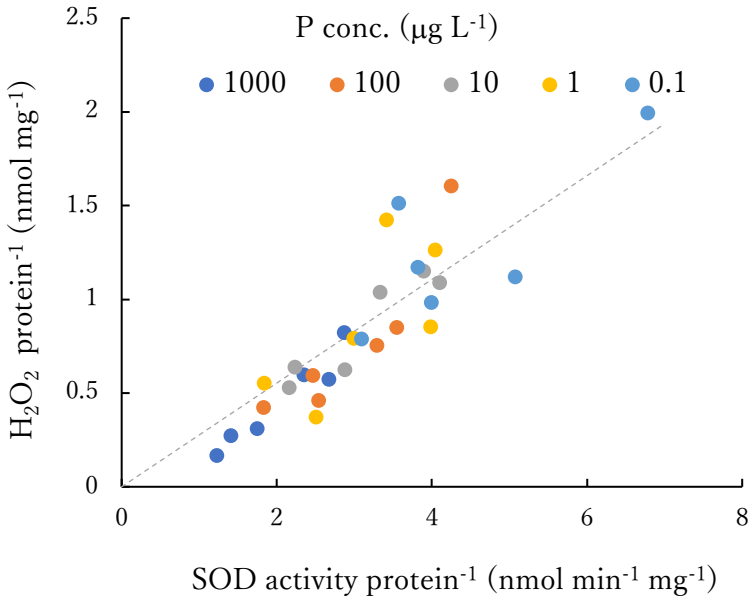



Figure 5.5.2.1. SOD activity per protein for different P concentration level (µgL$^{-1}$) and for each PAR intensity level (µmolm$^{-2}$s$^{-1}$). The approximate relation is shown by the diagonal line.

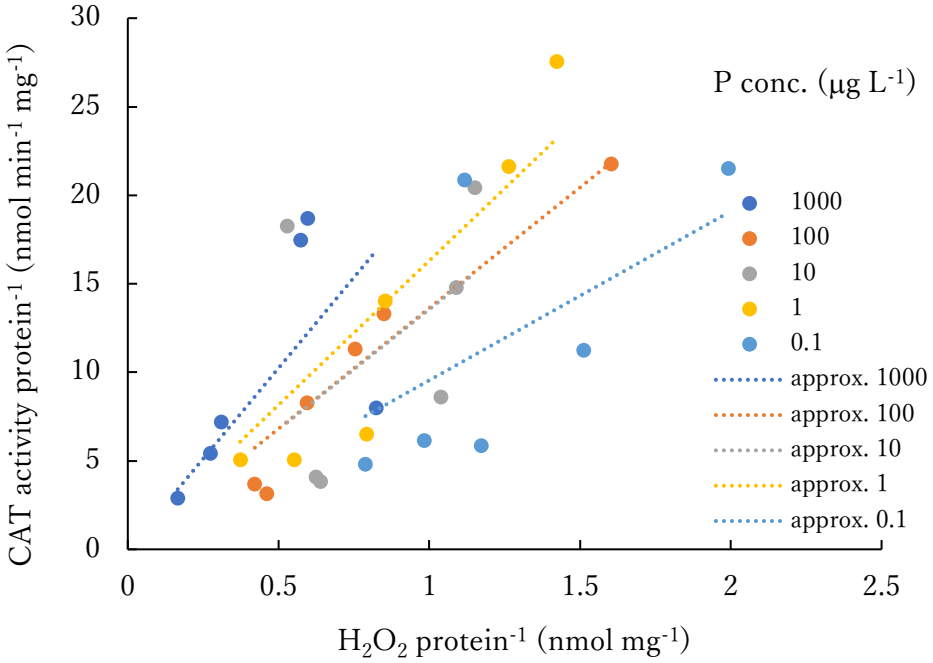



Figure 5.5.2.2. CAT activity per protein for different P concentration levels (µgL$^{-1}$) and for each PAR intensity level (µmolm$^{-2}$s$^{-1}$). Dotted lines indicate the approximate lines for each P concentration.

### 5.5.3 Discussion

#### 5.5.3.1 The effect of biologically produced $H_2O_2$ on the suppression of cyanobacterial blooms

The artificial endorsement of $H_2O_2$ has a high potential to suppress cyanobacterial blooms with less effect on other organisms compared to other controlling methods. Previous researchers obtained the lethal $H_2O_2$ dosage for cyanobacteria by laboratory incubations under different $H_2O_2$ concentrations; cyanobacterial chlorophyll declined to nearly half after an 18 h incubation with approximately 30 µmol$L^{-1}$ of $H_2O_2$ or after a 4 h incubation with 100 µmol $L^{-1}$ of $H_2O_2$. $H_2O_2$ delayed fluorescence decay with 0.1µmol of $H_2O_2$ $L^{-1}$. At the same time, the Fv/Fm value substantially declined with 100 µmol of $H_2O_2$ $L^{-1}$, and dead cells increased with 275 µmol of $H_2O_2$ $L^{-1}$. Cyanobacteria were in a lethal condition and sub-lethal at concentrations exceeding 50 µmol of $H_2O_2$ $L^{-1}$((Leunert et al., 2014). All previous experimental results reveal that cyanobacterial biomass is degraded with higher $H_2O_2$ concentrations; however, the $H_2O_2$ concentration threshold varies widely from 1 to 1000 µmol $L^{-1}$ (Leunert et al., 2014). In the present study, we used protein content as an indicator of biomass rather than the chlorophyll content of *Phormidium ambiguum* cells because chlorophyll-a can be expressed on a protein basis. However, the decreasing trend of protein content, which was seen with the Chl-a concentration, was also observed with $H_2O_2$ concentrations.

Natural $H_2O_2$ formation has been identified in aquatic ecosystems as photolysis of dissolved organic carbon (DOC) exposed to UV. Then it is reported that the $H_2O_2$ production varies with the nutrient content of the water body. However, the $H_2O_2$ concentration of these waters was in the magnitude of µmol $L^{-1}$. The comparison of these results indicates that the photolysis of organic carbon in natural water only is not sufficient to control cyanobacterial biomass.

$H_2O_2$ is also produced biologically and is accumulated in cells subject to high levels of environmental stress. In the present experiment, UV was limited. Accordingly, measured $H_2O_2$ was considered a biologically produced component in cells or cell surfaces, which was then released into the ambient water. In the present experiment, protein content was measured as a reference of the biomass of cyanobacteria. Cell biomass is two to three times larger with protein content.

As the buoyancy of the cells is nearly neutral, the $H_2O_2$ content per protein, ~1 mmol of $H_2O_2$ $kg^{-1}$, was generated and contained in the cell before release. This constitutes more or less the same level of the lethal $H_2O_2$ concentration in water.

The protein content in water declined with an increasing $H_2O_2$ concentration per protein up to 2 nmol $mg^{-1}$ protein. It was nearly same as the lethal level of the previous studies. A higher protein level was not observed with higher $H_2O_2$ concentration levels in the present study. The growth of cyanobacteria is suppressed by the generation of higher $H_2O_2$ levels.

The lethal $H_2O_2$ concentration obtained here corresponds well with about 5 µmol of $H_2O_2$ $g^{-1}$ FW of a threshold condition to grow *Egeria densa* in natural water, considering the weakness of cyanobacteria to $H_2O_2$ rather than other plant species (Foo et al., 2020).

### 5.5.3.2 The possible indicator of environmental stress and the effect of combined stress factors

The accumulation of ROS is reported to augment in parallel fashion to increased abiotic stress. In the present experiment, two types of abiotic stresses, phosphorous deficiency and high or low PAR intensities, were applied with different intensities of each.

Though $H_2O_2$ is produced under normal environmental conditions, its production is accelerated under high stress intensity. In natural water, cyanobacteria often suffer from a shortage of N and P. Stoichiometrically, the ratio of the N and P of cyanobacterial cells is approximately 16:1. Waters with an N:P ratio of <15 are most susceptible to cyanobacterial dominance. In the present experiment, the P concentration was changed with the fixed amount of an N concentration of 3000µg $L^{-1}$ (Wang et al., 2019). Thus, the P concentration becomes restrictive, except for 1000µg P $L^{-1}$ in the present study's conditions. A significant increasing trend was observed in $H_2O_2$ per protein with a decreasing P concentration. The deficiency of essential nutrients may increase oxidative stress and then deteriorate the growth rate of cyanobacteria.

Under all tested P concentrations, $H_2O_2$ per protein content decreased with increasing PAR intensity until 30 µmol$m^{-2}s^{-1}$, taking the lowest value there, then grew at higher PAR intensities, though the increasing rate gradually decreased. The enhanced production of $H_2O_2$ under prolonged low PAR conditions has not been sufficiently studied, though superoxide production in dark conditions is reported. With submerged macrophytes, *Egeria densa*, the $H_2O_2$ concentration was the lowest empirically under the prolonged exposure of a PAR intensity level of approximately 60 µmol$m^{-2}s^{-1}$ (Bouchard et al., 2011). The $H_2O_2$ concentration increased both with decreasing or increasing PAR intensities. However, the underlying mechanisms are unknown.

The increasing $H_2O_2$ concentration per protein over 30 µmol$m^{-2}$ $s^{-1}$ of PAR intensity is attributed to the excessive harvesting of PAR energy. In the thylakoid membrane, electrons are produced by solar energy and transmitted to plastoquinone in PSII, which are partially accepted for carbon dioxide fixation. More electrons are generated when exposed to higher levels of solar radiation. Consequently, the photoinhibition of photosystem-II (PS-II) is induced, leading to oxidative damage because of the generated ROS such as superoxide, hydroxyl radicals, and $H_2O_2$. It damages cellular components, such as the D1 protein, which otherwise mends the damaged photosynthesis apparatus.

The process comprises the direct reduction of $O_2$ by PS-I, resulting in singlet oxygen production followed by superoxide, which is converted to $H_2O_2$ by the activities of the enzyme SOD. In the present study, $H_2O_2$ per protein was proportionate with SOD activity, generating $H_2O_2$ from superoxide. CAT activity was far higher than other major antioxidant activities to decompose $H_2O_2$ and linearly increased with $H_2O_2$ concentration.

Though SOD and CAT activities demonstrated different dependencies on PAR intensity levels and the P concentration, their activities were evaluated by the single function with $H_2O_2$ per protein. A steady $H_2O_2$ concentration is sustained by balancing the generated $H_2O_2$ with different types of stresses and these antioxidant activities as a single function of $H_2O_2$ content per protein.

In natural water, cyanobacteria are exposed to various abiotic stresses that enhance oxidative stress, producing $H_2O_2$, which may deteriorate cyanobacterial biomass. A significant negative

correlation was recognized for protein content with respect to the $H_2O_2$ concentration (n=90, $R^2$=-0.712, p<0.01), irrespective of stress types (Drábková et al 2007a).
The production rate of $H_2O_2$ is not necessarily cumulative for different types of abiotic stresses. However, the $H_2O_2$ concentration was enhanced with increasing PAR intensity and decreasing phosphorus concentration, respectively, and the enhancement of the $H_2O_2$ concentration was independent of each other (p<0.01). The total $H_2O_2$ per protein is empirically given as the sum of $H_2O_2$ produced by the intensity of each stress component at least as a practical use level. Thus, the total $H_2O_2$ concentration is approximately provided by the sum of the $H_2O_2$ concentration attributed to each stress. The same trend was obtained for submerged macrophytes Consequently, a potential for using the $H_2O_2$ concentration to estimate cyanobacterial biomass exists (Diaz, J. & Plummer 2018).

### 5.5.3.3 The estimation of $H_2O_2$ concentrations produced by cyanobacteria under abiotic stresses

For the application of the empirically obtained results to practical use in the prediction of algal blooms in the environment where PAR and P concentrations are restricted factors for growth, the trend of the $H_2O_2$ per protein (nmol/mg) is obtained as a function of PAR ($\mu molm^{-2}s^{-1}$) and the P concentration, P ($\mu gL^{-1}$), as formulated by:

$$H_2O_2/protein = -312*PAR^2/(50^2+PAR^2)*((25/PAR)^4+1)*Log(P/133100) \quad (1)$$

where $0.1\mu g\ P\ L^{-1}<P<1000\mu g\ P\ L^{-1}$, $30\ \mu molm^{-2}s^{-1}<PAR$, and protein represents the amount of protein in $mgL^{-1}$.

The relationship is shown in Fig. 5.5.3.3.2 The simulated results of the $H_2O_2$ $protein^{-1}$ by Equation (1) compared with experimental results and a significant similarity was obtained ($R^2$ = 0.953, p = 0.012, for $1000\mu g\ P\ L^{-1}$, $R^2$ = 0.696, p = 0.0065 for $100\mu g\ P\ L^{-1}$; $R^2$ = 0.927, p = 0.023 for $20\mu g\ P\ L^{-1}$; $R^2$ = 0.982, p = 0.00289 for $1\mu g\ P\ L^{-1}$; $R^2$ = 0.024, and p = 0.024 for $0.1\mu g\ P\ L^{-1}$).

The protein content ($mgL^{-1}$) is shown in Fig. 5.5.3.3.3 The simulated results of protein content by Equation (2), as a function of $H_2O_2$ per protein, was shown to possess significant negative correlation ($R^2$ = -0.675, p<0.01), which is empirically formulated by:

$$protein = -192\ *Log((H_2O_2/protein)/4.1) \quad (2)$$

($R^2$ = -0.71, p<0.01).

With Equations (1) and (2), protein content is estimated as a function of PAR and the P concentration.
The estimated protein contents are denoted in Fig. 5.5.3.3.1. Protein content in water for different P concentration levels (mg/L) and for each PAR intensity level ($\mu molm^{-2}s^{-1}$). The concentration uniquely increased with increasing P concentrations.

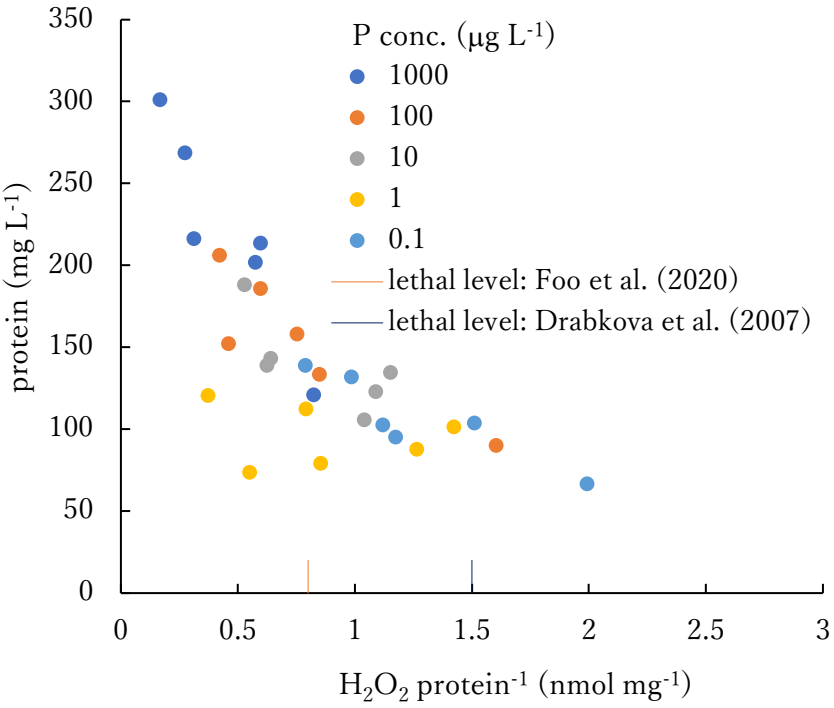



Figure 5.5.3.3.1 Protein content in water for different P concentration levels (μg/L) and for each PAR intensity level (μmolm$^{-2}$s$^{-1}$). Lethal level of cyanobacteria in the previous reports[35, 39] are shown for comparison.

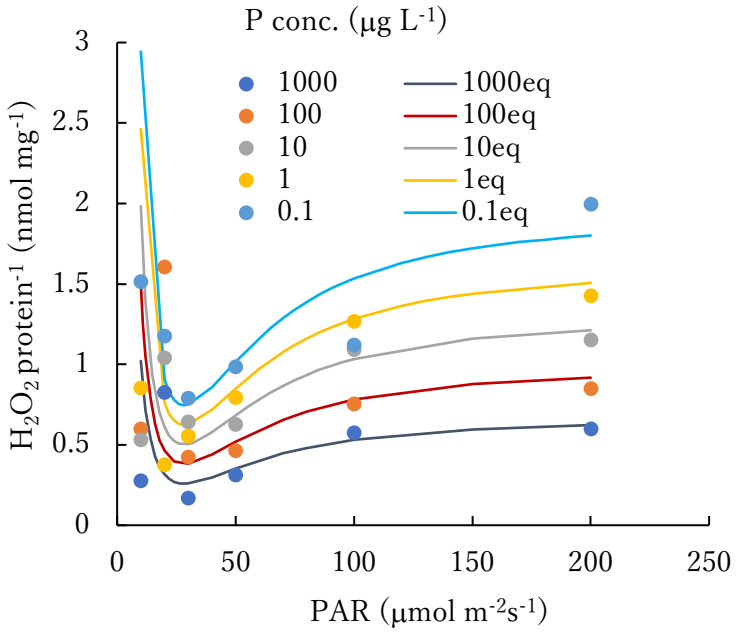



Figure 5.5.3.3.2 The simulated results of $H_2O_2$/protein by equation (1) compared with experimental results.

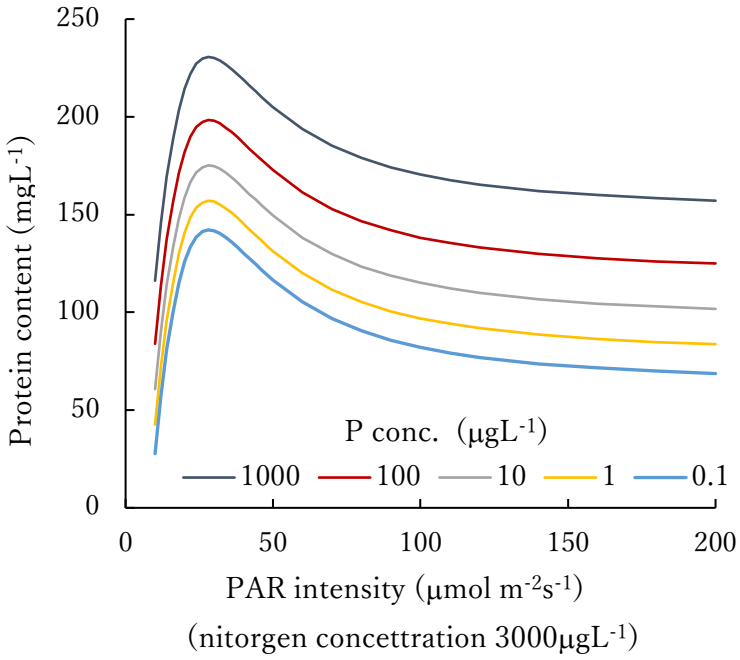



Figure 5.5.3.3.3 The simulated results of protein content.

The cellular growth rate gradually decreased with light intensity and the growth rate of cyanobacteria reached a maximum at 30 to 50 μmolm$^{-2}$s$^{-1}$ . *P. ambiguum* prefers relatively low light intensity at ~18 μmolm$^{-2}$s$^{-1}$. The diagram seems to provide reasonable results.

### 5.5.4 Conclusions

The endogenous $H_2O_2$ concentration is an effective tool to detect the stress level of cyanobacteria. Both PAR regimes and P concentration shortages are shown to enhance $H_2O_2$ concentrations in cyanobacterial cultures.

$H_2O_2$ per protein content declines in low PAR conditions and increases when exposed to higher PAR intensity levels while generally increasing as the P concentration decreases. $H_2O_2$ per protein for combined stresses is given by the sum of the amount produced by each stress. Protein content decreases uniquely following the value of $H_2O_2$ per protein in a cyanobacterial culture. Then the $H_2O_2$ per protein exceeds the threshold value, cyanobacteria will decline. As a result, cyanobacterial cell will lyse leading to death. Cyanobacterial cell biomass can easily be regulated by this way.
The prediction model was developed for the protein content to design management criteria for excessive cyanobacterial blooms in freshwaters.

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# Chapter 6 Conclusion and future research

## 6.1 Conclusion and future research

### 6.1. 1 Conclusion

Ecological balance depends on riparian plant species. Riparian areas are core habitats for a wide range of semiaquatic and terrestrial species. The riparian zone contains a range of ecologically important species. Therefore, it is important to monitor their habitat preferences, influencing environmental factors that affect their existence. Riparian plants is naturally adapted to abiotic conditions characterized by fluctuating water, sediment, nutrients, solar radiation, and drought. Due to frequent flood disturbances the sediment accumulations cannot develop properly. As a result, its nutrient level may decrease. The sediment nutrient level may also determine which species can distribute at specific-zones. The sediment nutrient level in riparian soil, especially TN or TP concentration, can filter and alter its biogeochemistry. When it comes to field sampling and ecological aspects, it is necessary to measure many samples over time. It is therefore important to avoid complex methods when analyzing data to ensure accurate results. The available methods to quantify plant stress level takes more than a year to assess as well as it takes a lot of budgets to implement. Hence, suitable and easy methods need to develop to quantify environmental stressors. When plants are subjected to environmental stress through metabolic and physiological adjustments, ROS is generated in different organelles depending on the stressor types. During exposure to different types of environmental stressors, $H_2O_2$ is generated. The $H_2O_2$ in plant tissues is relatively stable and can easily be measured. Therefore, quantification of $H_2O_2$ concentration could be a suitable way to determine environmental stressors. There are various factor that affect $H_2O_2$ quantification such addition of salicylic acid in tomato leaves $H_2O_2$ concentration increases from 0.15 μmol/gFW to 0.25 μmol/gFW. Various methods also give different results. For example, Apple leaves were estimated to contain 20–70 nmol/g FW of $H_2O_2$ using the $Ti(SO_4)_2$ assay, while 5–25 nmol/g FW was found using the Bioxytech $H_2O_2$-560 colorimetric assay. In order to achieve acceptable results, we need to develop a simple method. Therefore we aim to quantify $H_2O_2$ by evaluating the correlation between eFOX and $Ti(SO_4)_2$ assays using riparian plant species. Four riparian plants were collected namely *Ambrosia trifida*, *Solidago altissima*, *Artemisia princeps,* and *Sicyos angulatus* for this study. Each species showed a substantial correlation between the eFOX and $Ti(SO_4)_2$ assays in nonfrozen conditions (*Ambrosia trifida* ($r=0.767$, $p<0.001$), *Solidago altissima* ($r=0.583$, $p<0.001$), *Artemisia princeps* ($r=0.672$, $p<0.001$), and *Sicyos angulatus* ($r=0.828$, $p<0.001$)). Therefore, both methods can be introduced to quantify oxidative stress using $H_2O_2$ concentration easily. The eFOX and $Ti(SO_4)_2$ methods are broadly used to quantify $H_2O_2$ concentration of *Salix spp*., *Robinia pseudoacacia*, *Ailanthus altissima* with *Juglans mandshurica*, *Phragmites australis*, *Phragmites japonica*, and *Miscanthus sacchariflor*u, *Artemisia princeps and Sicyos angulatus*, and *Solidago altissima* riparian species. The leaf samples were collected of those species and soil also taken to measure moisture, soil TN or TP concentration, TN: TP. The results clearly indicate that soil moisture content is only significantly correlated with $H_2O_2$ concentration compared among parameters (TN or TP concentration, TN: TP). Therefore, it can be concluded that soil moisture content is the prime stressors for those riparian plants using $H_2O_2$ as an indicator.

Another application of Hydrogen peroxide measurement is applied in cyanobacterial bloom management. Cyanobacterial bloom becomes a world-wide problem especially in eutrophic lakes and reservoirs. Cyanobacteria are photoautotrophs in nature. The presence of light directly affects the growth of cyanobacteria, even moderate light changes are stressful. During

cyanobacteria growth, higher temperatures promote metabolic acceleration and lower temperatures augment them. Due to globalization the industrial wastewater consists of high amount of Nitrogen and Phosphorus which accelerate their growth. To suppress cyanobacteria, there is need to view their daily responses with light intensities in different temperatures and long-term effects can also be visualized. Nutrient depletion can also be a useful way to eliminate cyanobacteria. Hence, three experimental design was investigated using $H_2O_2$ as an oxidative marker. These are 1) One day exposure to different light intensities in temperatures alterations. 2) Long-term exposure high light intensities in different temperatures. 3) The nutrient level of Nitrogen and Phosphorus supplied shortage to their culture media. The representative cyanobacteria are *M.aeruginosa*, *P. ambiguum*, and *P. foetida* for this study. The eFOX method is used to quantify $H_2O_2$ concentration. In One day exposure, *M. aeruginosa* and *P. ambiguum* showed an increasing trend of $H_2O$, Chl-a, and CAT activity in different temperatures at 15:00. CAT activity was proportionate to the $H_2O_2$ increment, which implies the hysteresis effect of daytime PAR and antioxidant activity in the different temperatures. This study confirms that even in a single day exposure cyanobacteria feels stress. The long term effect of light intensities in temperature alterations confirms the proper cyanobacteria elimination process using $H_2O_2$ as an indicative marker. Photoinhibition can occur at 200 $\mu molm^{-2}s^{-1}$ and cyanobacteria cells growth decrease with more than 200 $\mu molm^{-2}s^{-1}$ light intensities. The algal biomass significantly lower at lowering temperatures. The Nitrogen and Phosphorus deficiency leads to cyanobacterial cell will lyse leading to death. $H_2O_2$ per protein content declines in low PAR conditions and increases when exposed to higher PAR intensity levels while generally increasing as the P concentration decreases. $H_2O_2$ per protein for combined stresses is given by the sum of the amount produced by each stress. Protein content decreases uniquely following the value of $H_2O_2$ per protein in a cyanobacterial culture. As a result, cyanobacteria can be controlled.
The new method of assessing plant oxidative stress is much more effective and time saving. A large-scale study is required to establish this method more appropriately. High light intensities, temperatures alterations, and nutrient depletion can be a countermeasure against algal blooms.

### 6.1. 2 Future research

We have considered a few abiotic stresses to know plants physiological condition considering $H_2O_2$ as an indicator. This study was developed in riparian plants and macrophytes. We are analyzing mangrove samples to know their stress conditions. Various biotic stressors can be applied, and also molecular mechanisms can be developed in future study.

In the cyanobacteria, we mainly developed laboratory-based experiments. We are analyzing filed samples such as Imperial palace pond (Tokyo) and Ojagaike pond (Chiba) to find the correlation between filed cyanobacteria samples with laboratory-based experiments. The non-chemical methods can be developed by considering our laboratory-based findings in the field analysis. There could be a possibility of increasing illumination of light intensities that is beyond the toleration of cyanobacteria.