EFFECTS OF WATER TURBULENCE AND MEAN FLOW ON GROWTH, METABOLISM AND ULTRA-STRUCTURAL VARIATIONS IN AQUATIC MACROPHYTE; ELODEA NUTTALLII

(水生植物コカナダモの生長、代謝、微細構造の変化における平均流動及び乱流成分の影響)

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by

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A dissertation submitted as a partial fulfillment of the requirement for the Degree of Doctor of Philosophy in Environmental Science and Engineering

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September 2015

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ACKNOLEDGEMENT

This research work would not have been accomplished unless generous support, guidance and encouragement of many individuals. First, I would like to express my profound gratitude to my supervisor Prof. Takashi Asaeda for his valuable guidance, suggestions and advices throughout the study. I would like to express the deepest appreciation to Prof. Yasuko Kaneko and Prof. Maki Kawai-Yamada for their guidance, encouragements and providing laboratory facilities for my study.

Special thanks are extended to Dr. Atzuko Miyagi and Dr. Kim Atzuzawa for their generous support and Dr. Harun Rashid is greatly acknowledged for his valuable suggestions and encouragements. I also extend my thanks to Ms. Taeko Oyama for her kind support in many directions throughout my study. Further, my sincere thanks are extended to all my lab mates for helping me in numerous ways.

It my great honor to thank to the Japanese government and the Ministry of Education, Culture and Sports, Science and Technology (MEXT), Japan for providing a scholarship to pursue my PhD at the Saitama University, Japan.

I humbly and affectionately admire the inspiration and much needed moral support given by my parents. At last but not least, with deepest love, I owe my gratitude to my loving wife Sandamali Sakunthala and my loving kid Senula Dinal for their never ending encouragements, sacrifices and moral support.

Finally, I would like to thank all who were important to the successful realization of my thesis, as well as expressing my apology that I could not mention personally one by one.

ABSTRACT

The array of functions provided by aquatic plants is essential to maintain the ecological balance of aquatic ecosystems. Aquatic plants experience various disturbances in aquatic systems for two reasons: the highly dynamic nature of the systems and the sedentary nature of the plants. The productivity of the plants is highly dependent on environmental stresses and a wide range of fluctuations in specific abiotic factors. Among the abiotic factors, water movement is the most important for submersed aquatic plants because the flow driven drag and lift forces have either a positive or a negative effect depending on the magnitude. The mechanisms governing flow-plant interactions are not yet fully understood, and the plant response to mean flow (without turbulence) against turbulence is largely unknown. Acquiring knowledge and clarifying the interactions between aquatic plants and environmental factors are important to understand how the functions of plants would benefit from the management of ecosystems. Although the stress responses of submersed plants to turbulence were reported previously, information on the plant responses to mean flow is scarce, to our knowledge. Further, effects of water movements on the ultra-structure of plant cells and amino acid metabolism are yet to be explored. This study hypothesized that, the water movements significantly impact to the plant metabolism, stress response and the ultrastructure of plant cells. Therefore in this study was designed to evaluate the variations in metabolism, stress responses and ultra-structures of aquatic plants responding to water movements. There were three laboratory experiments coupled with field observations.

In the first experiment, growth and stress responses of *Elodea nuttallii* exposed to either turbulence or main flow were compared to control plants grown in stagnant waters. Turbulence and main flow were generated using a vertically-oscillating horizontal grid and a specially designed re-circulating system, respectively. At the end of the experiment after three weeks, growth, chlorophyll fluorescence, concentrations of indole acetic acid, H_2O_2, chlorophyll, cellulose, lignin and antioxidant enzyme activities of plants exposed to turbulence or main flow were compared with control plants. A decrease in shoot elongation coupled with an increase in radial expansion was observed in plants exposed to turbulence and mean flow. The effects on the plants exposed to turbulence were further accompanied by significant increases in cellulose and lignin. Significant elevations in H_2O_2 and antioxidant enzyme activities were observed in

turbulence-stressed plants, and the lowest stress was observed in control plants. The effects of main flow-induced stress were similar to characteristics of control plants. Turbulence reduced total chlorophyll by approximately 40% compared to plants in the control and main flow. Mechanical stress induced by turbulence leads to increased oxidative stress and tissue rigidification in *E. nuttallii* and that turbulence triggered stress is more severe than that induced by main flow.

The second experiment investigated growth, metabolism and ultra-structural changes in response to turbulence in aquatic macrophyte; *Elodea nuttallii*, after exposure to turbulence for 30 days. The turbulence was generated with a vertically oscillating horizontal grid. The turbulence reduced plant growth, plasmolyzed leaf cells and strengthened cell walls, and the plants exposed to turbulence accumulated starch granules in stem chloroplasts. The size of the starch granules increased with the magnitude of the turbulence. Using capillary electrophoresis–mass spectrometry (CE-MS), an analysis of the metabolome found metabolite accumulation in response to the turbulence. Asparagine was the dominant amino acid that was concentrated in stressed plants, and organic acids such as citrate, ascorbate, oxalate and γ-Amino butyric acid (GABA) also accumulated in response to the turbulence.

The third trial was conducted to study the effect of mean flow on plant functioning and structure. *E. nuttallii* were exposed to flowing (~10 cms^{-1}) and stagnant waters for 30 days. At the end of the experiment, plant growth, chlorophyll and ultra-structural variations were compared. Final shoot length was not significantly different among two treatments while the plants grew in flowing waters consisted of comparatively longer internodes. Although the chlorophyll content of plants in the latter treatment was less than that of control group, the difference was insignificant. Electron microscopy observed thin, elongated chloroplasts in leaves of flowing treatments while, control plants with wide chloroplasts. Structure of leaf cells were affected by the water flow as the diameter of cells in control plants was bigger than that of flowing treatment. These results indicated that turbulence caused severe stress that affected plant growth, cell architecture and some metabolic functions of *E. nuttallii* than the mean flow.

A field investigation was conducted at Moto Arakawa, a tributary of the Arakawa River, Japanto study the consequences of turbulence induced stress response of aquatic macrophytes in natural condition. Six study sites were selected and the velocity fluctuations inside macrophytes stands were measured using a two dimensional electromagnetic current meter. Transects were significantly different (p<0.05) in terms of turbulence velocity. The location having the lowest turbulence was considered as the control turbulence. Plant stress responses to turbulence were compared by measuring the antioxidant activities (peroxidase, catalase and ascorbic proxidase), H_2O_2content and indole acetic acid (IAA). Antioxidant productions were significantly higher in plants exposed to higher magnitudes of turbulence compared to those grew in the control transect. Compared to control transect, an elevated level of H_2O_2 and low concentration of IAA were also measured in plants grown in turbulent environments. Decrease in IAA content was detected in response to increasing turbulence. Lignin and cellulose accumulation were significantly higher in plants exposed to elevated level of turbulence compared to control plants. Taken together, field observations suggest that mechanical stress induced by turbulence led to increase oxidative stress and tissue rigidification in *M. spicatum* and the laboratory findings were in consistent with field observations.

Findings of this study revealed that turbulence make significant negative impacts to plant functioning and cell ultra-structure, and the severity of the impacts increase with the magnitude of turbulence. Even though the effect of mean flow is stressful for aquatic plants, the impacts were small compared to that of turbulence. All together, present findings demonstrates the causes of water movements to aquatic plants and provides insights to clarify the interactions between aquatic plants and water movements to the benefit of aquatic ecosystem management.

Chapter 1. Introduction

The array of functions provided by aquatic plants is essential to maintain the ecological balance of aquatic ecosystems. Some of the important functions of aquatic plants include primary production, provisioning of food, habitat and refuge for different aquatic fauna, contributions to biogeochemical cycles, and regulation of sediment transport, among other functions (Bornette and Puijalon, 2011; Folkard, 2011; Nepf, 2012). Aquatic plants experience various disturbances in aquatic systems for two reasons: the highly dynamic nature of the systems and the sedentary nature of the plants (Chehab et al., 2009). The productivity of the plants is highly dependent on environmental stresses and a wide range of fluctuations in specific abiotic factors, such as water movement, sediment properties, macro-and micronutrient levels in the water column, availability of light, UV radiation, toxic metals and temperature, as reported in the literature (Bornette and Puijalon, 2011). Among the abiotic factors, water movement is the most important for submersed aquatic plants because the flow driven drag and lift forces have either a positive or a negative effect depending on the magnitude. For example, low flow is beneficial for photosynthesis(Westlake, 1967), whereas flow driven mechanical stresses alter growth, physiology and morphology in aquatic plants (Asaeda et al., 2010; Bornette and Puijalon, 2011). Even though most lake, rivers or estuarine flow motions are turbulent (Horne and Goldman, 1994), macrophytes are challenged by flow and turbulence simultaneously. Temporal variability at any point in the stream leads to rapid fluctuations of the flow velocity with the passage of turbulent eddies(Sand-Jensen and Pedersen, 1999), while flow turbulence and wave forces are extremely complex, and their magnitude and direction can change rapidly in many natural environments (Schutten et al., 2004). Despite flow extremes, flow regimes and hydraulics have been most commonly cited as abiotic determinants of macrophyte assemblages(Asaeda et al., 2004; Chambers et al., 1991; Green, 2005; Olson et al., 2012), few studies conducted to evaluate plant stress in response to turbulence(Champika Ellawala et al., 2011; Ellawala et al., 2011; Ellawala et al., 2012; Ellawala et al., 2013).

In addition to plant growth, biochemical evidence is also widely applied to detect plant stress. For example, the accumulation of reactive oxygen species (ROS) is one of the primary signals of stress(Apel and Hirt, 2004; Gill and Tuteja, 2010), and plants evolved defense mechanisms to counter the damage caused by excess ROS in stressful environments. One mechanism is the activation of ROS scavenging antioxidant enzymes such as catalase, peroxidase, superoxide dismutase, and ascorbic peroxidase (Apel and Hirt, 2004; Gill and Tuteja, 2010). Another defense mechanism is to accumulate multiple compatible solutes to protect the plant cell against oxidative damage(Liu et al., 2011). The osmolytes produced under stress are regarded as an osmoregulation mechanism that protects the cells from damage, and in addition to osmoregulatory protection, these osmolytes are also ROS scavengers (Bohnert and Shen, 1998). In this context, the accumulation of amino acids is widely thought to be a direct protective mechanism in many terrestrial plants (Rhodes et al., 1999). On the other hand, the ultra-structural changes in cells of submersed plants in response to turbulence are largely unknown. Ingber (2003) proposed that understanding the mechanisms behind the mechanical forces that regulate and integrate cellular activities in plants should be one of the new priorities in cell biology.

The mechanisms governing flow-plant interactions are not yet fully understood, and the plant response to main flow (without turbulence) against turbulence is largely unknown. Although the stress responses of submersed plants to turbulence were reported previously, information on the variation in amino acid metabolism with exposure to turbulence is scarce, to our knowledge. Acquiring knowledge and clarifying the interactions between aquatic plants and environmental factors are important to understand how the functions of plants would benefit from the management of ecosystems. Therefore, this research was designed to study the aquatic plant responses against the water movements.

1.1. Overall objective

Present study hypothesize that the water movements make significant impact to plant functioning and ultra-structure plant cells. Therefore, this study investigated the biochemical, physiological, structural and anatomical variations in aquatic macrophyte as a consequence of mechanical stress after exposure to water turbulence and mean flow.

1.2. Specific objectives

1. To compare the growth and stress response of aquatic plants after exposure to water movements.
2. To compare the ultra-structural variation in plant cells against mechanical stress induced by the water movements
3. To compare the variation in plant metabolism as a consequence of water movements
4. To study the significance of water turbulence as a stress vector of macrophytes in natural environment.

Chapter 2. Literature Review

2.1. Abiotic stress on aquatic plants

A significant deviation from the optimal condition of life is considered as the stress (Larcher, 2003).In natural ecosystems, distribution and growth of submersed macrophytes rely on a combination of factors including substrate characters, nutrient availability in sediment and water column, light penetration, pollutants, temperature, water movements etc (Figure 1). The organism under stress passes through a sequence of characteristic steps (Figure 2) that includes the alarming phase, the resistance phase and the phase of exhaustion (Larcher, 2003). Even though the plant age, adaptability, and seasonal changes are some of the important factors behind plant stress, response to a particular stress vector depends upon genetically determined reaction norms of plants. Morphological adaptations, biochemical alterations and biomass allocations between above ground and below ground tissues are some of the common responses of plants against the stress. However, plant growth and other biological functions would impair and eventually plants may injure and die after exceeding their tolerance limit.

In response to environmental concerns, alterations in plant physiology, morphology, histology and in biochemistry have also been reported in the literature (Wang et al., 2008; Wang et al., 2010; Zaman and Asaeda, 2013). Among these responses, biochemical variations were commonly employed to evaluate the plant stress. Generally, reactive oxygen species (ROS) are produced continuously in plant cells during various metabolic reactions take place in various cellular organelles such as chloroplast, mitochondria, peroxisome, plasma membrane, endoplasmic reticulum and cell wall (Mittler et al., 2004; Sharma et al., 2012). Despite low levels of ROS are important for acclamatory signaling (Dat et al., 2000; Sharma et al., 2012), overproduction of ROS makes phytotoxicity in plants leading to damage proteins, lipids, carbohydrates, chlorophyll and DNA (Gill and Tuteja, 2010; Rau et al., 2007). Molecular oxygen converts to highly reactive ROS after a stepwise reduction induced either by high-energy exposure or electron-transfer reactions.

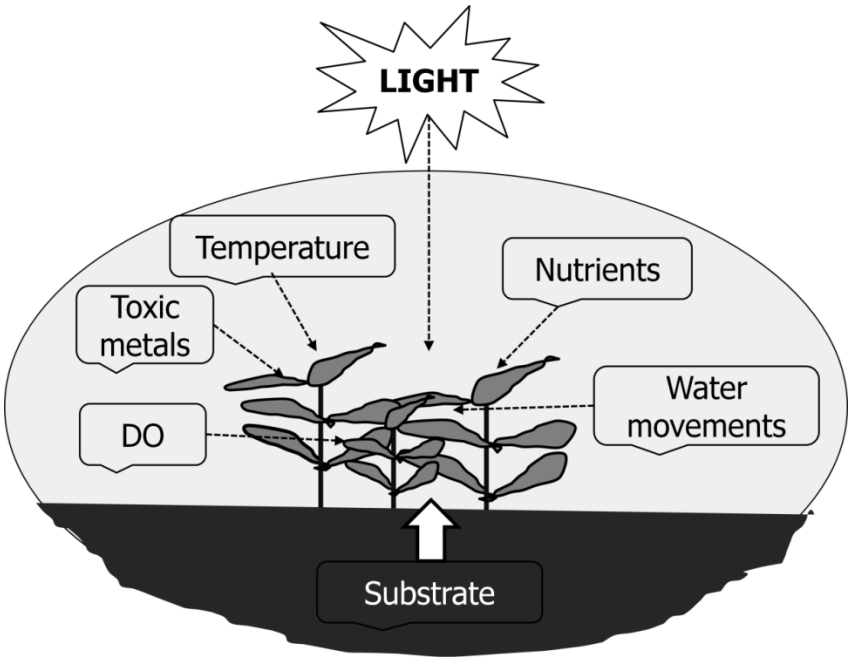

Figure 1. Abiotic stress factors on aquatic plants

ROS including superoxide radical ($O2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$) and as well as non-radical molecules such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) have potential to damage tissues and seriously disrupt plant metabolism via oxidation of membrane lipids, proteins, pigments and nucleic acids. H_2O_2 can diffuse across membranes resulting in the formation of destructive hydroxyl radicals during stress exposure (Wang et al., 2008).Therefore, plants are in need of rapid removal of $H_2O_2$for a better functioning. In a stress condition, plants seems to accumulate ROS in an elevated levels, and consequently activate the parallel up-regulation of ROS scavenging defense mechanism. ROS scavenging system of plants consists of enzymatic components such as catalase (CAT), ascorbic peroxidase (APX), guaicoal peroxidase (GPX) and superoxide dismutase (SOD), and antioxidants including ascorbate and gluthione(Noctor and Foyer, 1998).Therefore, the protective mechanisms evolved by the plants keep deleterious effects of the free radicals to a minimum level during stress.

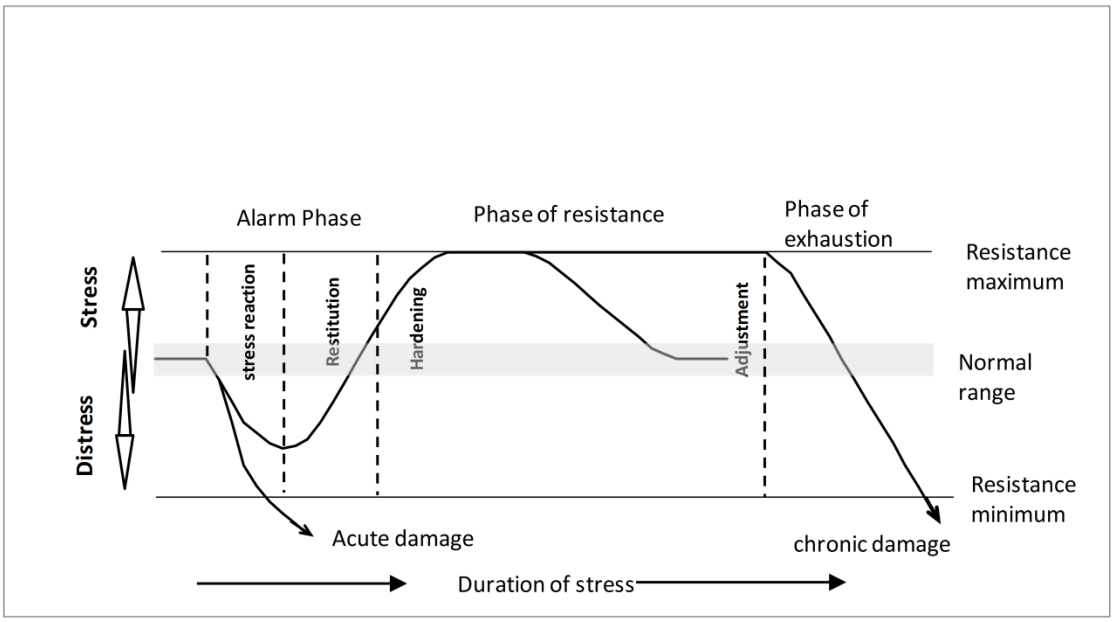

Figure 2. A Phase model of stress events and responses (Larcher, 2003)

Therefore, changes in the concentrations of antioxidant molecules are signals of plant tolerance/adaptation to stress conditions. The intracellular levels H_2O_2 is mainly regulated by a wide spectrum of enzymes, the most important being catalase and peroxidases. CAT and GPX scavenge H_2O_2 while SOD catalyzes the dismutation of two molecules of superoxide into O_2 and H_2O_2. Even though the scavenging of ROS prevents oxidative damage under steady state condition, the equilibrium between scavenging and ROS production in aquatic plants disrupt by an array of stress vectors such as water turbulence, NH_4^+, sediment anoxia, salinity, toxic heavy metal etc. (Table 1).

Table 1. Stress responses of aquatic plants for different abiotic factors

Stress vector	Plant Species	Plant responses															Reference
		Gt	H_2O_2	SOD	GPX	CAT	APX	GSH	GR	Chl-a	Chl-b	Chl-(a+b)	CT	MDA	Fv/Fm	SP	
Turbulence	*E. densa*	-	+		+					-		-					Champika Ellawala et al. (2011)
	V.spiralis	-	+		+												
	E.nuttallii	-	nc		+	+	+			-	-		nc		-		Ellawala et al. (2011)
	P.crispus	-	nc		+												Ellawala et al. (2013)
	M.spicatum	-	+		+	+	+			-	-						
NH_4^+	*M.mattogrossense*				+	+		+						-			Nimptsch and Pflugmacher (2007)
	H.verticillata		+	+	+	+	+	+				-	-	-			Wang et al. (2010)
	E.densa	-		+	+	+						-		+		-	Shengqi et al. (2012)
	C.demersum			+	+	+	+	+		-				+			Gao et al. (2012)
	L.minor	-								-	nc	-	nc				Wang et al. (2014)
		-		+	+/-							-	-	+		-	Huang et al. (2013)
	E.nuttalli	-	+	+										-			Zaman and Asaeda (2013)
Anoxia	*E.nuttalli*	-	+	+						-	-		-	+	-		Zaman and Asaeda (2013)

	M.spicatum	-	+		+	+	+		-	-		-	nc		-	Parveen (2014)
Salinity	*L. salicaria*										-	-		+		Zhao et al. (2013)
	H. verticillata			+	+	nc	+									Rout and Shaw
	N.indica			+	+	nc										(2001)
	N.gramenia			+	+	+										
	P.stratiotes		+	+	+	+	+	+				-	-	+		Upadhyay and Panda
	S.molesta		+	+	+	+	+	+				-	-	+		(2005)
	A.pinnata	-		+			+							+		(Masood et al., 2006)
	A.filiculoides	-		-			+							+		
	S.polyrhiza	-	+	+	+	+	+		+	-	-		-	+	-	Chang et al. (2012)
Heavy metal																
Cd	*P.australis*		nc		+	nc	+		+					n c		Ederli et al. (2004)
	P.australis(R)			+		+	+		+							Iannelli et al. (2002)
	P.australis(S)			+		+	+		+							
Cd	*B.monnieri*			+	+	-	+					-	-		-	Singh et al. (2006)
Cd	*L.minor*			+	+/-	+				-	-	-	-	+	-	Hou et al. (2007)
Cu	*L.minor*			+	+/-	+				-	-	-	-	+	-	

	H.verticillata	+/-	+	+/-	+/-	+		-	-	-	-	+	+/-	Srivastava et al. (2006)
	C.demrsum		+	+/-	+/-	+/-		-	nc	-				Rama Devi and Prasad (1998)
(Pb+Cd+Hg) mixture	*K.candel*(L)		+/-	+	+							+		Zhang et al. (2007)
	B.gymnorrhiza)		+/-	+	nc							+		
	K.candel(R)		+/-	+	+/-							-/+		
	B.gymnorrhiza(R)		+/-	+	+/-							-/+		
Cr	*N.indica*							-	-	-			-L	Sinha et al. (2002)
	V.spiralis							-	-	-			-L	
	A.sessilis							-	-	-			-L	
	N.alba							-	-				-	Vajpayee et al. (2000)
Pb	*C.demersum*	-	+/-	+/-	+/-	+/-	-	-	-	-	-	+	-	Mishra et al. (2006)

Hg	*P.crispus*				-	+	-	Ali et al. (2000)
Excess	*P.crispus*	+	+				-	Zhang et al. (2010)
nutrients	*C.demersum*	+/-	+/-	+		+		Xiong et al. (2010)

+: increase, -: decrease, +/-: increase followed by a decrease, -/+ decrease followed by an increase

Gt: plant growth, GSH: glutathione, CT: carotinoids, SP: soluble proteins, L- leave, R-Roots, Chl-a: chlorophyll-a, Chl-b: Chlorophyll-b, Chl (a+b): Total chlorophyll.

2.2. Water movements induced abiotic stress in aquatic plants

Water movement is the ubiquitous environmental concern which makes either positive or negative impact on plants depending upon the magnitude of the force (Chambers et al., 1991; Ham et al., 1981). For instance, enhanced nutrient uptake has been reported under low flow and small scale turbulence because of the reduction of in the thickness of boundary layer. The boundary layer (BL) is the water layer immediately adjacent to the leaves surface of aquatic plants where the materials flux across this BL by means of diffusion. Therefore the thickness of the BL which is governed by a combination of factors such as current velocity and surface roughness (Koehl and Alberte, 1988; Wheeler, 1980) plays an important role in macrophyte functioning.

Compared to other abiotic stresses, aquatic plants are persistently challenged by the flow driven mechanical stress i.e., the turbulence and main flow. Increase in flow velocity has both positive and negative impacts on aquatic plants depending upon the species being exposed. For example, increased flow velocity has reduced the BL thickness and consequently enhanced the productivity of *Zosterea marina* (Fonseca and Kenworthy, 1987). However, further reduction of BL wash off extracellular carbonic anhydrate which makes negative effect to the plant. Therefore the positive relationship between photosynthesis and current velocity applies up to a certain saturating velocity; i.e. above a critical DBL thickness (Fonseca and Kenworthy, 1987; Koch, 1993; Wheeler, 1980). The reason behind former reduction in photosynthesis is explained by the limitation of the enzymatic uptake system of plants and changes in the local hydrodynamic conditions (Koch, 1994; Wheeler, 1980). Theoretical diffusion BL requirements are based on smooth, epiphytic-free surfaces under steady, unidirectional velocities which are rarely occurs in natural conditions. Macrophytes in natural environment and also in laboratory cultures often consist of attached algae and debris which directly affect to the thickness of the BL. For instance, Sand-Jensen et al. (1985) reported 49% reduction in the BL thickness after removing epiphytes on the surface of *Zosterea marina*. Although the critical current velocity (CCV) was species specific, different CCV have been observed for the same species due to their physiological and/or morphological traits (Stewart and Ackerman, 2009; Westlake, 1967). For example, the CCV for *Ulva lactuca* to be 0.3 cms^{-1}(Koch, 1993), whereas former value was observed in the

the range from 2 to 6 $cms^{-1}$for the same species by other researchers (Hurd et al., 1996; Koch, 1994; Wheeler, 1980).

Aquatic systems are highly dynamic where water movements influence either directly or indirectly on aquatic plants in a complex way. Aquatic plants are typically occur as random patches or clumps in lotic ecosystems and the all flow related factors such as flow extremes, flow regimes and hydraulics are the most commonly cited abiotic determinants of their assemblages (Bunn and Arthington, 2002; Green, 2005; Schoelynck et al., 2013; Thomaz et al., 2006). Unsteady movement of air or water is referred as turbulence while it is normally associated with high flow velocities when no longer laminar flow exists. Temporal variability at any point in the stream leads to rapid fluctuations of the flow velocity with the passage of turbulent eddies (Sand-Jensen and Pedersen, 1999). Further, flow turbulence and wave forces are extremely complex, and their magnitude and direction can changed rapidly in many natural environments (Schutten et al., 2004). Under this context, plants in flowing waters would experience greater and more varied mechanical stress. Champika Ellawala et al. (2011) observed alterations in growth, morphology and biochemistry of plants after prolong exposure to water turbulence. Very few laboratory investigations explained the biochemical and morphological plasticity of macrophytes in response to turbulence (Table 1). In the former studies, the stress response of *Eigeria densa*, *Elodea nuttallii*, *Potamogetton pectinatus* and *Vallisnaria spiralis* were tested after exposure to different magnitude of turbulence and the oxidative stress was observed in response to turbulence. Particularly, accumulation of H_2O_2 was obvious in plants espoused to the high magnitude of turbulence (Table 1). However, plant responses to former stress in natural environment are not yet fully documented.

Basically two hypotheses have been proposed to explain the plant responses behind the mechanism stress. Morris and Homann (2001) proposed that, the intracellular pressure may play an important role in perceiving mechanical stimulus, and the alteration in intracellular pressure alter the downstream effects including translocation of subcellular organelles. Second hypothesis highlights, the changes in the membrane surface tension transmit the signals after receiving via mechanical stress and subsequently activate the stretch-activated channels, and oxidative stress in plants. According to second hypothesis, Ca^{2+} activated channels are important whereas the

observations have been reported using Ca^{2+} sensory proteins such as calmodulin in response to mechanical stress in *Arabidopsis*(Braam, 1992; Braam and Davis, 1990; Monshausen et al., 2009).

Chapter 3. Materials and method

This study consists of three main experiments. The aim of first experiment was to compare the oxidative stress of aquatic plants against the turbulence and mean flow. The second and third experiments evaluated the metabolism and ultra-structural variations in aquatic plants responding to turbulence and mean flow respectively (Figure 3).

3.1. Experiment-I

This research consists of two sub experiments followed by a field investigation. The aim of the first sub experiment was to select the suitable nutrition medium to be used in the subsequent main experiments. The second experiment was designed to compare the stress response of plants after being exposed to turbulence and main flow. Finally, a field investigation was conducted along Moto-Arakawa River (360 7′ 30.1″N, 1390 24′ 20″ E), which is a tributary of the Arakawa River flowing in southern Saitama, Japan in order to compare the experimental results with the field condition.

3.1.1. **Sub Experiment 1**

Hoagland nutrient solution (HNS) was selected as the culture medium to be used in the experimental cultures. HNS was prepared using pure analytical grade chemicals (Wako, Japan) according to Hoagland and Amon. (1938). There were six treatments (T1-T6) according to the percentage of HNS in the culture medium. T1 to T6 contained 0 (control), 5, 10, 20, 50 and 100 percent HNS respectively, while T1 contained only distilled water. Each treatment with two replicates (n=2) were randomly allocated into 12 (6x2) plastic beakers (2L) in a complete randomized design (CRD).

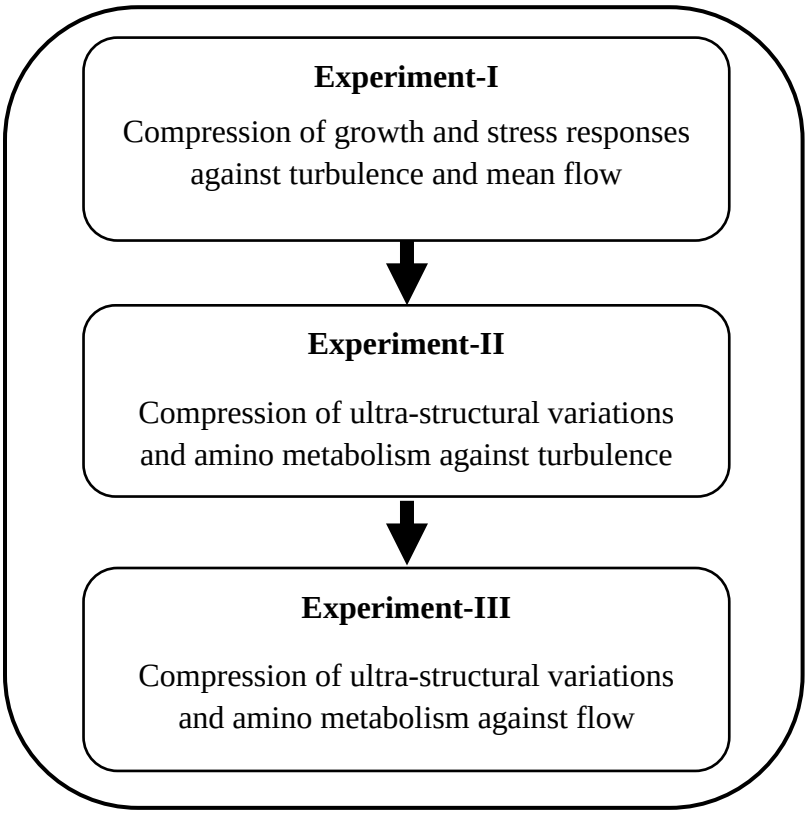

Figure 3. Flow diagram of the research plan

Approximately 500g of commercial river sand (90% < 1mm) was used as the substrate. Commercial sand was purchased from the local market (DIY, Doite, Japan) and washed several times using tap water and soaked in distilled water for 24 hours before being used. Experimental plants were obtained from a laboratory-maintained culture tank. Six similar size apical tips (initial length (IL) ~5cm) of *E. nuttallii* were planted in each beaker. Light intensity was maintained at ~100 µmol$m^{-2}S^{-1}$ using florescence lamps with a photoperiod of 12 h light and 12 h dark, and the experiment lasted for 30 days. At the end of the experiment, the final shoot length (FL), shoot elongation rate (SER); [SER=(FL-IL)/time)], the biomass and stress response were compared. Plant stress was tested by chlorophyll fluorescence and by measuring the concentration of H_2O_2 and peroxidase activity. The procedure for plant extraction, determination of H_2O_2 concentration and GPX activity are described in the Sub Experiment 2.

Chlorophyll fluorescence was determined using a chlorophyll fluorescence imaging technique (FC 1000-H; Photon Systems Instruments, Czech Republic) that involves auto-image segmentation. The cuttings of *E. nuttallii* were dark-adapted for 20 min and maximum quantum efficiency of PSII photochemistry (Fv/Fm) was calculated by using the following equation(DeEll and Toivonen, 2003).

$$\frac{Fv}{Fm} = \frac{Fm - F0}{Fm}$$

Where Fv, Fm, and Fo are the variable, maximum, and minimum fluorescence in dark-adapted states respectively.

3.1.2. **Sub Experiment 2**

There were four treatments (TR1-TR4) each with three replicates (n=3) as follows.

a) TR1: Plants were exposed to main flow (without turbulence) using a specially designed re-circulating system.
b) TR2: Controlled treatment for the TR1 in which plants were exposed to stagnant conditions using the same experimental setup as used in TR1.
c) TR3: Plants were exposed to turbulence (without main flow) using an oscillatory grid attached to a microcosm.
d) TR4: Control treatment of TR3 where plants were grown under stagnant water using the same microcosm as in TR3.

The turbulence velocity was decided after considering the velocity fluctuations in macrophyte stands in the natural environment, while the main flow velocity was designed to fall within the same magnitude of turbulence employed in TR1. All treatments were subjected to a photoperiod of 12 h dark and 12 h light regime. Light intensity was kept at 100-110 $\mu molm^{-2}s^{-1}$ and the temperature was maintained at 23-24 ^{0}C. On the basis of Experiment 1, 5% HNM was used as the nutrient medium for culturing plants. Over the experimental period, pH ranged from 7.7 to 8.2.

3.1.2.1. Experimental setup of TR1 (Flowing treatment)

Figure 4 shows the experimental setup employed to create a flowing condition in TR1. The setup consists of a transparent opaque pipe (length: 40 cm and diameter: 5 cm) attached to a small PVC pot (C), which contained substrate (commercial sand) for culturing plants. Diffusers were constructed using straws (McDonald-type) and placed near to the inlet and outlet of the transparent pipe to minimize turbulence (Binzer et al., 2005). The whole setup was fixed in a big glass tank (150×50×50 cm^3) and connected to the overhead tank (50L). Water circulation was maintained through a submersible water pump (S) and flow velocity was adjusted by using a valve (T) fixed at the outlet of the transparent tube. The water level inside the tank was (45 cm) kept above the transparent tubes. All replicates of TR1 and TR2 (six tubes) were fixed in a CRD in the same glass tank. Tubes of control treatment (TR2) were not connected to the overhead tank in to keep a stagnant condition.

Water flow velocity inside the transparent tube was measured at every 7.5 cm from the PVC pot (C) using a two-dimensional electromagnetic current meter (EMCM) (SF-5712, Tokyo-Keiosoku Corporation, Tokyo, Japan). For each point, velocity fluctuations were recorded at every 0.5 cm depth from the surface of the pipe for two minutes. The voltage signal was converted to velocity using the calibration graph after extracting data with GL200-820-APS software, Version 1.01 (Graphtec Corporation, Yokohama, Japan). The average main velocity (mean square root) and the turbulence velocity were calculated. Reynolds number at each depth was also calculated using the following equation.

$$Re = \frac{\rho UD}{\mu}$$

Where Re: Reynolds number (unit less), ρ: density (gm^{-3}), U: flow velocity (cms^{-1}), D: pipe diameter (cm), μ: dynamic viscosity ($gm^{-1}s^{-1}$).

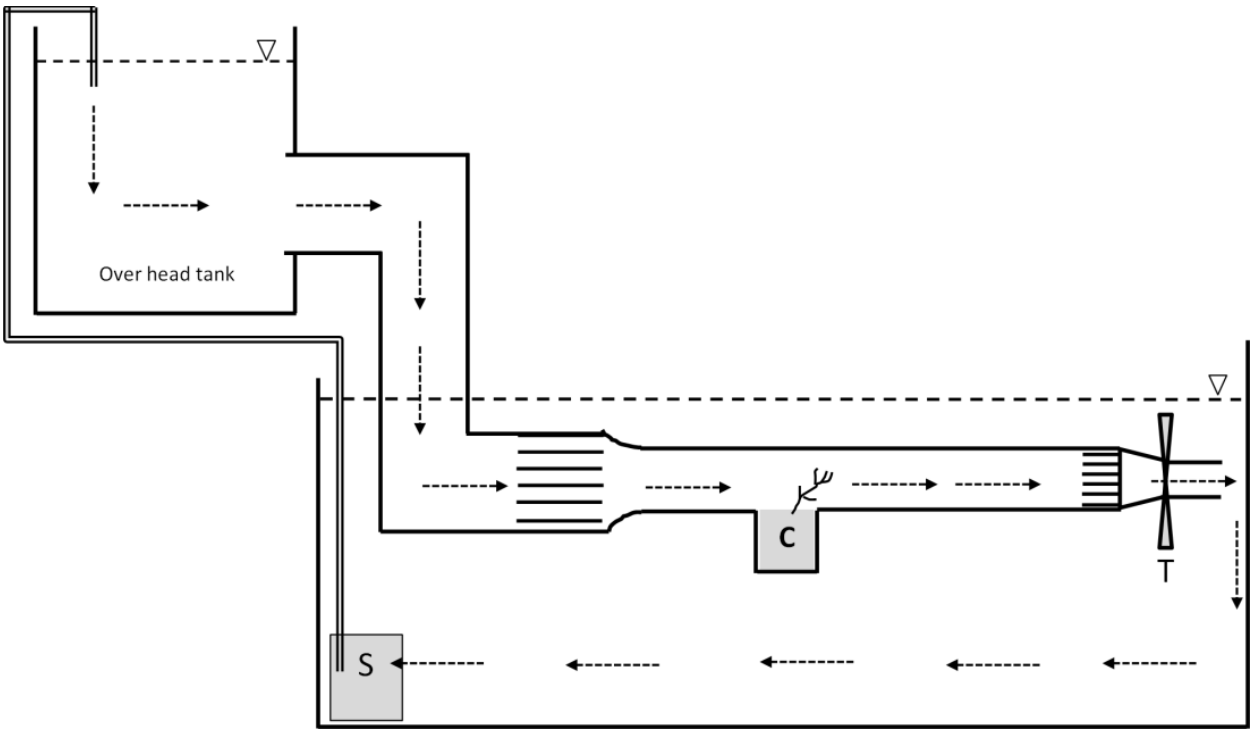

Figure 4.Diagrammatic representation of the experimental set up used in TR1 (arrow indicate water flow).

3.1.2.2. Experimental setup of TR2 (Turbulence treatment)

This treatment was conducted using a glass microcosm (15.7×15.7×24.5 cm^3) that contained a layer (~ 4 cm) of commercial sand as the substrate. The water level was maintained at 17 cm above to the substrate using 5% HNS. The turbulence condition inside the microcosm was generated using a vertically oscillating horizontal grid (oscillating frequency was 2 Hz) according to published literature (Champika Ellawala et al., 2011; Ellawala et al., 2011; Ellawala et al., 2012; Ellawala et al., 2013). The employed microcosm was relatively small for generating turbulence, but working under that particular scale was necessary in order to work with the DC motors under these laboratory conditions. The horizontal velocity profile of the microcosm was measured using the same EMCM at six different points, which were symmetrically distributed over the area. For each point, velocity fluctuations were monitored at three depths (5, 10 and 15

cm from the water surface) and the turbulence velocity was calculated as described previously for each depth by averaging all of six measurements. Each treatment with three replicate microcosms was randomly allocated in a CRD.

3.1.3. **Experimental plant and growing conditions**

E. nuttallii, one of the most common invasive submerged plants in lakes, ponds, rivers, and channels in Japan (Kadono, 1994; Kadono, 2004) was used as the experimental plant for the present study. Plant classification is given as follows.

Plantae
Tracheophyta
Liliopsida
Alismatales
Hydrocharitaceae
Elodea
Elodea nuttallii (Planch.) H. St. John

Figure 5. Nature of *Elodea nuttallii*

E. nuttallii plants were collected from Moto Arakawa River, Japan, and plants were grown in aquaria under laboratory conditions. After growing, six similar sized apical tips (3-5 cm) were planted in PVC pots (for TR1 and TR2) and in microcosms (for TR3 and TR4). Before starting the experiment, plants were acclimatized to experimental conditions for one week. After being acclimatized, plants were exposed either to main flow (TR1), turbulence (TR3), or control (TR2 and TR4) for 21 days. The experiment was stopped after 21 days as the apical tips of some plants in TR3 were close to touching the oscillating grid.

3.1.4. **Growth measurements and sampling**

At the end of the experiment, plant growth was compared by measuring the shoot length and stem diameter. The shoot length was measured individually with the aid of a ruler, and all shoots were clustered, and the longest shoot was measured when more than two shoots were found. Stem cross-sections were prepared for the terminal stem (~5 cm bellow to the apical tip) and basal stem (~5 cm above to the substrate) using a sharp razor blade. Stem diameter was measured using a microscope supported with the FLOVEL Image Filing System FlvFs (FLOVEL Inc., Tachikawa, Japan). For each replicate, six to eight clear transverse sections (TS) were measured for the stem diameter, and the average of three replicates was taken for the comparisons. From each TS, the maximum diameter of all cortex cells were measured (n=150-300), and the size class distributions were plotted. Some plants were oven dried to determine their cellulose and lignin.

3.1.5. **Pigment extraction, chlorophyll fluorescence and plant stress**

Plant shoots (~100 mg) were extracted to N-N dimethylformamide for 24 hours in darkness for pigment analysis. Total chlorophyll and carotenoids were analyzed spectrophotometrically (Shimadtzu, UV mini 1210) and quantified using coefficients published by Wellburn (1994). Chlorophyll fluorescence was measured as described previously. Plant stress responses were assayed by measuring the activity of CAT, APX, indole acetic acid oxidase (IAAO) and the concentrations of H_2O_2 and endogenous IAA.

3.1.6. **Sample preparation and stress assays**

For CAT, APX, GPX, H_2O_2 and IAAO assays, fresh plant shoots were extracted (~500 mg) to an ice cold phosphate buffer (50 mM, pH=6.0) which contained polyvinylpyrrolidone (PVP). IAA content in apical tips of *E.nuttallii* (~1g) was assayed after extracting to distilled water. After extracting, all extractions were centrifuged at 5000g for 20 minutes at 4^0C. The supernatant was separated and stored in -80^0C until analyzing. For every assay, each extract was analyzed in duplicate and the results are presented on a fresh weight (FW) basis.

3.1.7. **Enzyme assays**

3.1.7.1. CAT activity

CAT activity (EC 1.11.1.6) was assayed following Aebi (1984). Briefly, the reaction mixture contained 100 µL of 10 mM H_2O_2, 2.00 mL of 100 mM potassium phosphate buffer (pH=7.0) and 500 µL of enzyme extract. The decrease in absorbance at 240 nm was recorded for 0.5 min. CAT activity is given by the absorbance change (ΔOD) as ΔOD/min/ gFW.

3.1.7.2. APX activity

APX (EC 1.11.1.11) activity was determined according to Nakano and Asada (1981). The reaction mixture contained 100 µL of enzyme extract, 200 µL of 0.5 mM ascorbic acid in 50 mM potassium phosphate buffer (pH=7.0) and 2.00 mL of 50 mM potassium phosphate buffer (pH=7.0). The reaction was started after adding 60 µL of 1 mM H_2O_2. Decrease in the absorbance was recorded at 290 nm for every 10s. APX activity was calculated using the extinction coefficient of 2.8 mM/L^{-1} cm^{-1} and the activity is presented as the rate of H_2O_2 destruction (nM/min/gFW).

3.1.7.3. GPX activity

GPX activity was measured based on the guaicol oxidation according to MacAdam et al. (1992). The reaction mixture contains 3.0 mL of 50 mM potassium phosphate buffer (pH=6), 40 µL of 30 mM H_2O_2 and 50 µL of 0.2M guaicoal. The reaction was initiated by adding 100 µL of enzyme extract and the absorbance was measured immediately at every 15 seconds for three minutes. The rate of absorbance change (ΔOD) was calculated, and the GPX activity is expressed as ΔOD/min/ gFW.

3.1.7.4. Hydrogen peroxide content

The concentration of hydrogen peroxide was determined according to Jana and Choudhuri (1982). Briefly, 750 µL of enzyme extract was mixed with 2.5 mL of 0.1% titanium sulphate in 20% H_2SO_4 (v/v). The mixture was centrifuged at 5000×g for 15 minutes at room temperature, and the intensity of resulting yellow color was measured at 410 nm. H_2O_2 concentration was

estimated using the standard curve prepared with the known concentrations of H_2O_2. The concentrations of H_2O_2 are presented as µ mol/g FW.

3.1.7.5. IAA assay

The IAA concentration was also measured spectophotometrically by mixing an aliquot of enzyme extract (1.00 mL) with 2.00 mL of modified Salowski reagent (1.00 mL of 0.5M $FeCl_3$ in 50 mL of 35% perchloric acid) (Gordon and Weber, 1951). The intensity of resulting pink color was measured after one hour at 530 nm. The IAA concentration was calculated from the standard curve generated with the known concentrations of IAA, and the results are presented as µg/ gFW.

3.1.7.6. IAAO activity

IAAO activity was determined according to Beffa et al. (1990). Reaction mixture (1mL) contained 50mM phosphate buffer (pH=6.0), 5 mM $MnCl_2$, 5 mM 2-4 dichlorophynol, 14.2 mM IAA and 200 µL mL of enzyme extract. Assay tubes were incubated at 25^0C for one hour with shaking. The remaining IAA was determined spectrophotometrically as described previously and the IAAO activity was expressed as µmoles IAA destruction/min/g FW.

3.1.7.7. Cellulose and lignin

Cellulose content in plant shoots was measured according to Updegraff (1969) while lignin was assayed following the improved acetyl bromide procedure (Iiyama and Wallis, 1988; Morrison et al., 1995). For each treatment, replicates were pooled together and analyzed in triplicate due to lack of enough dry weight to analyze individually.

3.1.8. **Field investigations**

Field studies were also conducted along Moto-Arakawa River (360 7′ 30.1″N, 1390 24′ 20″ E), flowing in southern Saitama, central Japan. The river water originates from pumped-up groundwater and the catchment area was mostly urbanized with only about 1.4km^2 watershed areas, thereby the flow rate was relatively constant except during storms (Asaeda et al., 2010). Water temperature was relatively constant throughout the year as pumped-up groundwater was the original source, and the monthly averages ranged from 13.5 -14.0^0 C in November -April period

3.1.8.1. Field study-1

Two locations (L1 and L2) were selected along a stretch of the tributary on the basis of two factors, i.e., presence of *Elodea nuttallii* and the turbulence condition. For each location, two randomly selected quadrates (50×50 cm^2) were marked using poles and ropes. Surface velocity fluctuations inside the plant stands (3-4 cm below the surface) were recorded for five minutes using the EMCM to calculate the turbulence velocity. After recording velocity fluctuations, plants inside the quadrates were carefully removed to prepare plant extracts for the subsequent stress assays. All the extractions were conducted on an ice bath using a chilled mortar and pestle as in the field. After being extracted, all samples were immediately transported to the laboratory under freezing conditions (the extraction procedure was described previously).

3.1.8.2. Field study -II

Myriophyllum spicatum was the dominant macrophytes in the study site and therefore second field study was conducted to evaluate the stress responses of *M. spicatum* to turbulence. On the basis of turbulence and the abundance of macrophytes, observation was conducted at three upstream transects (L1-L3) and at three downstream transects (L4-L6) along a ~1 km stretch of the stream (Figure 6). Flow patterns were studied inside and outside plant stands at each transect using the same EMCM (SF-5712, Tokyo-Keiosoku Corporation, Tokyo, Japan).

Figure 6. Map of the study sites (L1-L6 indicates the sampling points)

For each transect two randomly selected quadrates (1×1 m^2) were marked using poles and ropes. Surface velocity fluctuations inside plant stands (3-4 cm below surface) were recorded for five minutes using the EMCM and the turbulence velocity was calculated. Similarly, velocity fluctuations were monitored outside of plant stands. The minimum turbulence was observed at L1 and it was considered as the control transect. After recording velocity fluctuations, all plants inside quadrates were carefully removed. Just after sampling, plant extracts were prepared for hormone and enzyme analysis as described previously. Species were identified using an aquatic plant identification manual (Kadono, 1994). Above ground and below ground plant materials were separately oven dried (80°C) until get a constant weight and the dry weight of roots and shoots were measured. Dried samples were ground using a Wiley mill and stored in sealed plastic bags for further analyses.

Depth measurements were taken in a cross section of each location at every 0.25 m distance using a depth gauge. Dissolved oxygen, pH and temperature were measured using a water quality monitor (Horiba, Kyoto, Japan) while two water samples were taken from each transect for further analysis. Water samples were transported to laboratory under cooling condition and stored under freezing condition after being filtered.

Plant extracts were prepared separately for each plant species for stress response assays as described in previous sections (3.1.7).

3.2. Experiment-II

The aim of this step was to evaluate the effects of turbulence on ultra-structure and the metabolism of *E. nuttallii*. This study consisted of two sub experiments. Electron microscopy was performed in the first sub trial to study the ultra-structural variations in cells of *E. nuttallii* leaves and stems caused by turbulence. The second experiment was conducted in the same conditions and measured the amino acid and other organic metabolite contents in plants response to the turbulence with capillary electrophoresis–mass spectrometric analysis (CE-MS).

There were three treatments according to the level of turbulence, i.e., control (stagnant water), low turbulence and high turbulence. The three replicates of each treatment were randomly allocated to nine (3 x 3) glass microcosms (15.7 x 15.7 x 24.5 cm^3) in a completely randomized design for experiment-1, whereas experiment-2 was conducted using four replicates per treatment (n = 4).

The turbulence was generated using a vertically oscillating horizontal grid attached to DC motors, as previously reported (Champika Ellawala et al., 2011; Ellawala et al., 2011; Ellawala et al., 2012; Ellawala et al., 2013). Each microcosm consisted of a layer of commercial sand (~4 cm) and 5% Hoagland's nutrient solution as the culture medium, and the water level was maintained at 17 cm above the substrate. The pH of the microcosm ranged from 7.7 to 8.2, and the temperature was maintained at 23-24°C during the experiment. All treatments were subjected to a photoperiod of 12 h dark and 12 h light. The light intensity was maintained at 100-110 µmol m^{-2} s^{-1} by white fluorescent lamps. The velocity of the turbulence was chosen based on the velocity fluctuations in macrophyte stands in the natural environment. The velocity profile of the microcosm was measured using a two-dimensional electromagnetic current meter (SF-5712; Tokyo-Keisoku Corporation, Tokyo, Japan) at seven randomly selected points, which were symmetrically distributed over the area of the microcosm. At each point, the velocity fluctuations were recorded at 5, 10 and 15 cm depths for 120 s (Table 2). The velocity readings were transferred to the data logger (GL200A; Graphtec Corporation, Yokohama, Japan) and were analyzed with GL200-820-APS software (Version 1.01). The 1200 x and y velocity readings that were recorded at each point were used to calculate the turbulence velocity (mean root velocity) (Green, 2005).

Table 2. Fluctuations in turbulence velocity in the microcosms (mean ± SD, n=3)

Depth (cm)	Turbulence velocity (cm s^{-1})		
	Control	Low	High
5	nd	2.5 ± 0.4	5.7 ± 1.7
10	nd	1.5 ± 0.5	3.1 ± 0.7
15	nd	1.6 ± 0.4	2.6 ± 0.1

(nd: not detected)

Six similar sized apical tips of *E. nuttallii*(Initial length (IL) ~3-5 cm) were clipped from the stock tank and planted in the microcosms. These plants were acclimated to the experimental conditions for one week. After acclimation, the plants were exposed either to stagnant (control), low turbulence or high turbulence conditions for 30 days. Both experiments (1 and 2) were completed after 30 days because the apical tips of some of the plants in the low turbulence treatments were close to contact with the oscillating grid. At the end of the experiments, the anatomical variations, final shoot lengths (FL), shoot elongation rates [SER; SER= (FL-IL)/time] and the concentrations of major metabolites were compared among treatments.

3.2.1. **Microscopic study**

The effects of turbulence on apical leaves and basal stems were observed in the previous study (Experiment-I). Therefore, in this study, the cells of the apical leaves and basal stems (~5 cm above the substrate) were analyzed with microscopy. A conventional electron microscopic study (Seki et al., 2014) was conducted to study the ultra-structural changes caused by turbulence. Briefly, the leaves and stems were fixed in 2% glutaraldehyde in 0.05 M potassium phosphate buffer (pH 7.0) for 2 h at room temperature followed by overnight fixation in a refrigerator. After a rinse with buffer, the plant materials were postfixed in 2% OsO4 in 0.05 M potassium phosphate buffer (pH 7.0) for 2 h at room temperature. Then, the plant materials were dehydrated using an acetone series and were embedded in Spurr's resin. Ultra-thin sections were prepared for observation under the electron microscope (Hitachi, H-7500, Tokyo, Japan), following the method of Seki et al. (2014). The electron microscope images were enlarged and the thicknesses of the epidermal cell walls were measured with a ruler. The maximum length and width of starch

granules were measured, and the values were used for comparisons. For each epidermal cell, at a few randomly selected points (n= 4-10), the cell wall thickness was measured. Stem transverse sections (TS) were photographed under a light microscope with a Nikon DXM 1200C digital camera at the highest resolution (4116 x 3072 pixels). The images were analyzed to quantify the number of cells with starch granules among the stem cortex cells, and 30 cells were randomly selected in each stem TS to determine the number of starch granules per cell.

3.2.2. **Metabolome analysis with CE-MS**

The metabolites were analyzed with a capillary electrophoresis-mass spectroscopy (CE-MS) system (Agilent Technologies, Waldbronn, Germany), following the procedure in the literature (Miyagi et al., 2010; Miyagi et al., 2013). In brief, plant materials (shoots) were homogenized with liquid nitrogen. Approximately 50-100 mg of plant sample was put into a microtube, and 200 µL of methanol was added to inactivate enzymes. Then, 200 µL of MilliQ water with 100 µM 1, 4-piperazine diethane sulfonic acid (PIPES) and 100 µM methionine sulfone as internal standards was added. The mixture was vortexed for 5 min, followed by centrifugation at 15,000 rpm at 4°C for 5 min. After centrifugation, the supernatant was transferred to a 3 kDa cutoff filter (Millipore, Billerica, MA, USA) and centrifuged at 12,000 rpm for 30 min. The filtrate was stored at -80°C until analysis. The CE-MS analysis was followed according to the method of Miyagi et al. (2010) and the concentrations of metabolites are presented as µmol/g FW.

3.2.3. **Determination of starch content**

Starch content was measured spectrophotometrically following the anthrone method(Hansen and Møller, 1975). Approximately 20 mg of dried plant sample was extracted into 80 % ethanol. The mixture was centrifuged and the supernatant was decanted. The residue was mixed with 1.1% HCl and the mixture was heated in a water bath at 100^0C for 30 min. The mixture was diluted to 10 mL using distilled water. An aliquot of 1 mL was put into a centrifuge tube and cooled rapidly to 0^0C using ice. After that, 4 mL of anthrone reagent (1g of anthrone in 500 mL of 72% H_2SO_4) was added and the mixture was heated for 11 minutes in a water bath. The intensity of resulting blue colour was read at 630nm. Starch content was quantified using the standard calibration curve prepared using known concentrations of starch (Wako chemicals, Japan). For

each treatment, replicates were pooled together and analyzed in triplicate (n=3) as those samples did not include enough dry weight to analyze individually.

3.3. Experiment -III

The experiment-III was designed to study the effect of mean flow on aquatic plant function and cell ultra-structure. There were two treatments as plants exposed to flow (~10 cms^{-1}) and plants exposed to stagnant waters (control). The same experimental setup employed in the flowing treatment in the experiment 1 was used to create flowing condition with comparatively elevated magnitude of mean flow.

The experiment was conducted for 30 days. At the end of the experiment, plant growth, stress responses, metabolism and anatomical variations were compared. Plant growth was compared by measuring the shoot length and shoot elongation rate (SER). Stress responses, metabolome analysis and microscopic observations (both light and electron) were also done as explained in the previous steps.

3.4. Statistical Analysis

The data were tested for normality using the Shapiro-Wilk test before statistical analyses. The results are presented as the mean ± SD (n=3 or 4). The data were subjected to one-way analysis of variance (one-way ANOVA) followed by Duncan's multiple range tests to evaluate the differences among means at a significance level of 0.05. The numbers of starch granules in the cortex cells were compared with Chi-square tests. Student t-test was used in the experiment-II to compare the flowing and control treatments. The graphics were prepared with Microsoft Excel (2010), and the statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS) v16.0.

Chapter 4. Results

4.1. Experiment-I

4.1.1. **Sub experiment-1**

The observed trend in plant growth and stress response in *E. nuttallii* after growing at different nutrient conditions are presented in Table 3. In terms of biomass, low concentrations of HNM (5-20%) provide significantly better growth compared to the control and high nutrition concentrations (50% and 100%). On the other hand, SER closely agrees with the former observation. Similar to growth response, plants grown in low nutrient media contained significantly low concentrations of H_2O_2 and GPX activity. On the other hand, the average maximum quantum efficiency of photo-system II photochemistry (Fv/Fm) of 0, 5, 10, 20, 50 and 100% treatments were 0.76±0.00, 0.79±0.01, 0.79 ±0.01, 0.76 ± 0.01, 0.76±0.02 and 0.074±0.01 respectively. The highest Fv/Fm value which is nearly 0.80 was observed for plants grown in 5% and 10% nutrition media compared to control and other treatments. On the basis of this experiment, 5% HNS was selected as culture medium for the subsequent main experiment.

Table 3.Growth and stress responses of *E. nuttallii* grown in different nutrient concentrations (values are mean ±SD, n=2)

Response	Treatments (% of HNM)						Test statistics	
	0	5	10	20	50	100	F	*p*
Biomass (g FW/plant)	30.8 ± 3.7 ^{b}	42.6 ± 2.3^{a}	41. 5 ± 3.0 ^{a}	41.38 ± 3.1 ^{a}	24.5 ± 6.7^{b}	25.1 ± 1.8 ^{b}	9.97	*p*<0.05
Shoot length (cm)	8.6 ± 0.2^{a}	10.3 ± 0 .4^{a}	8.9 ± 0.3 ^{a}	8.6 ± 1.6 ^{a}	5.6 ± 0.7 ^{b}	5.7 ± 0.3 ^{b}	11.3 3	*p*<0.05
SER (mm/day)	2.6 ± 0.6^{abc}	3.9 ± 0.1^{a}	3.3 ± 0.1^{a}	3.1 ± 0.7^{ab}	1.8 ± 0.7^{c}	1.9 ± 0.2 ^{c}	5.30	*p*<0.05
H_2O_2 concentration(µ mol/ g FW)	1.69± 0.77^{c}	1.99 ± 0.33^{c}	2.73 ± 0.33^{c}	3.37± 0.30^{bc}	4.84± 0.30^{ab}	6.33± 0.01^{a}	9.98	*p*<0.05
Peroxidase activity(Δ OD/ min/ g FW)	2.54 ± 0.32^{b}	2.16 ± 0.19^{b}	3.22 ± 0.93^{ab}	2.77 ± 0.68^{a}	4.74 ± 0.30^{a}	4.81 ± 1.04^{a}	5.87	*p*<0.05

Different superscripts in the same raw indicate the significant different at 0.05 significant level.

4.1.2. **Sub Experiment 2**

4.1.2.1. Velocity profiles

Velocity profiles of the experimental units are presented in Figure 7Figure 7.Velocity profiles of experimental units (A: main velocity of TR1, B: turbulence velocity of TR1, C: Reynolds's numbers of TR1, D: turbulence velocity in microcosm used in TR3).. The maximum flow velocity (2.5-2.7 cms^{-1}) was observed in the middle region of the transparent tube (Figure 7. A), while the turbulence inside the pipe was comparatively very small (<0.22 cms^{-1}, Figure 7B).

Calculated Reynolds numbers were less than 2000 (Figure 7C) and therefore, flow in the tubes of TR1 could be considered as a laminar flow at all depths. The average turbulence velocity of the microcosm used in TR3 is presented in Figure 7D.

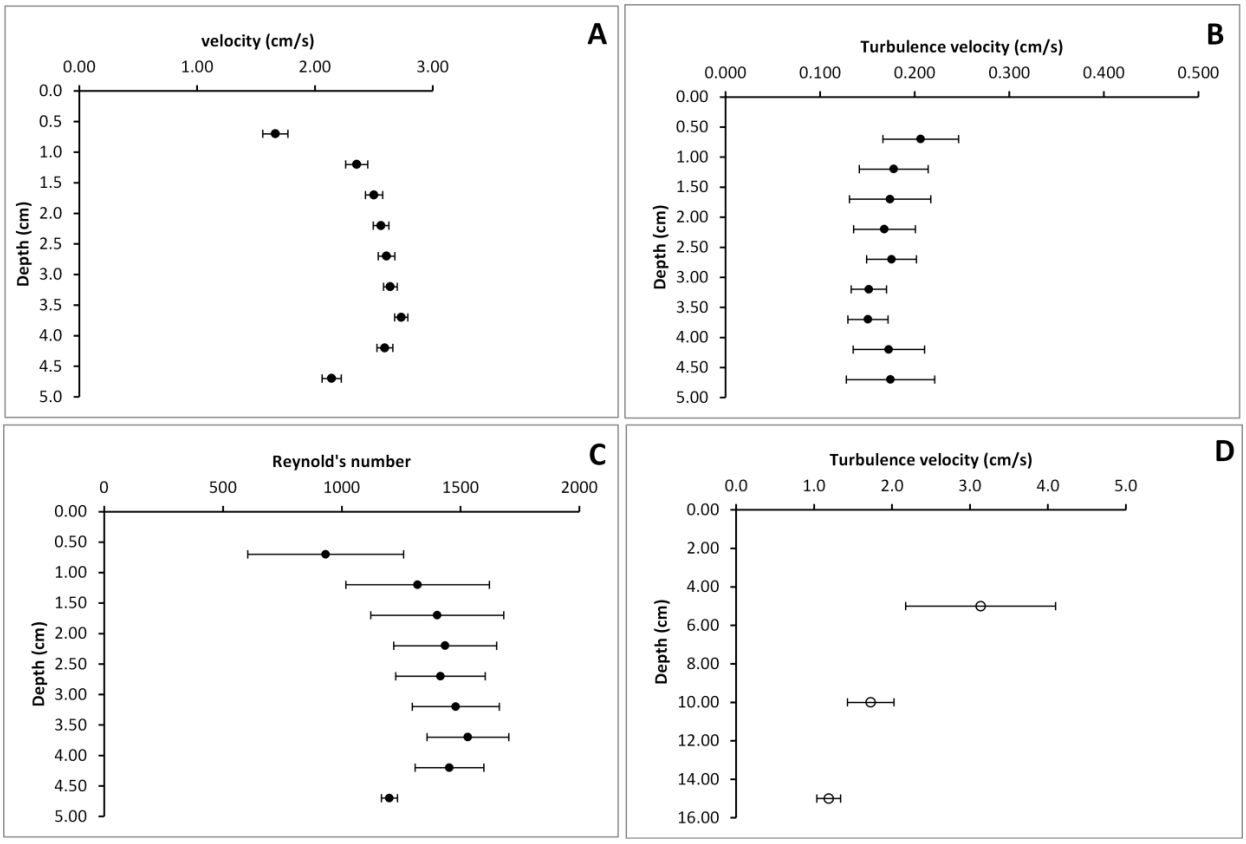

Figure 7.Velocity profiles of experimental units (A: main velocity of TR1, B: turbulence velocity of TR1, C: Reynolds's numbers of TR1, D: turbulence velocity in microcosm used in TR3).

4.1.2.2. Plant growth

Figure 8 compares the shoot length ratio (initial length: final length). Water movements significantly affected the shoot length ($F=9.68$, $p<0.05$) as plants exposed either to turbulence or main flow had a significantly shorter shoot length compared to the control treatments.

The final shoot length of control plants and stressed plants were approximately four times and three times longer than the initial length respectively. Variations in the stem diameter of *E. nuttallii* are presented in Figure 9.

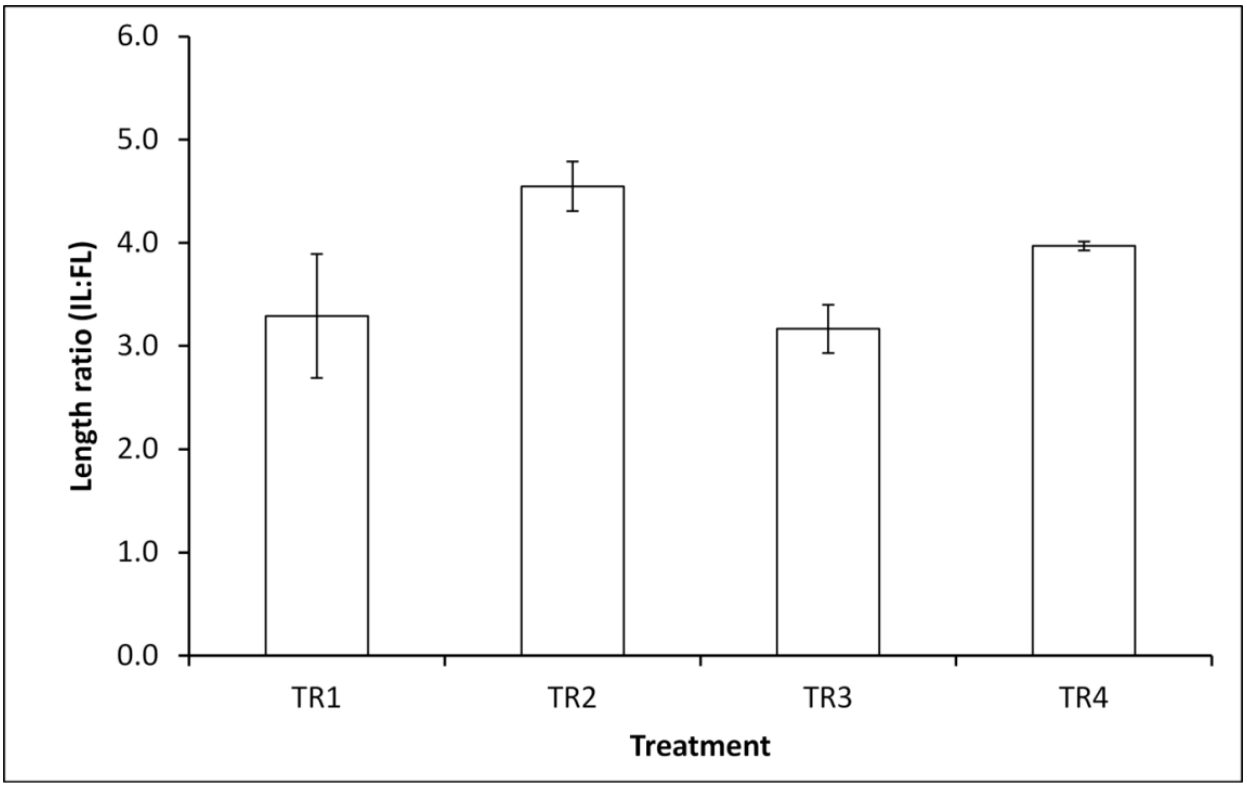

Figure 8.Final length to initial length ratio of *E.nuttallii* after being treated with turbulence and main flow

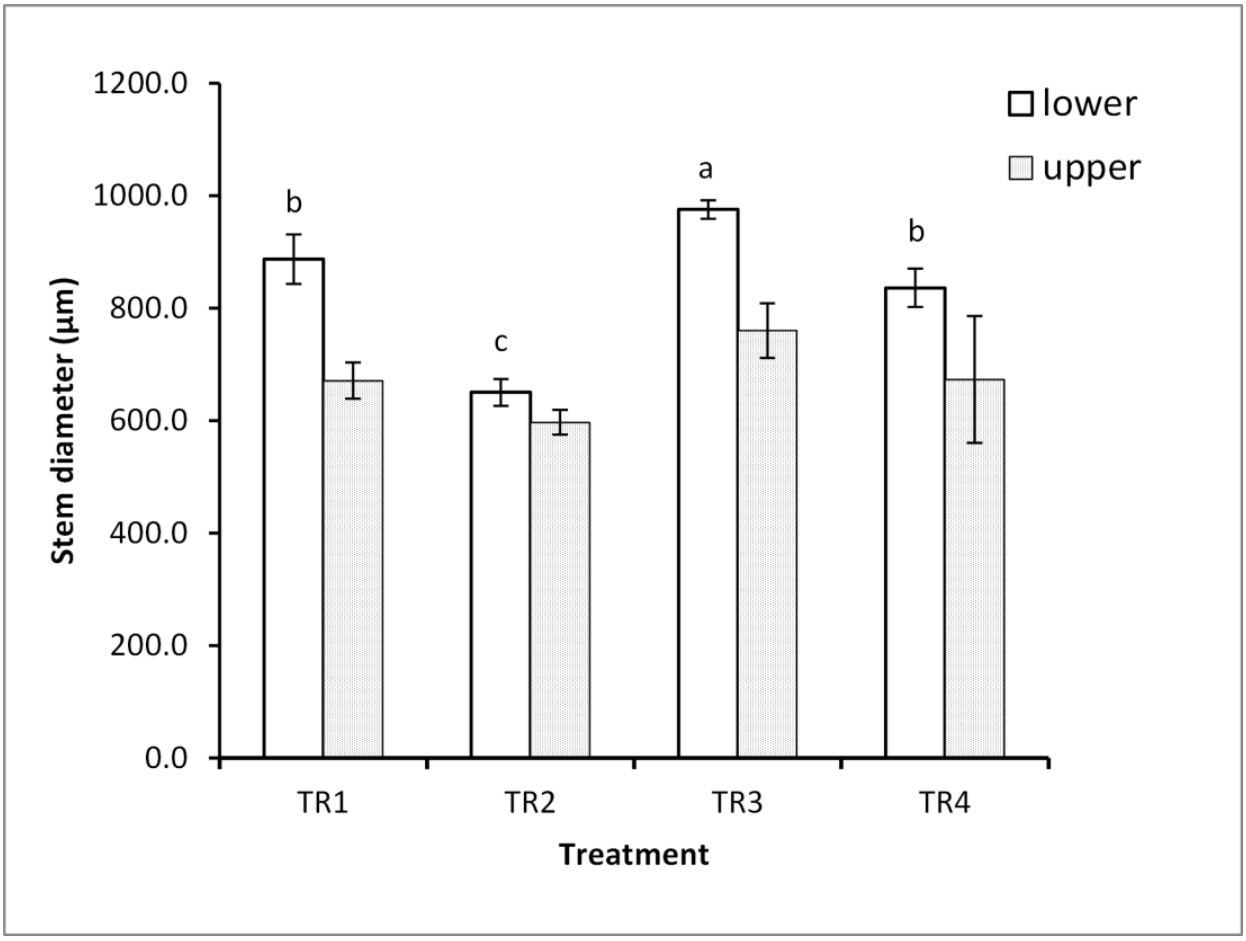

Figure 9.Variation in the stem diameter of *E.nuttalli*

Even though the diameter of the apical stem was statistically similar among treatments, the turbulence and main flow significantly increased the thickness of the basal stem ($F=43.68$, $p<0.001$). Plants consist of short and wider stems when exposed to water movements while plants grew in stagnant waters characterized with a thing-long shoot. However, wider stems observed in plants exposed to water movements (TR1 and TR3) consist of wider cortex cells compared to those in control plants. Fifty percent of cells in the cortex of plants exposed to main flow were less than 40 µm while the same percentage of cortex cells in control plants was less than 35 µm (Figure 10). A similar trend was observed for the TR3 and TR4 where 50% of the cortex cells in TR3 were less than 40 µm while it was less than 45 µm in those plants exposed to turbulence.

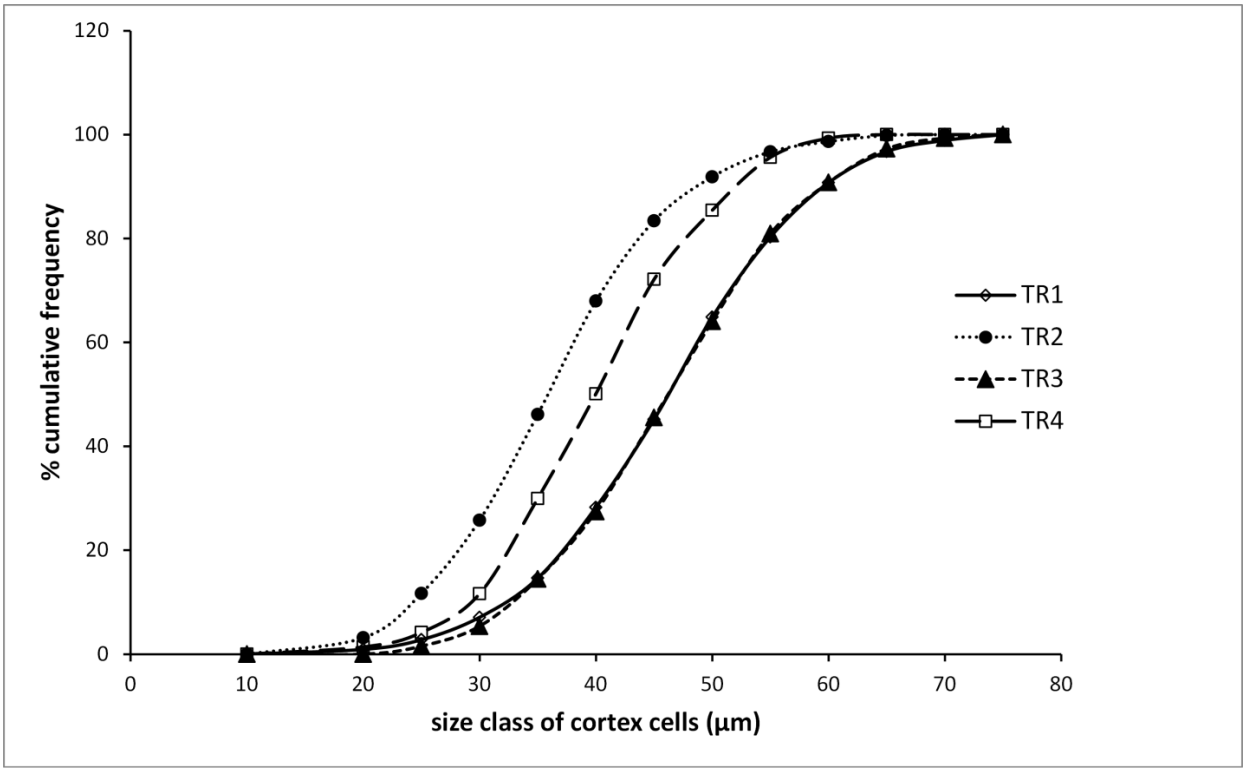

Figure 10.Size class distribution of stem cortex cells

4.1.2.3. Stress response

Results presented in Table 4 summarize the biochemical responses of *E. nuttallii* for the water movements. The highest concentration of H_2O_2 was observed in plants exposed to turbulence followed by main flow and control. However, control plants (TR 2 and TR4) contained similar concentrations of H_2O_2. Similar to H_2O_2, antioxidant enzyme activities of plants exposed to turbulence were significantly higher than that of control plants. CAT activity of TR2 and control were similar, but the activity of turbulence-stressed plants showed significantly higher CAT activity. Both main flow and turbulence induced the activity of APX and plants exposed to water movements showed elevated levels of APX activity compared to their respective control treatments. The highest APX activity was observed in plants exposed to turbulence followed by TR2, TR1 and TR3.

Table 4. Biochemical responses of *E. nuttalli* to turbulence and main flow

Responses	Treatment				$F_{(3,8)}$	*p*
	TR1	TR2	TR3	TR4		
H_2O_2 content (µ mol/g FW)	15.86±0.41[b]	12.36±0.72[c]	17.27±0.21[a]	12.83±0.55[c]	64.73	**
CAT activity (ΔOD/min/ g FW)	0.16±0.03[b]	0.15±0.05[b]	0.27±0.03[a]	0.10±0.02[b]	9.79	*
APX activity (H_2O_2 destruction nM/min/g FW)	48.70±0.72[b]	34.61±5.36[c]	65.86±4.78[a]	15.01±0.98[d]	69.55	**
IAA content (µg/ g FW)	80.04±2.88[a]	86.80±4.08[a]	67.51±1.19[c]	72.57±0.65[b]	54.77	**
IAAO activity (µmoles IAA destruction/min/g FW)	1.04±0.17[b]	0.87±0.20[b]	1.73±0.22[a]	1.00±0.08[b]	11.99	*
Cellulose (%)	23.47 ± 7.55[b]	21.8 ± 0.42[b]	50.03 ± 5.58[a]	14.35 ± 0.73[b]	16.87	*
Lignin (g/ kg DW)	93.77 ±1.57[b]	77.38 ± 5.5[bc]	121.95 ± 11.23[a]	69.91 ± 5.74[c]	21.11	*

(Different superscripts at the same raw indicate the significant difference at 0.05. * and **indicate the significant difference at 0.05 and 0.001 levels respectively).

IAA production in *E. nuttallii* was severely affected by the turbulence as the lowest IAA content was observed in plants exposed to former treatment (Table 4). However, main flow did not make negative impact to the IAA production in *E. nuttallii* as turbulence. Further, the observed trend in IAAO closely agrees with the former observation made for IAA.

It was clear that *E. nuttallii* strengthens the plant body against the water movements by accumulating cellulose and lignin. Specifically, plants exposed to turbulence (TR3) contained significantly high concentrations of cellulose where the cellulose content of the former plants were more than threefold higher than that of control plants (TR4) and twofold higher than plants exposed to main flow induced plants (Table 4). A similar trend was also observed for lignin, where the highest lignin content was observed in plants exposed to turbulence followed by main flow and control. However, lignin content in plants exposed to main flow was not significantly different from control plants.

4.1.2.4. Chlorophyll and chlorophyll fluorescence

Variations in the total chlorophyll and chlorophyll fluorescence are presented in the Figure 11. There was a significant effect of turbulence on the chlorophyll content ($F = 25.41$, $p<0.05$). However, the chlorophyll content of plants exposed to main flow was statistically similar to that of control plants. The lowest chlorophyll concentration was observed in TR3 where the plants were exposed to turbulence. On the other hand, former stress reduced the chlorophyll in turbulence-stressed plants approximately 40% compared to the control group (TR4).

Fv/Fm values closely agreed with the former observations as the lowest chlorophyll fluorescence was found in turbulence-induced plants ($F = 46.56$, $p<0.001$). However, the Fv/Fm ratio of plants grown in main flow and stagnant conditions were statistically similar and therefore, turbulence created severe stress on *E.nuttallii* compared to main flow. However, the average carotenoid contents of TR1-TR4 were 15.84±0.39, 10.07±0.08, 17.83±0.72, 12.82±6.88 µg/g FW respectively, and it was not significantly different among treatments ($F=1.93$, $p>0.05$).

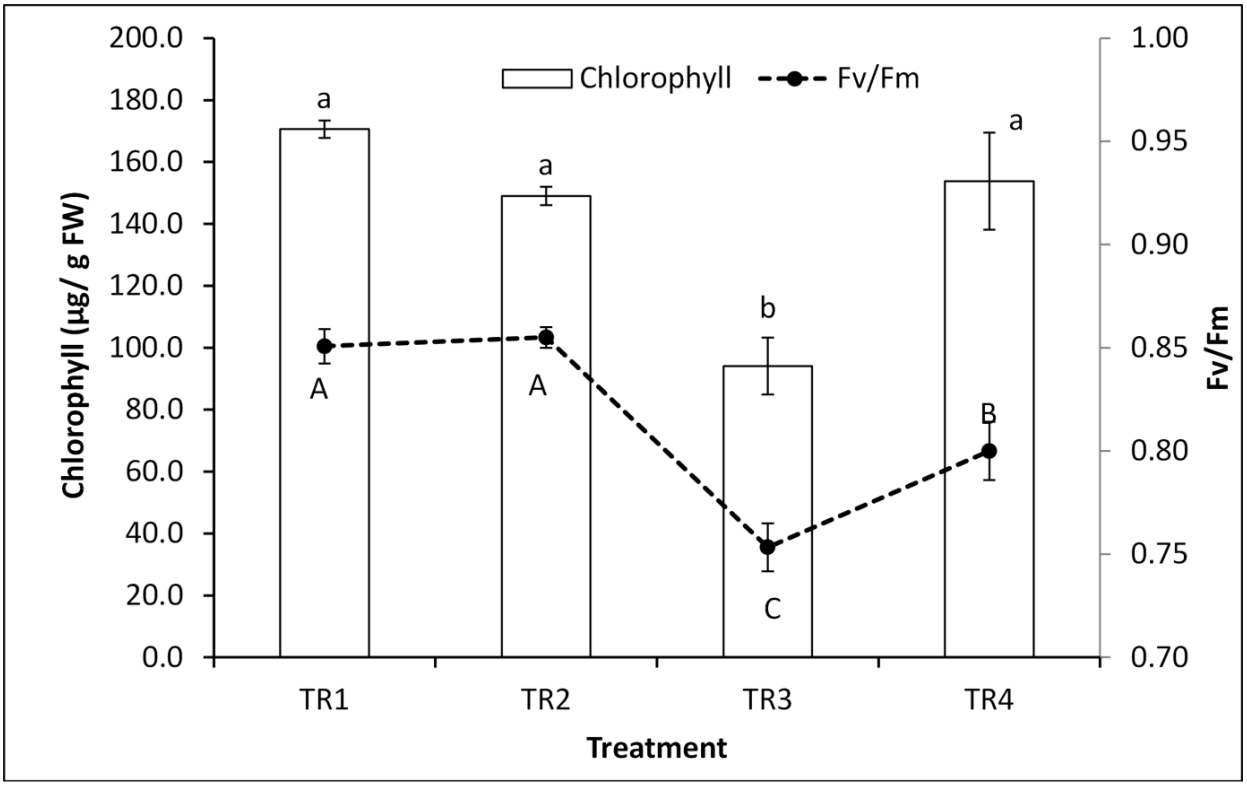

Figure 11.Total chlorophyll and chlorophyll fluorescence of *E. nuttallii*, different superscripts in the same category indicate the significant difference at 0.05 significant level (lowercase and upper case letters represents total chlorophyll and chlorophyll fluorescence respectively).

4.2. Field investigations

4.2.1. **Field study-1**

L1 is a pool-like environment where water flows smoothly while an elevated level of turbulence was observed in L6. The average turbulence velocity in L1 and L2 was 1.55 ±0.68 and 4.07 ± 0.00 cms^{-1} respectively where the L2 has a significantly high magnitude of turbulence compared to L1 ($t = 5.17$, $p<0.05$). Plants in L2 showed a significantly elevated level of stress response compared to plants grown in L1 (Figure 12).

CAT activity of plants exposed to elevated levels of turbulence was significantly higher than that of plants (Figure 12A) grown in a low turbulence environment ($t =11.89$, $p<0.05$). A similar trend was also observed for H_2O_2 ($t =60.56$, $p<0.05$) and APX ($t =10.09$, $p<0.05$). Even though the IAA contents of *E.nuttallii* (Figure 12C) were significantly different ($t = 4$, $p<0.05$), the observed trend in a former response was contradicting the experimental results.

Similar to experimental results, a significantly high cellulose content ($t = 15.68$, $p<0.05$) was found in plants exposed to high turbulence under field conditions (Figure 12E). Plant stress responses observed under experimental conditions and field conditions are within the same range, and the observed trends under an experimental condition were supported by the field investigations.

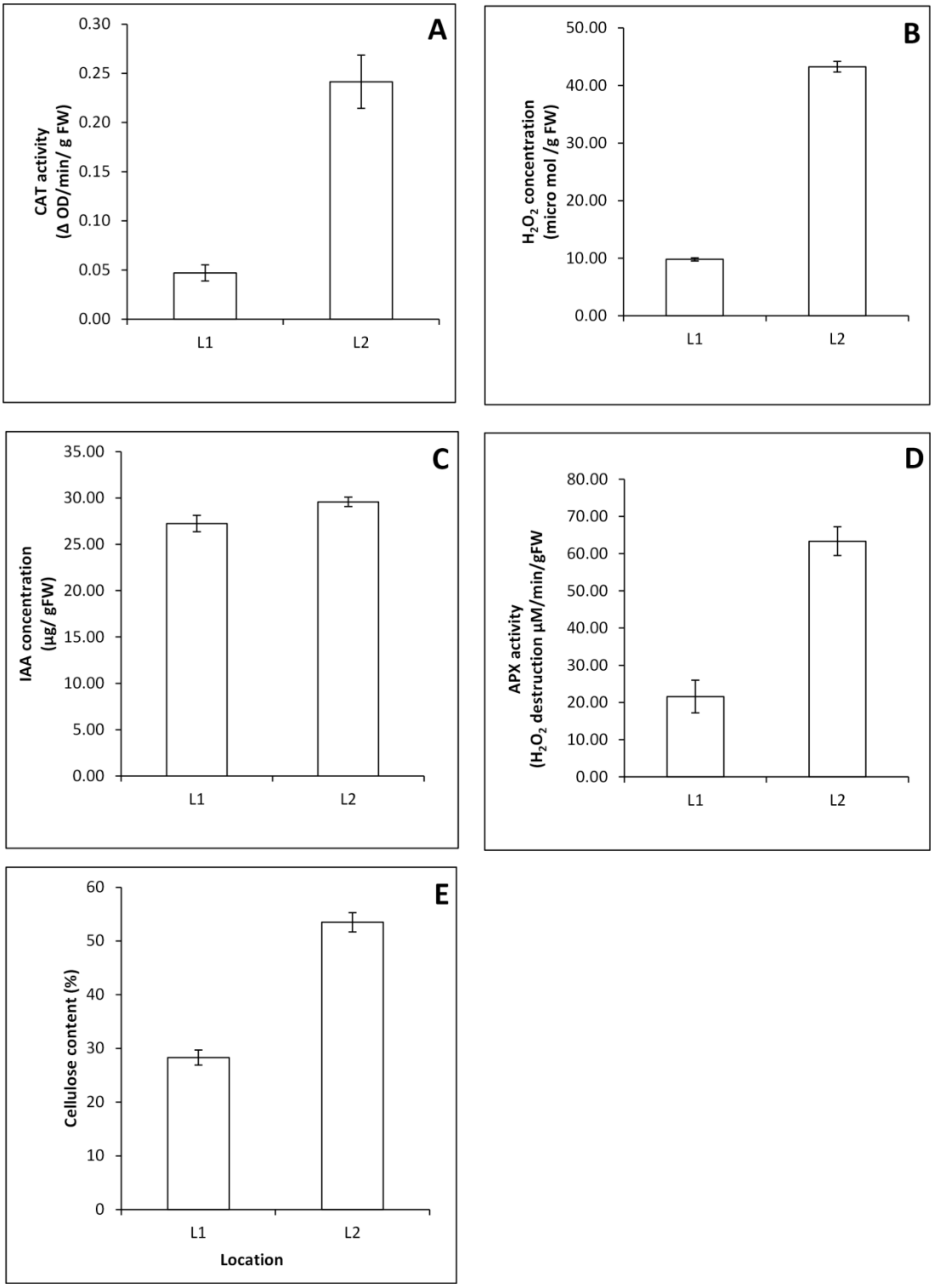

Figure 12.Stress response of *E.nuttallii* in natural environment (L1: low turbulence site, L2: high turbulence site, A: CAT activity, B: H_2O_2 concentration, C: IAA concentration, D: APX activity and E: cellulose)

4.2.2. **Field study-II**

4.2.2.1. Turbulence condition of study sites

The observed turbulence conditions are presented in the Figure 13 where the lowest turbulence was found in the control transects (L1). Compared to control transect, water flow remained comparatively high turbulent in other locations.

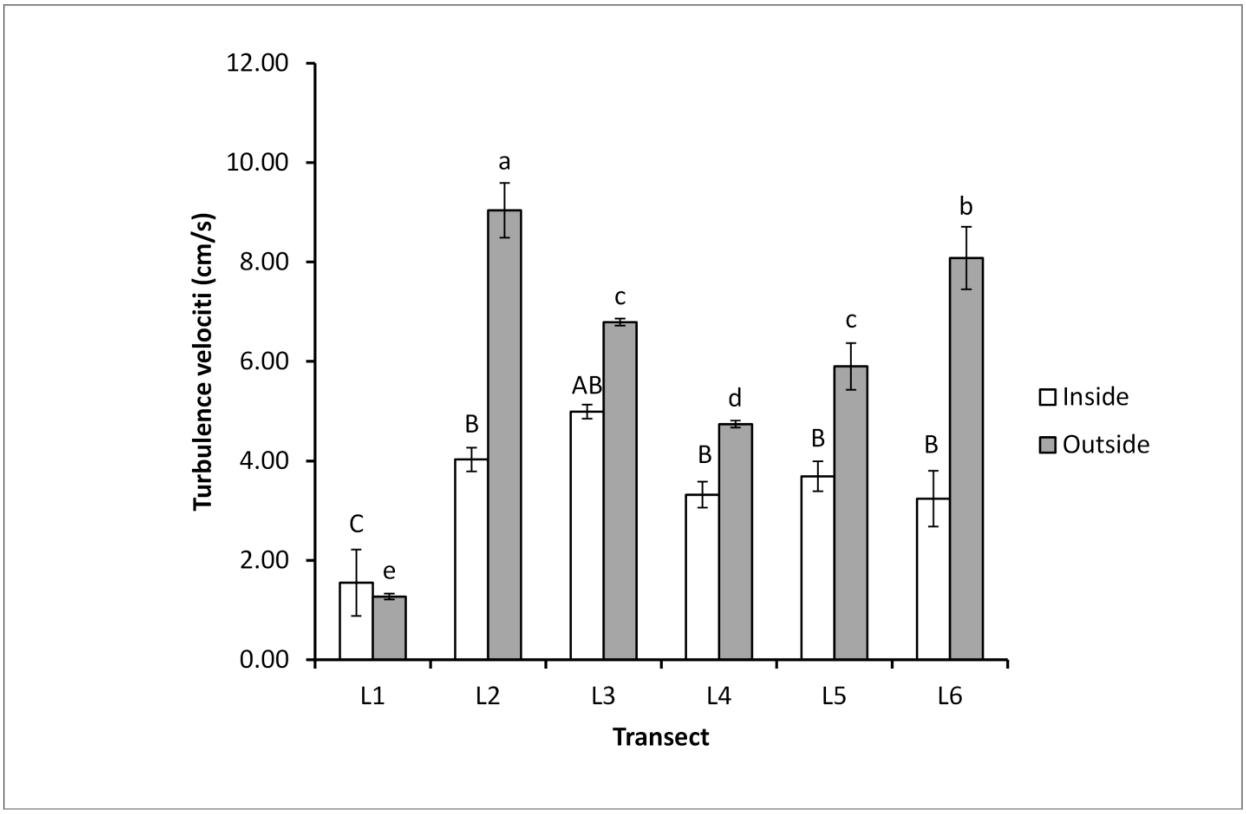

Figure 13.Turbulence velocity of study sites (different lower case letters within group indicate significantly different values, p<0.05).

There was a significant difference in inside turbulence velocity among study sites ($p < 0.05$) where the highest and the lowest inside turbulence were observed in L3 and L1 respectively. Even though outside and inside turbulence were similar in control transect (L1), outside turbulence was comparatively higher than that of inside condition in other locations. Turbulence velocity of L2 was statistically similar to that L3 while condition in former two sites was approximately two fold higher compared to control site. However, the inside condition of L4, L5 and L6 were higher than the control site and independent from the location. Similarly outside

turbulence were also significantly different among study sites (F =95.39, $p< 0.001$) where the maximum outside turbulence was observed in L2 followed by L6, L3, L5, L4 and L1.

4.2.2.2. Species distribution and depth profile

Four macrophyte species were found in study sites, *Myriophyllum spicatum*, *E. nuttallii*, *Spaganium erectum* and *Vallisnaria spiralis*. According to visual observation *E. nuttallii* was totally covered the bottom of control transect whereas M. *spicatum* and *S. erectum* were rarely found in the L1. However, the dominant species in other locations was *M. spicatum*. In addition to L1, *S. erectum* was also found in L5 and L6 while *V. spiralis* was observed only in latter two sites (Figure 14). Macrophytes occurrence and the depth profiles of the study sites are presented in Figure 14. L1 has a smooth bottom with a maximum depth (77 cm) while other locations were less than half of the depth of control site. Further, irregular bottoms were observed in latter locations and particularly, substrate of L2 and L3 were totally covered with a gravel bed.

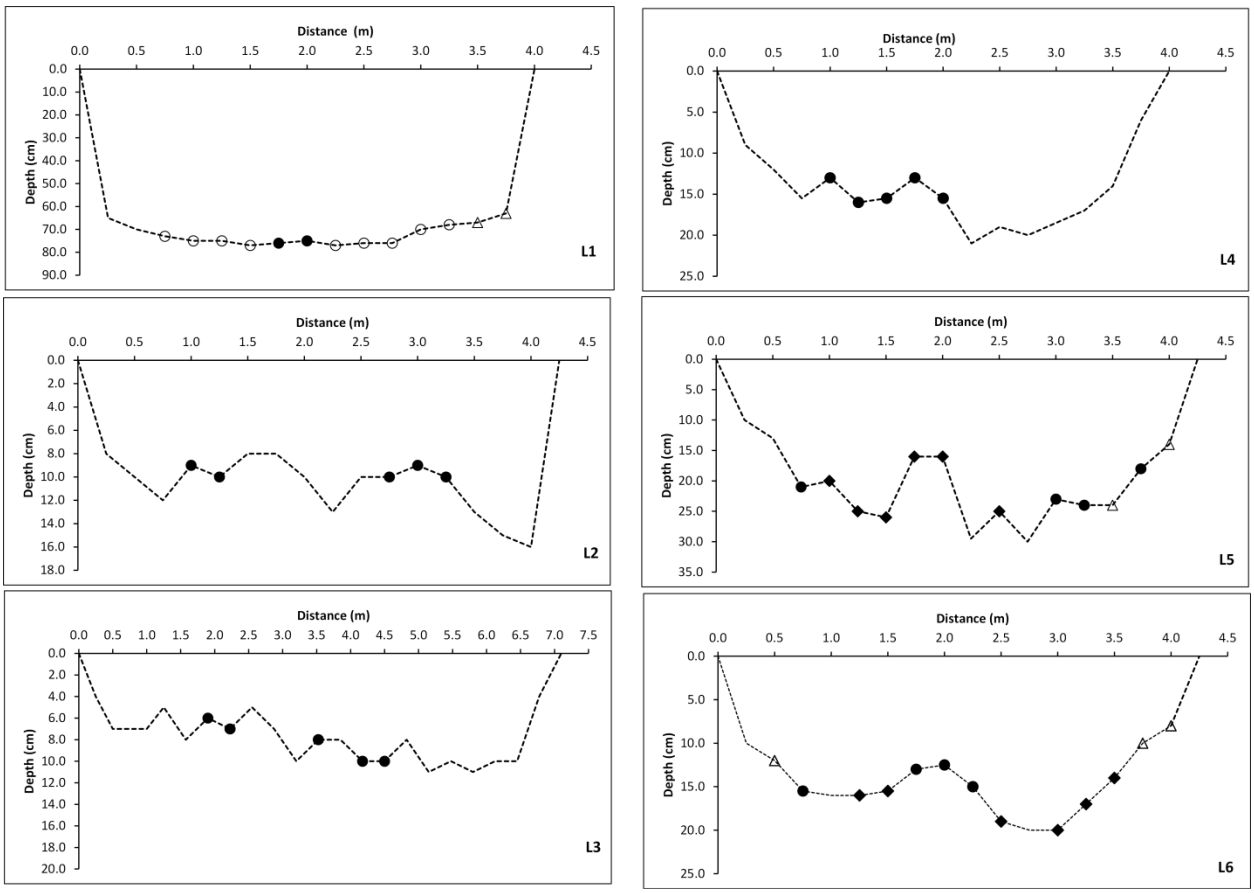

Figure 14. Species occurrences and the depth profile of study sites.
(○:*E.nuttallii*, ●: *M. spicatum*, ▲: *S.erectum, and* ♦: *V. spiralis*)

4.2.2.3. Stress response of macrophytes

Results presented in Table 5 summarize the biochemical responses of aquatic macrophytes *E .nuttallii*; (EN) *M. spicatum*; (MP), *S. erectum*; (SP) and *V. spiralis*; (VS) with respect to their location. The observed data in this field study (Table 5) provide evidences for the activation of antioxidant mechanisms in studied plants and plant responses were species specific. For instance, *E. nuttallii* showed comparatively high concentration of hydrogen peroxide compared to other species within the same environment (L1). Further, CAT activity of former species was also higher than that of *M. spicatum*.

Table 5. Comparison of the concentration of H_2O_2 and IAA, and the antioxidant enzyme (CAT, GPX and the APX) activity of plant species

Response	plant	Transect						$F_{(5,6)}$	*p*
		L1	L2	L3	L4	L5	L6		
H_2O_2content	*EN*	33.72 ± 0.60							
(µ mol/g	*MS*	21.75 ± 0.30^{c}	41.22 ± 1.55^{ab}	45.51 ± 0.00^{a}	44.06 ± 4.93^{ab}	25.66 ± 2.91^{c}	38.91 ± 0.20^{b}	34.0	*P*<0.001
FW)	*SP*	11.10 ± 0.22^{b}				40.96 ± 3.61^{a}	33.29 ± 0.27^{a}	109.6	*P*<0.05
	VS					26.20 ± 0.36	39.55 ± 2.30		NS
IAA (µg/	*EN*	7.83 ± 0.21							
gFW)	*MS*	16.28 ± 0.19^{a}	12.27 ± 0.16^{b}	7.67 ± 0.19^{d}	7.65 ± 0.33^{d}	9.96 ± 0.76^{c}	9.84 ± 0.87^{d}	79.2	*p*<0.001
	SP	15.67 ± 0.16				13.07 ± 1.9	17.94 ± 0.33	8.9	NS
	VS					26.20 ± 0.36	39.55 ± 2.30		NS
CAT activity	*EN*	0.034 ± 0.008							
(ΔOD/ gFW)	*MS*	0.021 ± 0.008^{b}	0.068 ± 0.004^{a}	0.049 ± 0.021^{a}	0.019 ± 0.004^{b}	0.025 ± 0.001^{b}	0.072 ± 0.023^{a}	6.54	*p*<0.05
	VS					0.05 ± 0.002	0.056 ± 0.000		NS
GPX activity	*EN*	0.202 ± 0.016							
(ΔOD/ gFW)	*MS*	0.140 ± 0.007^{e}	0.46 ± 0.049^{a}	0.365± 0.007^{b}	0.270 ± 0.014^{c}	0.350 ± 0.000^{b}	0.205 ± 0.007^{d}	58.2	*p*<0.001
	SP	0.37 ± 0.083^{b}				0.68 ± 0.002^{a}	0.829 ± 0.026^{a}	41.7	*p*<0.001
	VS					1.16 ± 0.14	1.44 ± 0.75		NS
APX activity	*EN*	0.021 ± 0.004							
(H_2O_2	*MS*	0.34 ± 0.05^{b}	0.83 ± 0.04^{a}	1.01 ±0.16^{a}	0.46 ± 0.02^{a}	0.80 ± 0.19^{b}	0.54 ± 0.15^{b}	13.8	*p*<0.05
destruction									
mM/min/gF	*SP*	0.16 ± 0.00^{b}				0.41 ± 0.07^{a}	0.58 ± 0.047^{a}	39.5	*p*<0.05
W)	*VS*					0.28 ± 0.16	0.29 ± 0.01		NS

(EN: *E. nuttallii*, MS: *M. spicatum*, SP: *S. erectum*, VS: *V. spiralis*, different superscript at the same raw indicate the significant difference at 0.05, NS: Not significant).

M. spicatum and *S. erectum* observed in high turbulence environments showed significantly elevated level of H_2O_2, APX and GPX compared to the same plants in control site which experienced minimum turbulence. In addition, *M. spicatum* showed a similar trend for CAT activity. Further, the lowest activity of APX in *M. spicatum* and *S. erectum* was also found in L1 where the lowest stress was observed. Turbulence led to significant increase in antioxidant activity in *M. spicatum* and *S. erectum* while the stress response of *V. spiralis* was relatively independent from the location.

Present findings showed that *M. spicatum* in control site (L1) and as expected, the content of IAA was found to be higher than that of plants found in high turbulent environments. Turbulence led to a decrease in IAA content by two fold in L3-plants which exposed to the highest turbulence as compared with the plants in control site.

4.2.2.4. Biomass lignin and pigments

Table 6 summarizes biomass, lignin and cellulose contents observed at studied transects. The highest total above ground biomass was found in control transect where the minimum turbulence was occurred. Specially, transects having elevated magnitudes of turbulence (L2 and L3) contained low ABG biomass and it is clear that, the turbulence led to decrees in biomass. However, the BGB was independent from the location.

Table 6. Biomass, cellulose and lignin (Different superscript in the same raw indicate the significant difference at 0.05 level)

Parameter	Transect						F	p
	L1	L2	L3	L4	L5	L6		
ABG biomass (g DW/m^2)	546.8 ± 11.3^{a}	33.65 ± 6.5^{c}	49.7 ± 20.7^{cd}	83.2 ± 9.1^{bc}	101.9 ± 11.8^{b}	59.8 ± 29.9^{bcd}	273.3	*
BG Biomass (g DW/m^2)	2.5 ± 1.7	2.5 ± 0.7	1.4 ± 0.7	2.8 ± 0.1	2.7 ± 0.9	2.1 ± 0.7	0.629	*ns*
Cellulose (mg/g DW)	230.3 ± 5.9^{e}	320.8 ± 4.4^{c}	351.5 ± 8.9^{b}	296.6 ± 0.0^{d}	384.6 ± 4.9^{a}	383.3 ± 0.0^{a}	265.6	*
Lignin (mg/g DW)	40.1 ± 0.2^{e}	49.2 ± 3.4^{e}	54.4 ± 4.1^{cd}	60.8 ± 0.2^{bc}	42.8 ± 0.3^{de}	52.7 ± 5.7^{a}	18.8	*

(BG biomass data were ln transformed, *ns*: not significant, *: significant at 0.05 level)

There was a negative correlation between turbulence and the above ground bio mass (r=-0.88, p<0.001) and the bio mass ratio (ABG to BGB) was significantly correlated with inside turbulence (r=-0.874, p<0.05). Even though the biomass ratio was negatively correlated with the inside main velocity, the relationship was not significant (r=-0.512, p>0.05).

Turbulence driven mechanical stress led to significant increase in cellulose and lignin contents in *M. spicatum* shoots (Table 6). Plants grown in control transect contained the lowest level of cellulose and lignin compared to *M. spicatum* in other transects. Significantly elevated levels of lignin and cellulose in plants exposed to high turbulence could be an adoptive mechanism to strength plant shoots against turbulence.

Total chlorophyll and carotenoids in plants were influenced by turbulence and the observed pigment concentrations are presented in Figure 15.

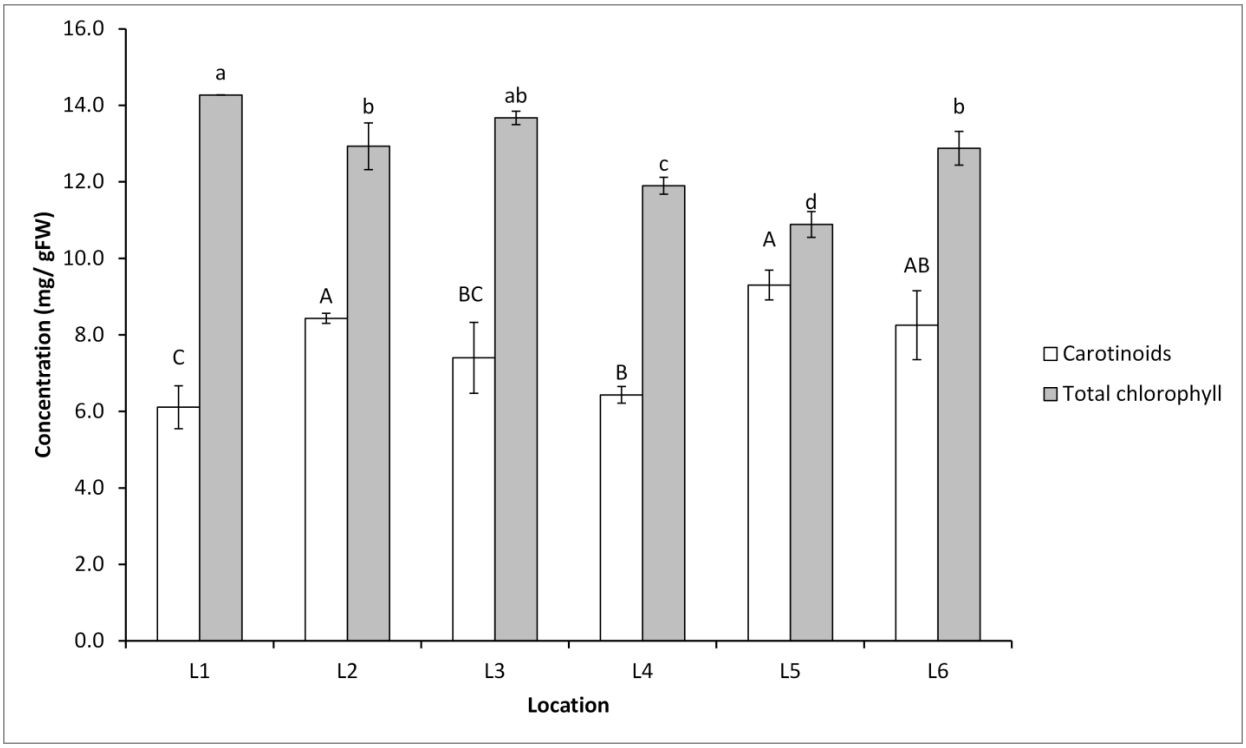

Figure 15.Total chlorophyll and carotenoids content of *M. spicatum* (different superscript in the same category indicates the different at 0.05 significant level)

Except for L3, total chlorophyll content of *M. spicatum* was significantly (p<0.05) lower than that of L1 plants while chlorophyll content of L3 plants is statistically similar to that of plants in L1. Compared to plants in the control site, significantly high content of carotenoids were also found in plants in other sites. However, the carotenoid concentration of L3-plants was statistically similar to that of control plants.

4.2.2.5. Water quality

Dissolved oxygen content in study sites remained in a similar range (6.25-6.35 ppm) and temperature ranged from 15^{o}C to 16^{o}C. Observed pH range was 6.1-7.0 while the NO_2^-, NO_3^- and PO_4^{3-} concentrations are presented in the Table 7. Nitrite (NO_2^-) and nitrate (NO_3^-) concentrations were statistically similar among study sites while the phosphate concentration is significantly different.

Table 7: water quality of study sites (different superscripts in the same raw indicate the significant difference at 0.05 level)

Parameter	Transect						$F_{(5,6)}$	p
	L1	L2	L3	L4	L5	L6		
NO_2^-	0.016 ±	0.022 ±	0.025 ±	0.017 ±	0.016 ±	0.02 ±	0.25	$p>0.05$
(ppm)	0.001	0.007	0.017	0.006	0.013	0.00		
NO_3^-	1.101 ±	0.691 ±	0.904 ±	0.912 ±	0.732 ±	0.88 ±	1.94	$p>0.05$
(ppm)	0.248	0.061	0.192	0.102	0.014	0.14		
PO_4^{3-}	0.011 ±	0.016 ±	0.013 ±	0.017 ±	0.024 ±	0.018 ±	62.3	$p<0.05$
(ppm)	0.007^c	0.007^b	0.00^c	0.001^b	0.007^a	0.001^b		

4.2.2.6. Relationship between turbulence and plant responses

Inside turbulence was significantly correlated with the stress response measurements; GPX (r=0.551, $p<0.05$), H_2O_2 (r=0.859, $p<0.001$) and APX (r=0.794, $p<0.001$). Further, significant positive correlations were found between turbulence and cellulose (r=0.465, $p<0.05$). Moreover, IAA content of *M. spicatum* was negatively correlated with the turbulence (r=-0.916, $p<0.001$) while CAT activity did not show a significant correlation to turbulence (r=0.342, $p>0.05$). These findings clearly indicate the turbulence triggered oxidative stress in *M. spicatum* in natural environment. Even though a negative correlation was observed between turbulence and chlorophyll, the relationship was not significant (r=-0.370, $p>0.05$). Linear regression analysis (y=a+bx) showed significant ($p<0.05$) relationships between stress response and turbulence (Table 8).

Table 8. Results of linear regression analysis for the effect of turbulence on plant responses

Response	Model statistics			Parameter estimates (significance level, SE)	
	R^2	F	p	a	b
IAA	0.839	52.09	**	19.86 (**, 1.33)	-2.39 (**, 0.33)
GPX	0.432	7.60	*	-2.13 (*, 0.32)	0.22 (*, 0.08)
APX	0.719	25.54	**	-1.58 (**, 0.28)	0.28 (**, 0.06)
H_2O_2	0.761	31.76	**	2.78 (**, 0.15)	0.22 (*, 0.03)
Cellulose	0.682	8.69	*	5.38 (**, 0.14)	0.11 (*, 0.03)

Except IAA, all other responses are in the form of ln transformed, ns: not significant, * and ** indicate the significant difference at 0.05 and 0.001 levels respectively; a and b are constant (y=a+bx).

Even though the total chlorophyll negatively correlated with the main flow (r=-0.665, p <0.05), all other responses were not correlate significantly with the main flow i.e, IAA (r=-0.578, p<0.05), H_2O_2 (0.037, p>0.05), Lignin (0.53, p>0.05), CAT (-0.162, p>0.05), GPX (-0.268, p>0.05).

4.3. Experiment -II

4.3.1. **Plant growth and cell ultra-structure**

The plants were all alive in the microcosms, and mortality was not observed during the experiment. The growth of the plants was severely affected by the turbulence, as measured in the final shoot length Figure 16.

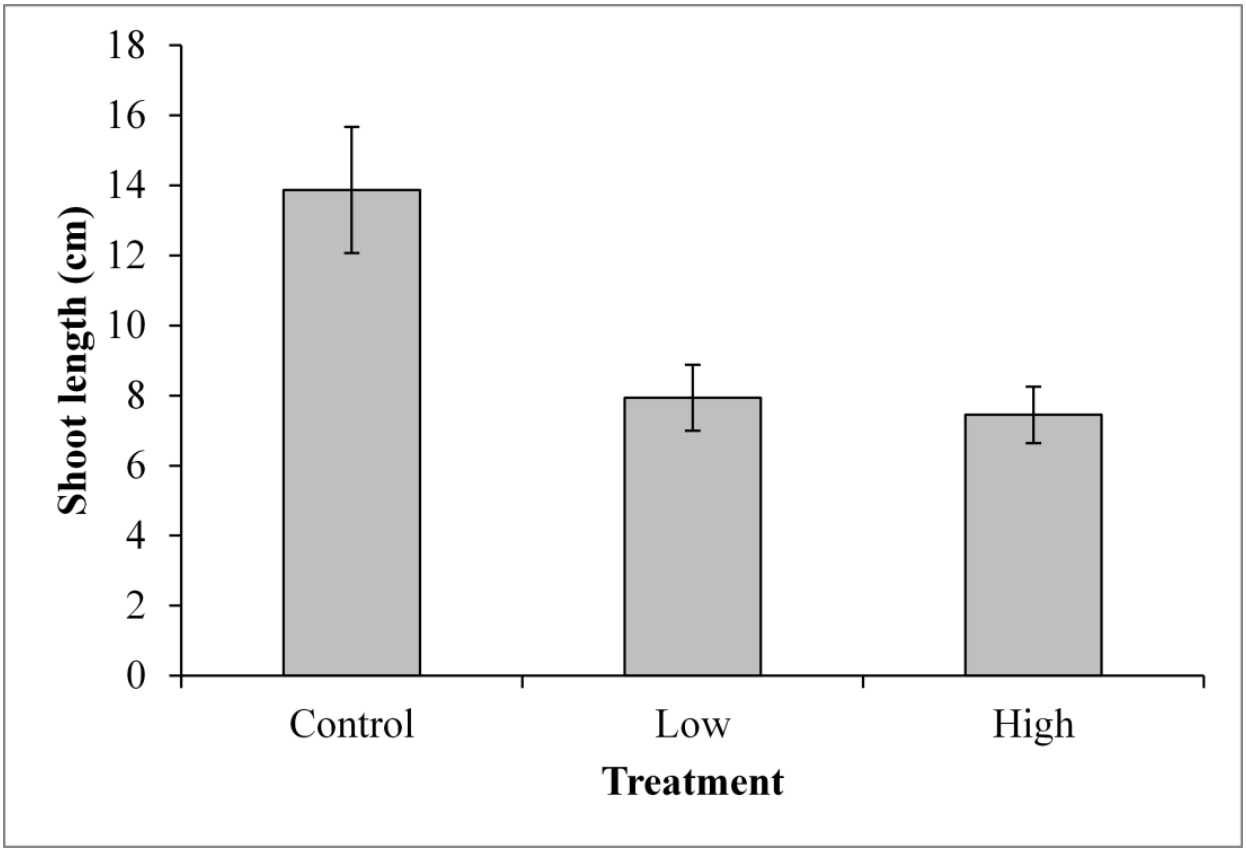

Figure 16.Shoot length of *E. nuttallii* after exposure to stagnant (Control), low turbulence (Low) and high turbulence (High) for 30 days (Values are mean ± SD, n=4)

The final shoot lengths of *E. nuttallii* were significantly different ($p< 0.05$) among the three treatments, and the final length of plants grown in stagnant waters (control) was approximately twofold longer than that of the plants exposed to turbulence (Figure 16). Similarly, the shoot elongation rate (SER) was significantly different ($p< 0.05$) among the three treatments. The

highest SER was observed in plants in stagnant (control) conditions (3.8 ± 0.6 mm day^{-1}), which was followed by low turbulence (1.7 ± 0.3 mm day^{-1}) and high turbulence (1.6 ± 0.3 day^{-1}).

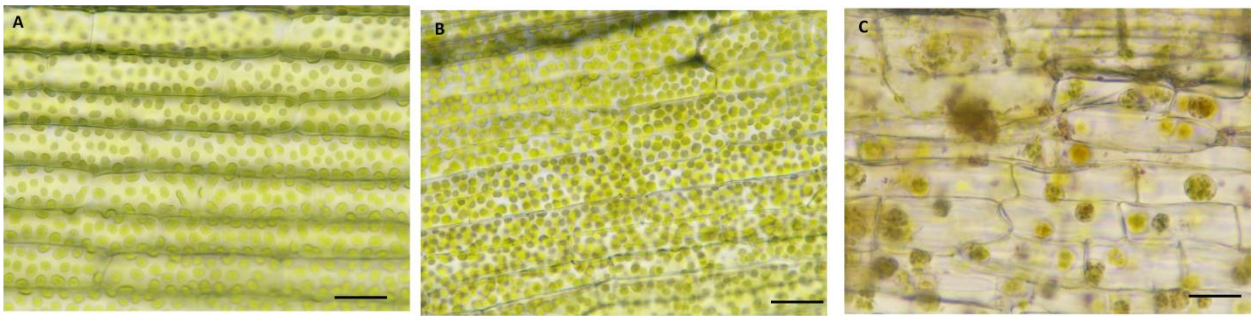

Figure 17.Appearance of *E. nuttallii* leaves after 30 days of each treatment with light microscopy. (A: Control, B: Low turbulence and C: High turbulence. Bar is 50 µm)

The similar SERs that were observed for the plants grown in low and high turbulence conditions were not significantly different. Furthermore, plants in the control treatments grew to the surface of the microcosms; whereas the plants exposed to turbulence grew to approximately half of the height of the microcosms. In addition to differences in plant growth, the apical tips of plants exposed to turbulence became yellowish with time; whereas the apical tips of plants grown in stagnant waters (control group) remained green at the end of the experiment. The yellow apical leaves observed in the turbulence-stressed plants were explained by the destruction of chloroplasts and plasmolyzed leaf cells (Figure 17).

A gradual destruction of the chloroplasts occurred in parallel to the increase in the magnitude of turbulence, and turbulence caused plasmolysis in the leaf cells of *E. nuttallii* (Figure 18C and Figure 17C).

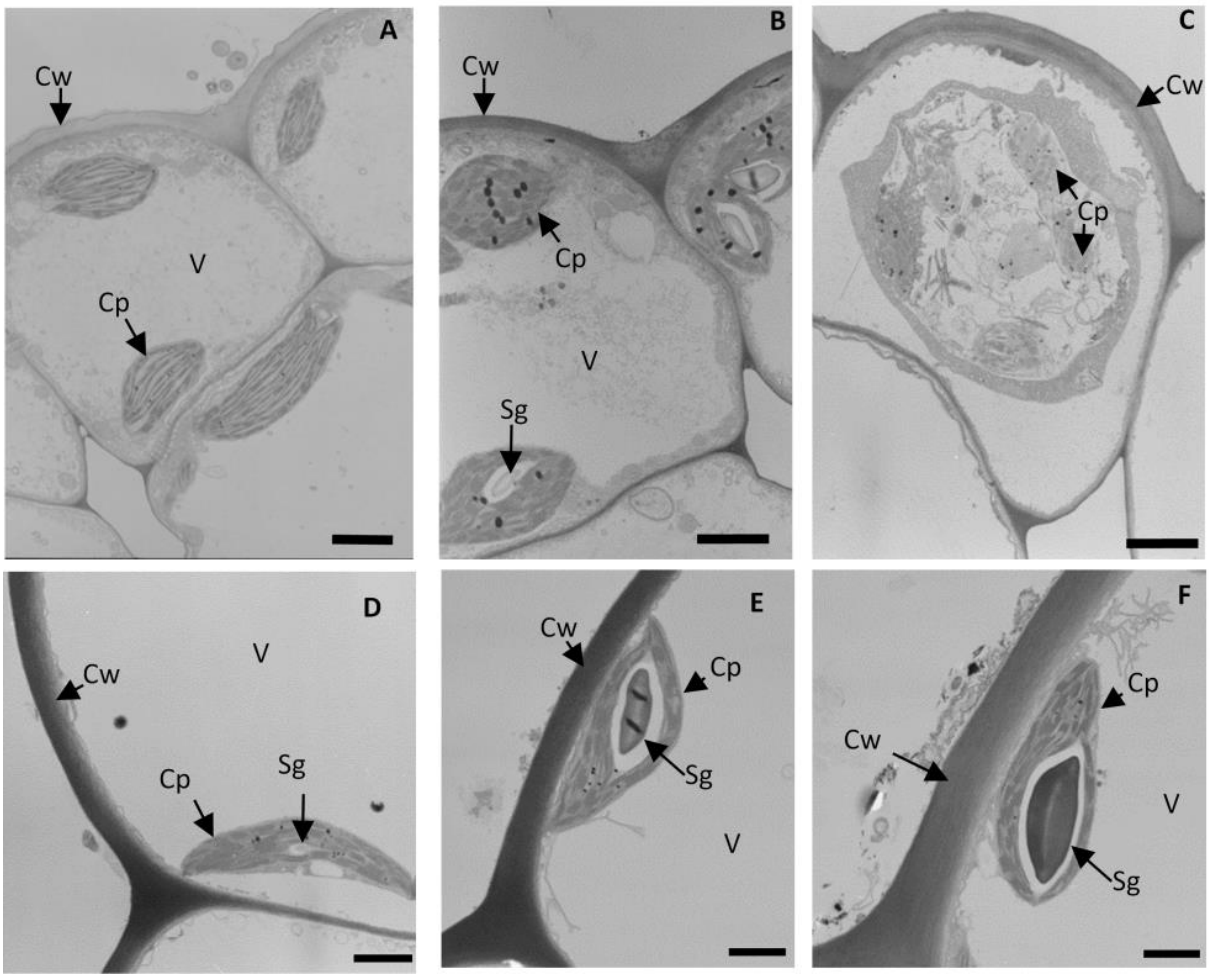

Figure 18.Ultra-structural changes of *E. nuttallii* leaf and stem. Pictures in the upper and lower panels show the transverse sections of the upper cell layer of a apical leaf and the epidermal cells of the basal stem (~5 cm above to the substrate), respectively. A and D: Control, B and E: Low turbulence, and C and F: High turbulence. (Cw: cell wall; Cp: Chloroplast; Sg: Starch granules; and V: Vacuole. Bar is 2 µm).

With electron microscopy, the plasmolysis observed under light microscopy was also clearly observed (Figure 18C); the plasma membranes of the leaf cells were detached from the cell walls under conditions of high turbulence (Figure 18C). We did not observe plasmolysis in *E. nuttallii* leaf cells in either stagnant (control) or low turbulence conditions. The average diameter of a cell in the upper cell layer of *E. nuttallii* leaves (Figure 17A-C) of control, low and high turbulence conditions was 48.6 ± 6.8, 55.7 ± 13.0 and 52.5 ± 12.0 µm (n = 20), respectively, and the diameter of those cells was not significantly different among the three treatments (p>0.05).

However, the cell wall thickness was significantly different among treatments ($p< 0.05$), and the thickness of the stem epidermal cell walls increased with the turbulence (Figure 19D-F and Figure 19). The control stems had the lowest cell wall thickness followed by low and then high turbulence.

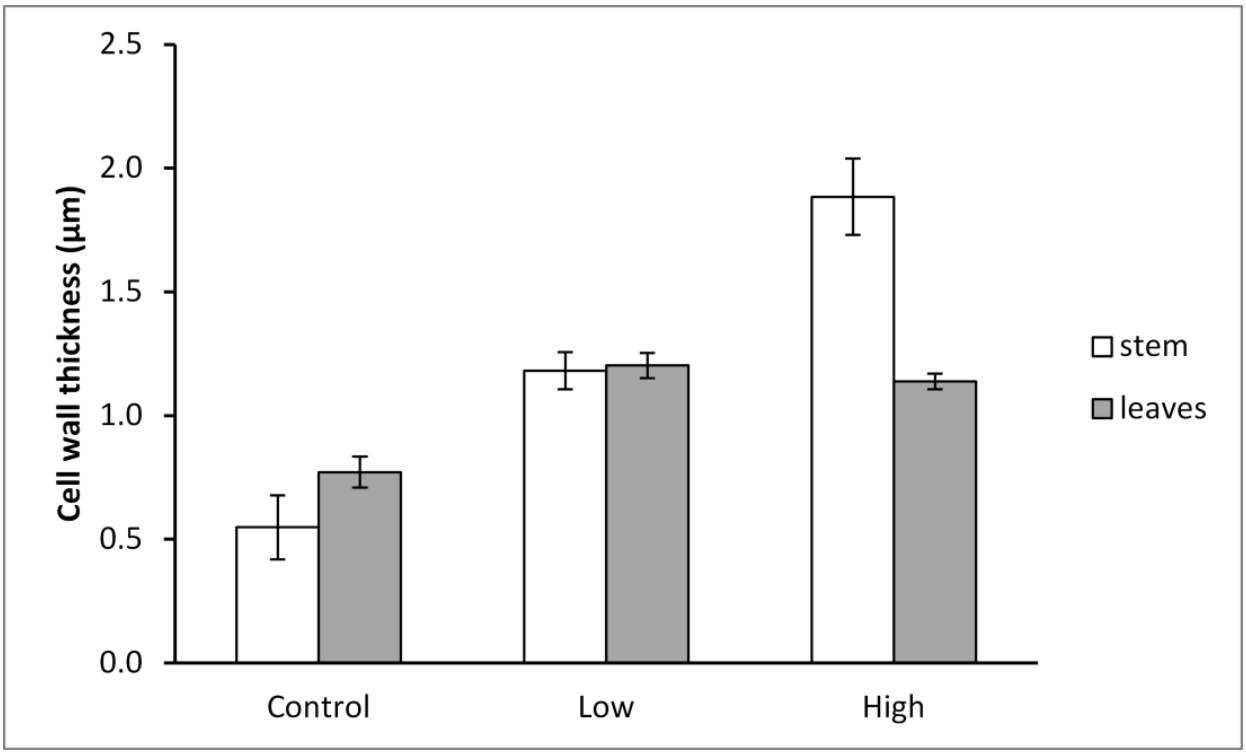

Figure 19.Cell wall thicknesses of the epidermal cells of apical leaves and basal stem (~5 cm above to the substrate) of *E. nuttallii* (Values are mean ± SD) after 30 days.

Moreover, the epidermal cell wall thickness in stems of plants exposed to high turbulence was approximately fourfold thicker than that of the cells of control plants (Figure 18). In leaves, the epidermal cell walls of plants exposed to turbulence were significantly thicker ($p < 0.05$) than those of the control leaves (Figure 18A-C and Figure 19). Although the cell wall thickness of epidermal cells of leaves in plants grew in low turbulence (1.20 ± 0.05) was higher than that of plants in high turbulence (1.10 ± 0.03), the difference was not statistically significant. Additionally, finger-like ingrowths of the cell wall were observed in all treatments, but these ingrowths were prominent in epidermal cells of leaves exposed to low turbulence (Figure 20).

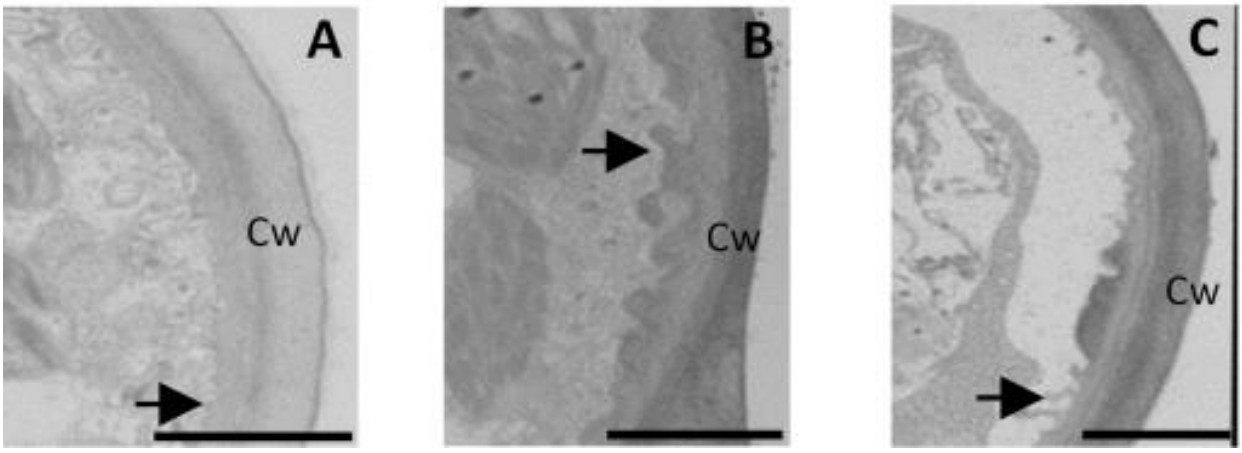

Figure 20.Closer view of the cell wall of epidermal cells of apical leaves (A: Control, B: Low turbulence, and C: High turbulence. Cw: cell wall; and arrow indicates the finger-like ingrowths. Bar is 2 µm)

E. nuttallii accumulated more starch granules in the chloroplasts with exposure to turbulence (Figure 18). Although starch granules were found in leaf chloroplasts under low turbulence (Figure 18B), those granules were rarely found in the leaf chloroplasts of plants grown in the control and high turbulence treatments. The starch granules were found in stem chloroplasts of all plants (Figure 18D-F).

However, the number of starch granules per cortex cell was significantly different among the three treatments (Pearson Chi-square = 39.17, $p< 0.01$). Notably, the turbulence-stressed plants contained a significantly larger number of starch granules in cells in the cortex, and almost all cortex cells of the plants exposed to high turbulence contained starch granules (Table 9). Moreover, the size of the starch granules found in the stem chloroplasts of plants exposed to high turbulence was significantly larger than those in the other two groups (Figure 18, Table 9). Total starch content of plants exposed three different conditions were significantly different ($p<0.05$), i.e.; the starch content of plants exposed to control, low turbulence and high turbulence were 37.97 ± 6.05, 58.48 ± 2.42 and 81.22 ± 3.48 µg gDW^{-1} respectively. Starch content in plants exposed to high turbulence was approximately two-fold higher than that of control plants.

Table 9. Characteristics of starch granules (SG)

Observation	Plant tissue	Turbulence condition		
		Stagnant	Low	High
Length (µm)	Leaves	< 0.7	0.8 - 1.7	< 0.7
	Stem	0.5 - 3.7	3.1- 4.2	1.9 - 4.8
Width (µm)	Stem	0.2 - 1.0	0.8 - 1.5	1.0 - 2.5
	Leaves	< 0.5	0.4 - 0.6	< 0.5
Cells with SG per section (%)	Stem	16	60	97
Number of SG (per cell section)	Stem	0 - 1	0 - 4	0 - 6

(Except for cells with starch granules, all other parameters are presented as min-max range.)

4.3.2. **Amino acid metabolism**

Figurev21 summarizes the observed variation in metabolite contents of *E. nuttalli* responding to water turbulence. The contents of 3-phosphoglyceric acid (3 PGA), phosphoenolpyruvate (PEP) and pyruvate were not significantly different among the three treatments (Figurev21). The citrate content was significantly higher in plants exposed to the turbulence than in the control plants grown in stagnant waters (Figurev21, citrate). Furthermore, the content of citrate was more than twofold higher, approximately, in the turbulence-stressed plants than in the control plants. The isocitrate-oxalate pathway was apparently affected by the turbulence (Figurev21). The isocitrate content was significantly higher in the control plants than in the plants exposed to turbulence (Figurev21, isocitrate). The oxalate content was significantly different among treatments, and the response observed for oxalate to turbulence was opposite the trend observed for isocitrate, i.e., the highest oxalate content was found in the plants exposed to the turbulence followed by the control plants (Figurev21, oxalate). The ascorbate content in the plants exposed to turbulence increased significantly compared with that in the control plants (Figurev21, ascorbate). Thus, the ascorbate-oxalate pathway could explain the increase in the content of oxalate (Figurev21). Although the content of 2-oxoglutarate (2OG) was not significantly different among the treatments, a significant decline in the content of glutamate (Glu) in response to turbulence was observed (Figurev21, Glu). The highest Glu content was found in the control plants followed by

the plants grown in low and high turbulence. There were significant differences in the contents of glutamine (Gln), histidine (His) and γ-Amino butyric acid (GABA) among the three treatments. The content of proline (Pro) was also significantly different and followed the same trend that was observed for the Gln. Although an increasing trend in the content of arginine (Arg) with turbulence was observed (Figurev21, Arg), the differences were not significant among the three treatments. Therefore, the Glu-His, the Glu-GABA and the Glu-Pro pathways in *E. nuttallii* were influenced by the mechanical stress of turbulence.

The contents of succinate, fumarate and malate were not significantly different among the three treatments. Notably, there was clear evidence for the effect of the turbulence on the aspartate (Asp)-asparagine (Asn) pathway. Significant differences in the Asp and the Asn contents were found among the three treatments, and the highest Asp content was found in the control plants followed by the plants exposed to low and high turbulence (Figurev21, Asp). The Asn content of *E. nuttallii* in the control, low and high turbulence treatments was 58, 73 and 75%, respectively. The trend observed for the Asn content was opposite to the trend observed for Asp in which the highest Asn content was in plants exposed to the high turbulence followed by low turbulence and control plants (Figurev21, Asn). The content of methionine (Met) declined in response to the turbulence, whereas the contents of lysine (Lys), isoleucine (Ile) and leucine (Leu) in *E. nuttallii* were not significantly different among the treatments (Figurev21). The content of alanine (Ala) (Figurev21, Ala) declined with the turbulence.

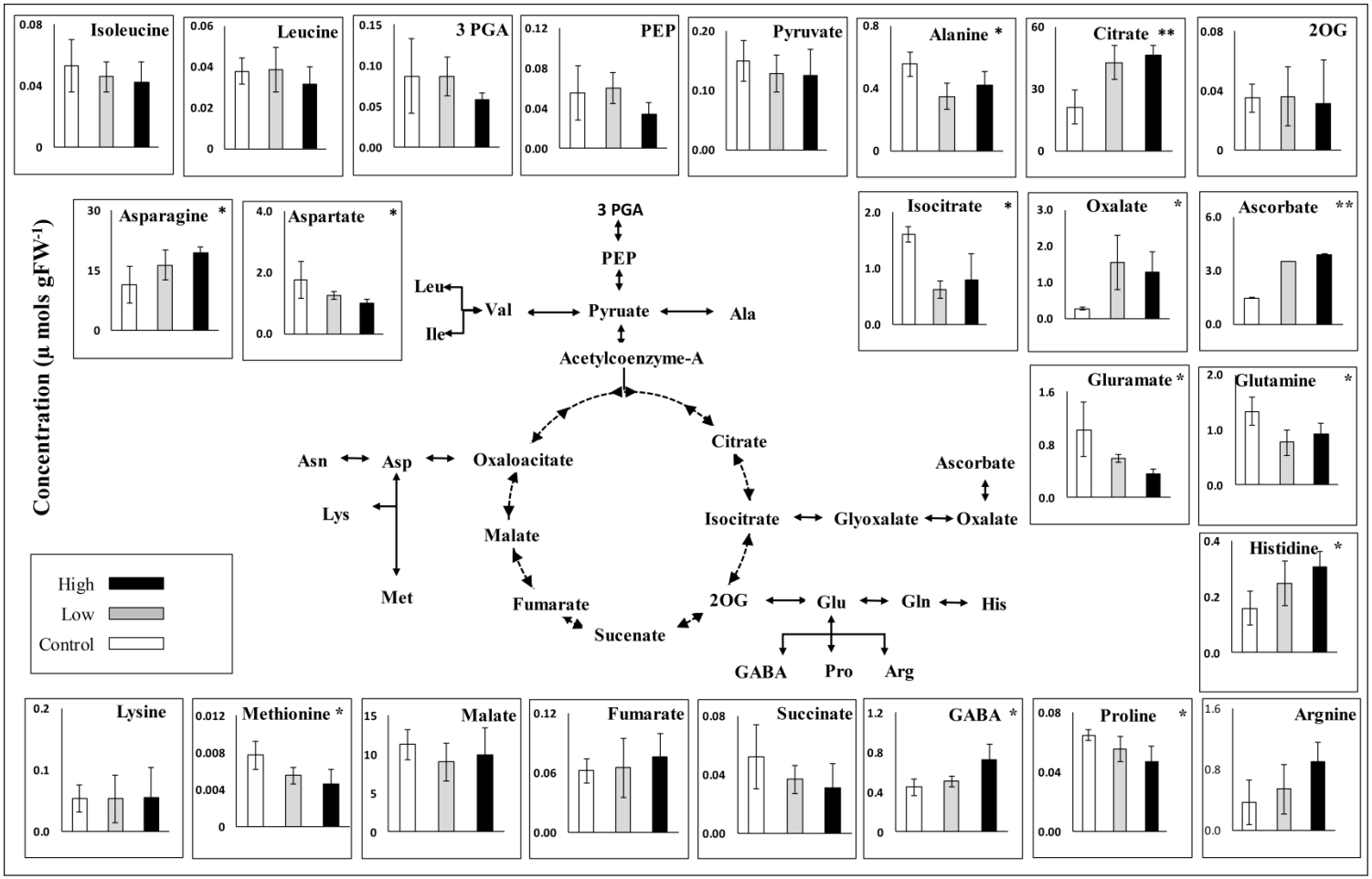

Figure 21.Alterations of primary metabolites in *E. nuttallii* in response to turbulence, 3-phosphoglyceric acid (3 PGA), phosphoenolpyruvate (PEP), γ-Amino butyric acid (GABA), 2-oxoglutarate (2OG). (Values are mean ± SD, for GABA and oxalate, n=3 and for the other metabolites, n=4. * and ** indicates the significant difference at 0.05 and 0.01 levels respectively.).

4.4. Experiment-III

4.4.1. **Plant growth and ultra-structural variations**

Although plants grew into both directions in control tubes, all plants in flowing tubes showed flow directional growth (Figure 22). In control tubes *E. nuttallii* strands grew either upwards and downwards freely inside the transparent tube. However, plants in flowing tubes were affected by the force generated by water flow whereas those plants grew as straight strands in the middle part of the tube in parallel to water flow.

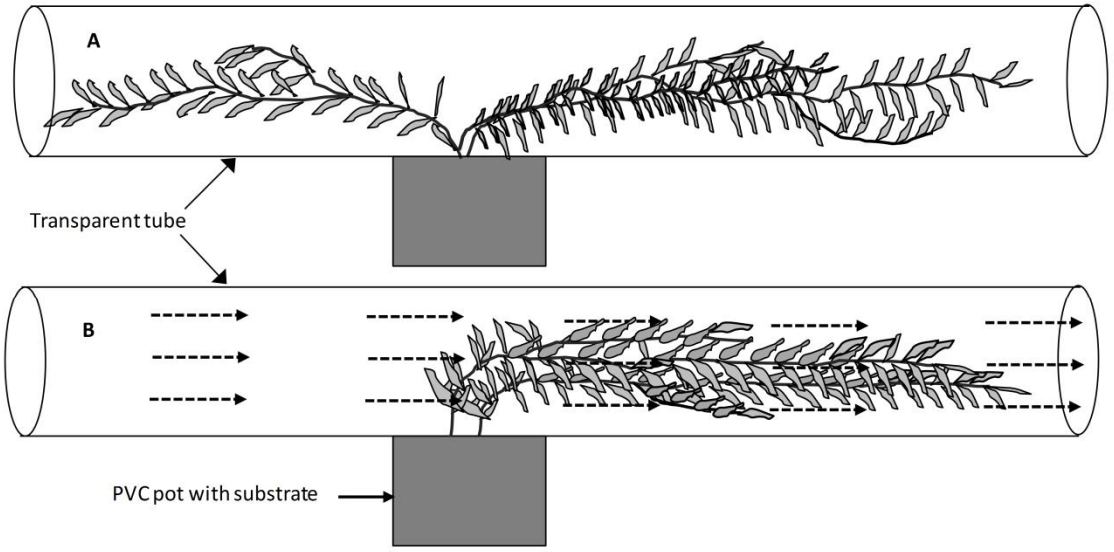

Figure 22. Diagrammatic representation of the plant growing inside the experimental units (A: Control, B: Flowing, arrows inside tube B indicate the water flow).

Average final shoot length of *E. nuttallii* grown in flowing (28.7 ± 2.6 cm) and control conditions (28.6 ± 2.0 cm) were not statistically significant ($p > 0.05$). However, majority of the internodes of plants in the flowing treatments are longer than those in control plants; i.e., only 12% and internodes were longer than 6 mm in control plants while that percentage of plants in flowing treatment was 37% (Figure 23).

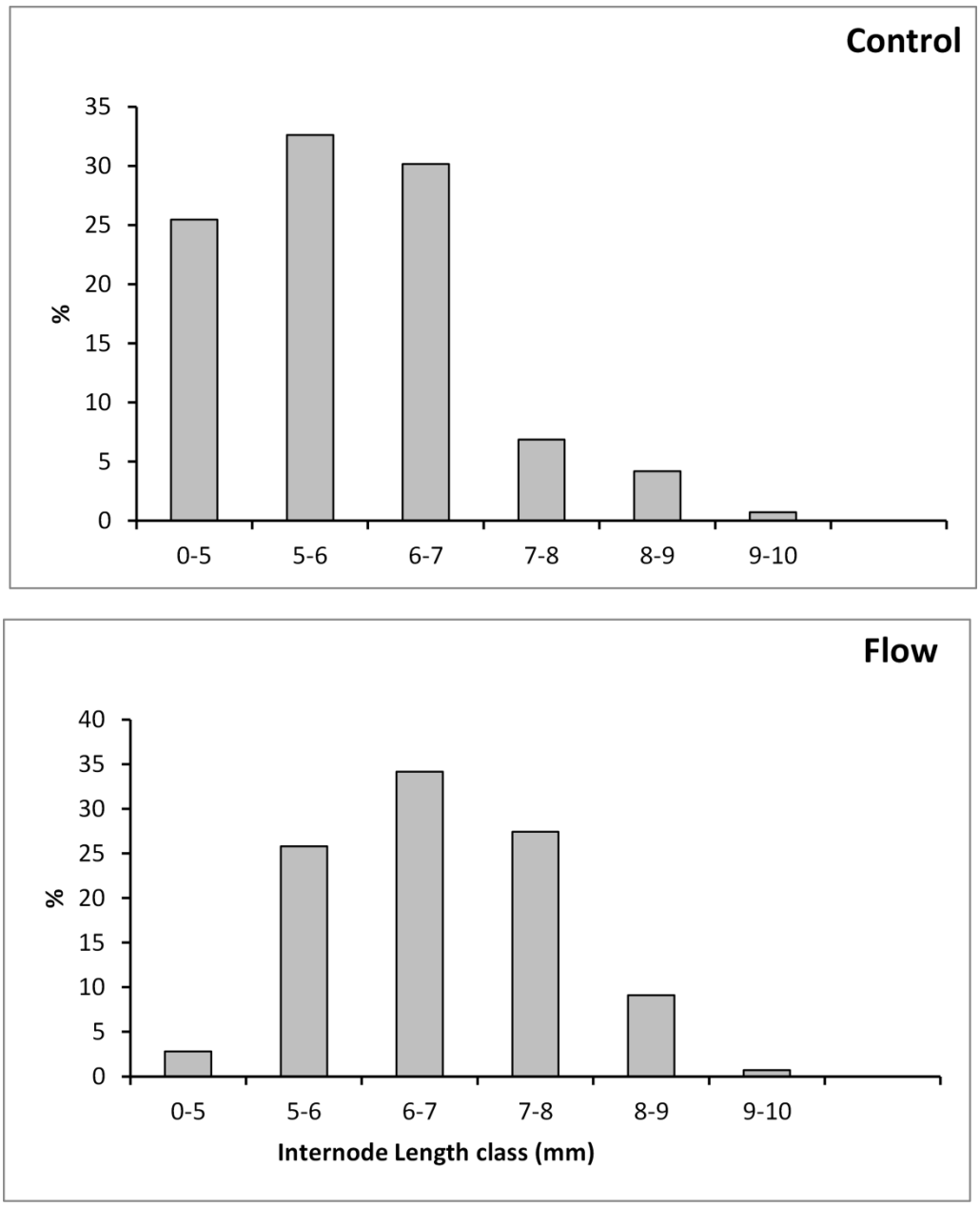

Figure 23. Length frequency distribution of *E. nuttallii* internodes after exposure to stagnant (control, n = 105) and flowing (n = 120) for 30 days.

The average diameters of the leaf cells exposed to stagnant and flowing conditions were 20.97 ± 0.98 and 19.09 ± 0.76 µm respectively, where the control plants consist of significantly wider stem cortex cells. Further, in control plants, diameter of approximately 50% of cortex cells were bigger than 20µm whereas the 70% of cortex cells of the plants exposed to control were less than 20µm (Figure 24-leaf). However, an opposite trend was observed for the stem cortex cells (Figure 24-stem).

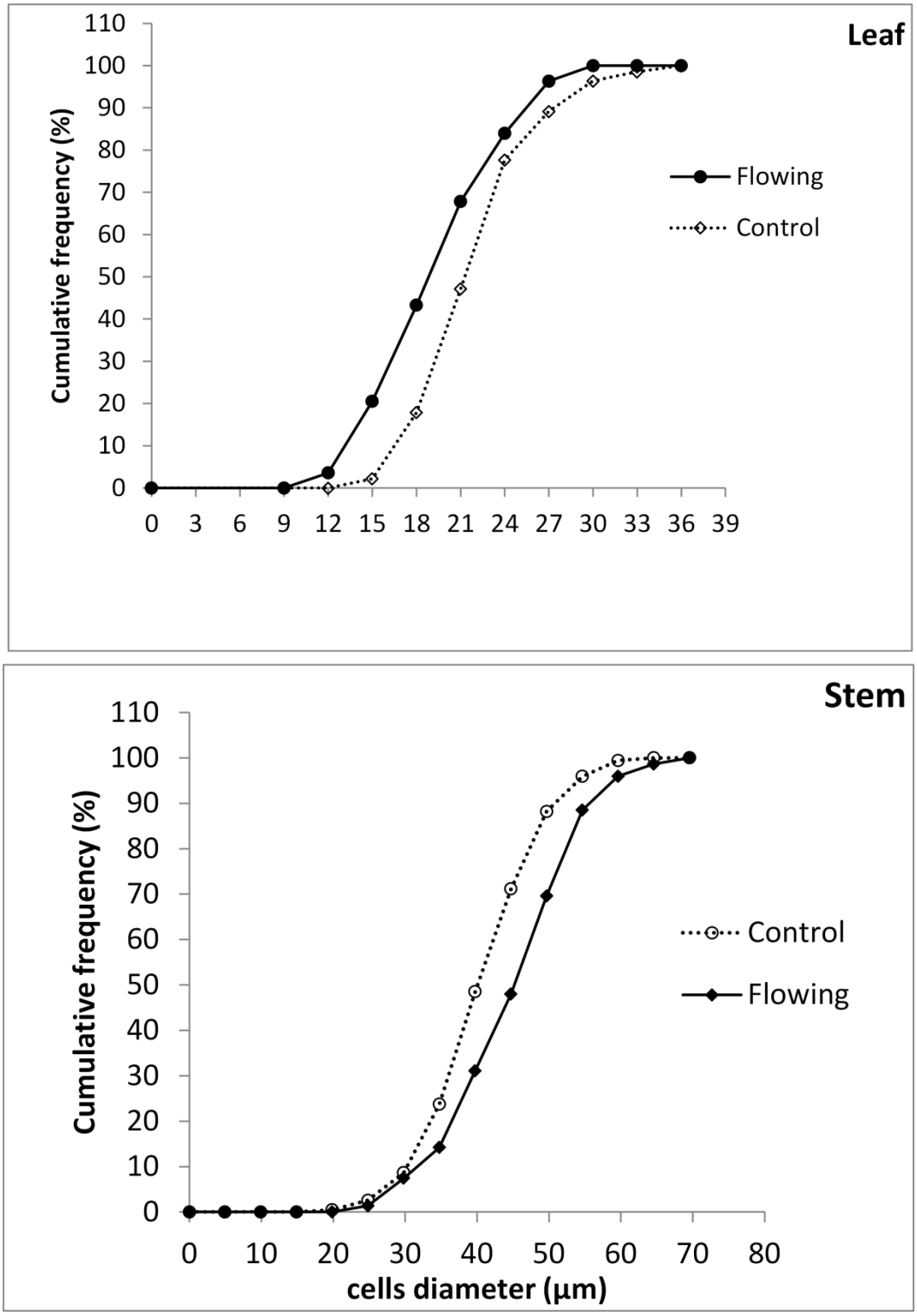

Figure 24. Size class distribution of leaf and stem cortex cells

In the control plant stems, approximately 71% of cells were bigger than 45 µm while only 52% of cortex cells in control stems were bigger than the former size.

Ultra structural changes in leaf and stem

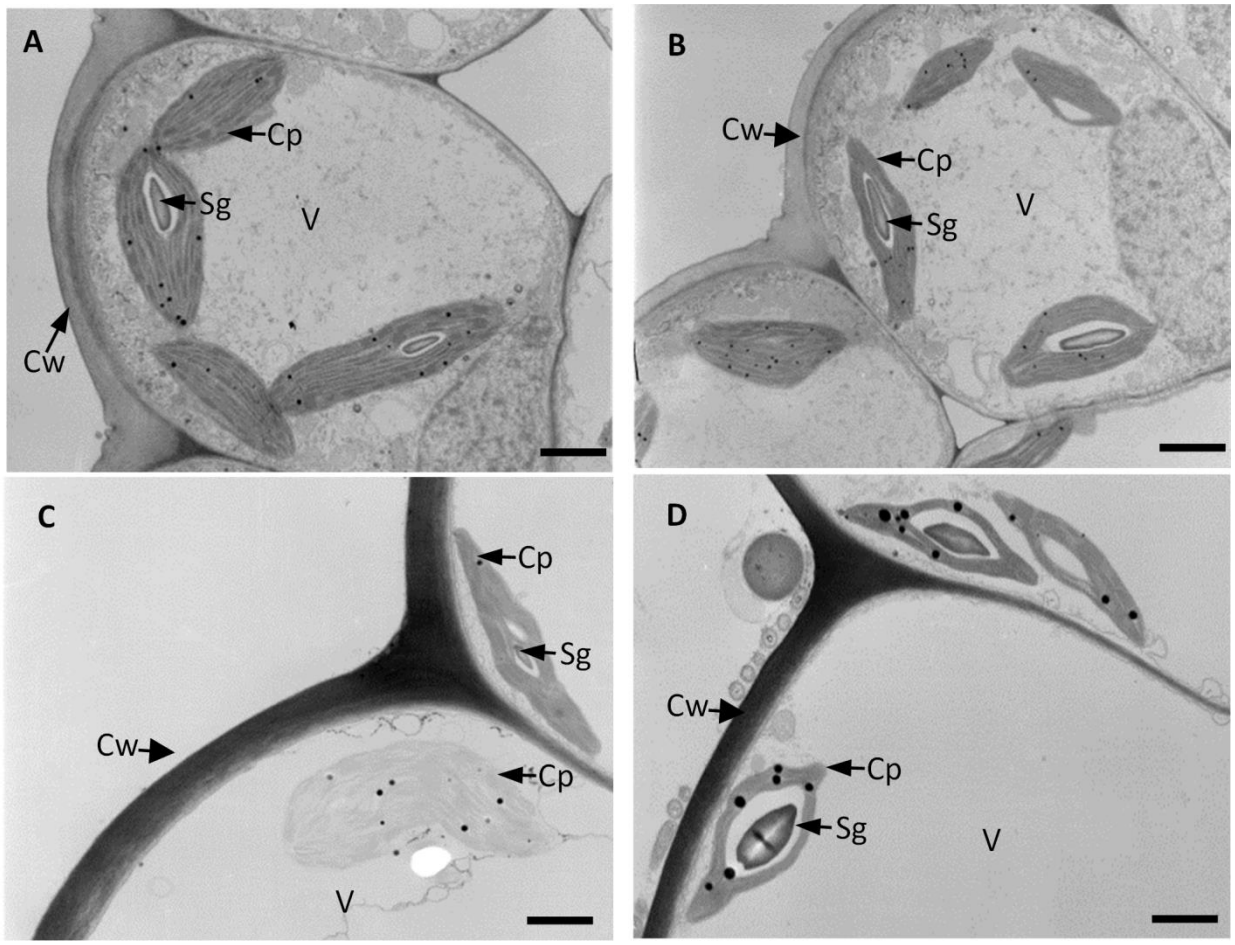

Figure 25.Ultra-structural changes of *E. nuttallii* responding to water flow.

(pictures in the upper and lower panels show the transverse sections of the lower cell layer of a apical leaf and the epidermal cells of the basal stem (~5 cm above to the substrate), respectively. A and C: Control, B and D: flowing treatment (Cw: cell wall; Cp: Chloroplast; Sg: Starch granules; and V: Vacuole. Bar is 2 µm).

Epidermis of the cells in control leaves (1.03 ± 0.02 µm) was significantly (p<0.05) thicker (Figure 25-A) than that of plants (0.84 ± 0.04 µm) exposed to water flow (Figure 25-B). A similar trend was also observed for the stem epidermis whereas the control plants consists of thicker epidermis than plants exposed to flow (Figure 25 C-D) ; i.e. the stem epidermal thickness of the plants exposed to control and flowing treatments were 1.41 ± 0.09 µm and 1.08 ± 0.09µm respectively. Similar to previous experiment1, stem diameter of plants exposed to water

movements (867.1 ± 5.7 µm) were significantly (p< 0.05) wider than that of control plants (817.2 ± 3.4 µm).

Although there were declining trends in Chlorophyll-a (Chl-a) and chlorophyll-b (Chl-b) after exposure to high water flow (Figure 26), the differences in this regards among two treatments were not significantly different (p> 0.05). Morphometric features of the chloroplast have been affected by the mean flow as it observed in length: width ratio. This ratio was significantly different (p< 0.05) among two treatments whereas chloroplast in control plants were wider and short (length: width ratio was 2.17 ± 0.06 µm) while elongated chloroplasts (length: width ratio was 3.14 ± 0.02 µm) were observed in plants exposed to water flow.

Chlorophyll contents

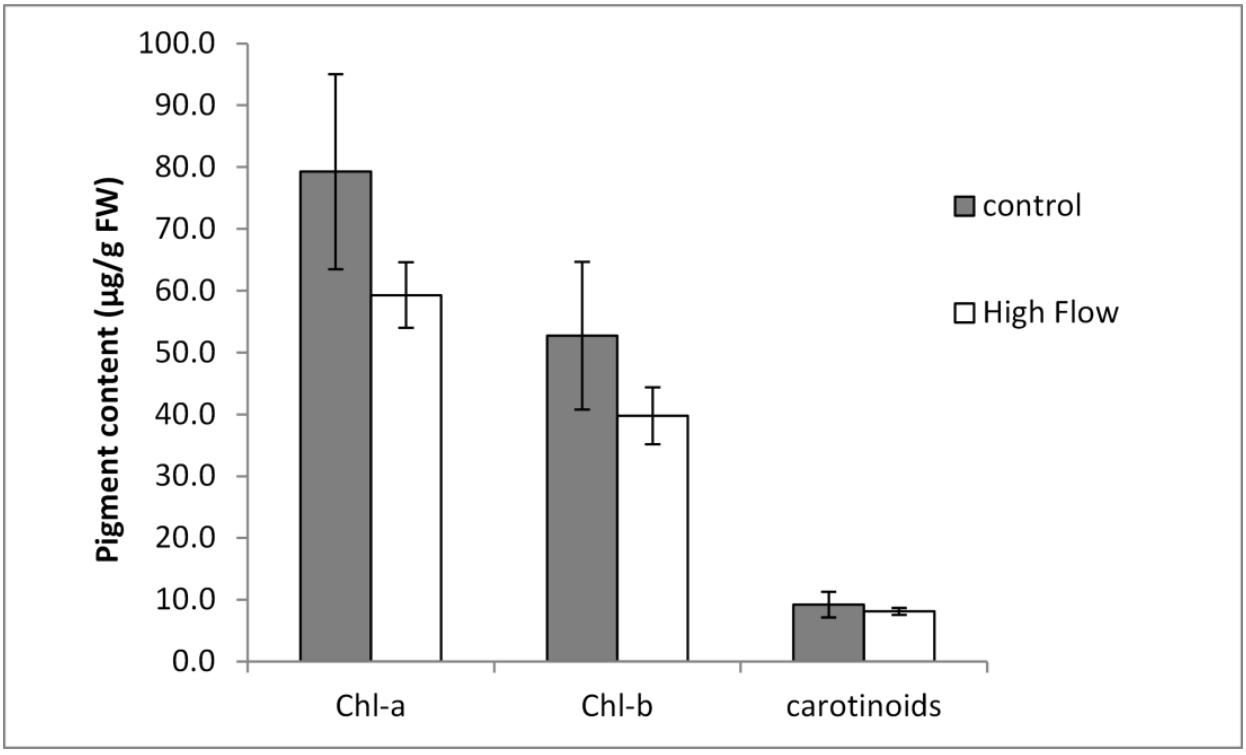

Figure 26. Pigment contents in *E. nuttallii* in response to stagnant and high flow

Compared to control plants, starch accumulation in stems was clear in plants after exposure to mean flow. However, starch granules were observed in leaves of both stagnant and flowing treatments (Figure 25).

4.4.2. **Effects of water flow on plant metabolism**

Table 10 shows the contents of major amino acids in plants exposed to flow and control. All most all amino acid (except Glu) contents were statistically similar among two treatments.

Table 10. Amino acids contents of *E. nuttallii* after exposure to mean flow and control for 30 days (values are in mean ± SD, n=3)

	Content (nmol gFW^{-1})	
Amino acid	Control	Control
Ala	206.45 ± 59.60	211.58 ±15.94
Arg	250.67 ± 111.21	242.72 ± 85.95
Asn	8667.36 ± 2061.78	9138.52 ± 877.83
Asp	1023.25 ± 220.70	1104.02 ± 134.38
Gln	733.10 ± 167.96	949.82 ± 9.45
Glu	407.42 ± 92.20	636.15 ± 74.59
Lys	29.71 ± 10.69	31.29 ± 5.63
Orn	34.63 ± 9.07	27.52 ± 7.43
Cys	0.15 ± 0.06	0.17 ± 0.12
Gly	185.46 ± 83.95	133.72 ± 37.49
His	95.62 ± 35.93	111.91 ± 25.19
Ile	27.07 ± 5.69	28.86 ± 6.44
Leu	30.55 ± 7.48	30.52 ± 9.28
Met	2.08 ± 1.52	2.38 ± 0.50
Phe	47.34 ± 7.99	55.61 ± 15.41
Pro	26.42 ± 7.27	30.61 ± 5.03
Ctl	5.78 ± 1.79	5.15 ± 0.78
GABA	117.33 ± 24.17	156.34 ± 70.90
Ser	762.02 ± 189.27	848.04 ± 31.08
Thr	270.64 ± 55.00	306.21 ± 50.42
Trp	10.52 ± 2.84	11.21 ± 2.49
Tyr	18.99 ± 4.36	19.83 ± 3.52
Val	41.82 ± 8.77	46.06 ± 9.54
TOTAL	12994.39	14128.24

Similar to previous experiment-II, the asparagine represents comparatively larger amount compared to other amino acids. Percentage contribution of Asn in control and flowing treatments were 66% and 64% respectively. Even though the total amino acid content in plants exposed to

mean flow was higher than that of control plants, it was not statistically significant. Contents of other major metabolites are presented in Table 11.

Table 11.major metabolites contents of *E. nuttallii* after exposure to mean flow and control for 30 days (values are in mean ± SD, n=3)

	Content (nmol gFW^{-1})	
Metabolite	Control	Flowing
2OG	1360.19 ± 602.79	1951.97 ± 41.81
Aconitate	118.86 ± 35.10	146.44 ± 15.33
Ascorbate	1059.56 ± 216.68	1530.82 ± 207.51
Citrate	5384.39 ± 1432.72	6587.34 ± 982.07
Fumarate	48.20 ± 16.87	58.54 ± 6.15
Isocitrate	57.22 ± 14.03	64.12 ± 2.12
Lactate	1298.29 ± 571.31	1433.03 ± 870.18
Malate	5301.99 ± 1387.32	6480.53 ± 894.41
Succinate	28.25 ± 8.55	36.31 ± 2.78
3PGA	74.61 ± 15.78	123.34 ± 41.71
DHAP	5.06 ± 4.02	11.89 ± 5.95
F6P	19.95 ± 9.34[b]	47.57 ± 12.41[a]
FBP	1.64 ± 0.67	3.70 ± 1.73
G1P	4.51 ± 2.01[b]	15.55 ± 4.52[a]
G-3-P	68.02 ± 21.17	41.70 ± 26.64
G6P	126.79 ± 43.45[b]	216.06 ± 31.78[a]
Pyruvate	135.15 ± 80.35	137.09 ± 61.15
PEP	27.43 ± 14.99[b]	83.23 ± 18.96[a]
6PG	6.22 ± 4.67	3.88 ± 1.58
Cinnamate	13.33 ± 1.79[b]	18.95 ± 2.40[a]
R5P	2.68 ± 1.19	1.39 ± 0.65
Ru5P	2.51 ± 0.41[a]	1.21 ± 0.61[b]
RuBP	14.19 ± 6.81	9.26 ± 2.18
Shikimate	10.42 ± 4.85	12.54 ± 3.91

Plants exposed to water flow accumulated significantly high contents some metabolites i.e. F-6-P, G-1-P, G-6-P, PEP and cinnamate (Table 11). However, the Ru-5-P content of control plants was significantly higher than that of plants exposed to water flow (Table 11). Therefore, water flowing basically causes to the glycolysis than on TCA cycle as observed in the same plants under water turbulence.

Chapter 5. Discussion

5.1. Experiment 1

5.1.1. **Selection of suitable nutrient condition for *E. nuttallii***

High nutrient concentration has been reported as one of the main factors that induces the oxidative stress in submerged macrophytes in eutrophic waters (Cao et al., 2007). Particularly, GPX, superoxide dismutase (SOD), CAT and APX prevent the oxidative damage in plants by scavenging reactive oxygen species (ROX). Therefore, an elevated level of GPX in plants exposed to a high nutrient concentration provides an evidence for the oxidative stress. Similar to present study, Zhang et al. (2010) observed an elevated level of GPX activity in *Potamogetton crispus*a fter growing under high nutrient condition. Further, the observed trend in plant growth responses agrees with the previous literature as inhibition of macrophytes growth was reported under elevated level of nutrients(Best, 1980; Smolders et al., 1996; Zhang et al., 2010). However, accumulation of H_2O_2 has been observed in different macrophytes species under various stress conditions (Ellawala et al., 2013; Wang et al., 2008; Zaman and Asaeda, 2013). As there was absent any stress in the control treatment, the stress responses of plants grown in a former condition were similar to that of plants exposed to low nutrient media. However, the observed stress in *E.nuttallii* under high nutrient concentration was further confirmed by the chlorophyll fluorescence analysis. Moreover, visual observation also confirmed that algae growth was prominent in a high nutrient condition and excessive growth of algae hampers the growth of macrophytes. Therefore, low nutrient concentrations of HNS (<5 %) are suitable to grow *E.nuttallii* in experimental culture systems due to their better growth and less oxidative stress.

5.1.2. **Stress responses of *E.nuttallii* against turbulence and mean flow**

Mechanical stress induced by turbulence and main flow reduced plant length (Figure 8), and the observed trend for shoot length in turbulence and control treatments agree with the previous observations reported for some other aquatic plant species (Ellawala et al., 2011; Ellawala et al., 2012; Ellawala et al., 2013) Growth response to mechanical stimuli is termed as thigmomorphogenesis and retardation of tissue elongation coupled to an increased radial

expansion is one of the common responses in terrestrial plants for mechanical stress. Therefore, radial expansion of stems observed in plants exposed to water movement could be a morphological adaptation to withstand against the hydrodynamic forces exerted on plants either by turbulence or main flow. Similar observations have been reported for terrestrial plants against mechanical stress (Biddington, 1986; Braam, 2005). Increased stem thickness could accompany the morphology of stem cortex cells (Figure 10) as the cortex cells of stressed plants were significantly bigger than those of control plants. Further, lignification has been identified as an adoptive defense mechanism against mechanical stress (Jaegher et al., 1985; Saidi et al., 2009). Therefore, increased lignin and cellulose levels in plants exposed to turbulence (Table 4) could be an adaptive mechanism which minimizes the mechanical damage caused by turbulence. Consequently, the elevated level of cellulose and lignin in plant shoots is thickening and the local cell wall is strengthening, which led to increased flow resistance in *E.nuttallii*. Our findings agree with the previous observation reported for young *V. spiralis* tested under laboratory conditions for turbulence (Ellawala et al., 2013).

The ROS levels in plants are increased by most biotic and abiotic stress vectors and consequently induce the activity of antioxidant enzymes. Under this context, phytohormones and antioxidants play an important role in the diverse processes as well as in stress responses in plants (Bari and Jones, 2009). Depending upon the severity of stress, ROS activates the signaling system either to suppress or activate the antioxidant enzymes in plants (Apel and Hirt, 2004; Delledonne et al., 2001; Jayakumar et al., 2006). Mechanical stress has an ability to induce the activity of GPX, CAT, APX and SOD in plants. For instance, Ellawala et al. (2011) observed an elevated level of antioxidant activity in some aquatic macrophytes after exposure to turbulence, while changes in antioxidant enzymes in tomato plants were observed by Saidi et al. (2009) in response to mechanical stress. Compared to control plants, the accumulation of H_2O_2 and the elevated levels of CAT and APX activity in turbulence induced plants (TR3) indicate the turbulence triggered oxidative stress in *E. nuttallii*.

Auxins are considered to be the prime candidate to control a stress-induced morphogenic response in plants (Kawano et al., 2003; Potters et al., 2007). Further, decline in IAA following a mechanical stress has also been reported for both terrestrial and aquatic plants(Champika

Ellawala et al., 2011; Saidi et al., 2010). Therefore, the lowest IAA concentration observed in plants exposed to turbulence agrees with the former findings. Further, the low concentration of IAA in turbulence-stressed plants could be responsible for their impaired growth compared to a control and flowing treatment. A significant negative correlation between IAA and shoot elongation has been reported for *V. spiralis*, and *E. nuttallii*(Champika Ellawala et al., 2011). Saidi et al. (2010) reported that the concentration and the distribution of IAA could be trigged by H_2O_2. In the present study, the highest H_2O_2 concentration and the lowest IAA were found in plants exposed to turbulence stress. APX is thought to play the most essential role in scavenging ROS and protecting cells in higher plants, algae, euglena and other organisms (Gill and Tuteja, 2010). APX consumes H_2O_2 along the Halliwell-Asada pathway to catalyze ascorbic acid forming monodehydrosacorbate (Apel and Hirt, 2004). Consequently, the elevated levels of APX in response to turbulence might influence the Halliwell-Asada pathway regulated activity in *E. nuttallii*. However, further studies are needed to explore this phenomenon for better clarification.

Compared to main flow, turbulence suppressed chlorophyll production in *E .nuttallii* because of the lowest chlorophyll content was observed in later plants. Accumulation of ROS might be a possible explanation behind this reduction as chloroplast is one of the main organelle that produces ROS in plant cells (Demidchik, 2015). Further, environmental stress has an ability to down regulation of photosynthetic gene and photosynthetic decline leading to expression of leaf senescence-related genes and senescence induction (Saibo et al., 2009; Sillanpää et al., 2005). Visual observation noticed that leaves of plants exposed to turbulence quickly became yellow with time. The reduction of the photosynthesis ability in plants exposed to turbulence compared to main flow was further explained by the observed trend in Fv/Fm values. Similar trends in chlorophyll and chlorophyll fluorescence have been observed in previous studies for *Eigeriadensa*, *V. spiralis*, and *Charafiriosa* against turbulence (Ellawala et al., 2011). Even though rapid photosynthesis was observed under low flow (0.02-0.5 cm/s) (Westlake, 1967), flow velocity employed in flowing treatment (TR1) was higher than that tested in the former study.

Gravity on the downstream slope of the water surface makes unidirectional flow in streams, and the flow pattern has implications on the community's structure, metabolism, physical resistance

and developments of aquatic macrophytes (Madsen et al., 1993; Sand-Jensen and Pedersen, 1999). In reality, mechanical stress induced by turbulence might have an impact on plants in any direction as turbulent fluctuations are independent from the direction. Therefore, the stress induced by turbulence might become more severe than that of main flow as the latter force is unidirectional. As a consequence, plants are in need of a strong adoptive defense mechanism that works against the mechanical stress driven by water movements, particularly against the turbulence. The present study observed increasing trends in antioxidant enzyme activities, H_2O_2, cellulose and lignin in response to main flow and turbulence under experimental conditions, and the observed trends were further supported by the field investigation.

5.2. Stress response of *M. spicatum* against turbulence in field condition (Field study-II)

Macrophytes were affected by a different degree of turbulence depending on their location. Control site (L1) was a pool where a smooth flow with the lowest turbulence was observed. All the other locations showed comparatively high magnitude of turbulence making a severe stress on plants compared to the control site. However, plant communities reduced flow turbulence inside and therefore different turbulence velocities were observed between inside and outside of plant stands where the similar observations reported in the literature (Sand-Jensen, 2008). Fluctuation in flow turbulence has ability to makes changes in growth and several other biochemical aspects of aquatic plants. However, plants are able to cope with such circumstances using various adaptive mechanisms such as morphological and biochemical alterations. Doyle (2001) reported that plants allocate a greater biomass to below-ground organs under mechanical stress and our findings agree with the former observation, as present study observed a strong negative correlation between biomass ratio and turbulence.

The elevated antioxidant activity and ROS in response to turbulence (Table 5) provides a clear evidence for the turbulence driven oxidative stress in macrophytes. However, the hydrodynamic forces exert on rooted aquatic plants is the main impact of water movement and the former impacts are relative to plant size and species. As an example, *E. nuttallii* showed an elevated level of antioxidant activity and the lowest concentration of IAA compared to other plants in control site. Therefore it can be suggested that the tolerance limit of *E. nuttalli* for turbulence might lower than that of other species. On the other hand, former findings suggested that the

preference of *E. nuttallii* for the stagnant waters rather than turbulent flowing condition. *V. spiralis* and *S. erectum* showed higher level of tolerance than other two species. Probably the morphological adaptations such as well-developed root system, ribbon like leaf structure which support to withstand in flowing waters (Doyle, 2001; Ke and Li, 2006) could explain former observation. Further, flow directed growth pattern of *V. spiralis* and *S. erectum* reduces the drag force depending on the angle between plant and the substrate (Madsen et al., 2001). Moreover, *S. erectum* grow as single shoot in flowing waters, which reduces the drag (Sand-Jensen, 2008) and it is an emergent aquatic plant having a hard shoot structure than other studied plants. However, *V. spiralis* was independent from the location in terms of stress response. Therefore, the plant morphology plays a critical role to overcome the mechanical stress induced by water movements where the present findings agree with the previous literature (Bornette and Puijalon, 2011; Nielsen et al., 2006).

Auxins are considered to be the prime candidate to control stress-induced morphogenic response in plants (Kawano et al., 2003; Potters et al., 2007). Decrease in IAA following a mechanical stress has been observed for both terrestrial and aquatic plants (Saidi et al., 2009) and a similar trend was observed in response to turbulence in the present study (Table 1). Turbulence might affect to the growth of *M. spicatum* as concentration of IAA content negatively correlated with the turbulence. Even though the growth responses were not observed in the present study, a significant negative correlation between IAA and shoot elongation in *V. spiralis*, and *E .nuttallii* was reported by Champika Ellawala et al. (2011). On the other hand, concentration and the distribution of IAA can be trigged by H_2O_2 while auxin catabolism in plants is hastened by the mechanically stimulated peroxidases (Saidi et al., 2010). We observed a significant correlations between IAA and H_2O_2 ($r=-0.712$, $p=0.009$) and IAA and APX ($r=-0.615$, $p<0.05$) for *M. spicatum* under for field conditions.

CAT and GPX activities of plants exposed to elevated level of turbulence were significantly higher than that of plants subjected to less turbulence (Table 5). The elevated level of peroxidase is a response indicator enzyme with higher affinity for H_2O_2 than CAT (Andrews et al., 2002). Further, peroxidases are involving in the cross-linking of hydroxyproline-rich glycoproteins with phenolic acids (Andrews et al., 2002; Zhang et al., 2007). Therefore peroxidase might contribute

to enhance plant defense mechanism against turbulence driven mechanical stress by scavenging ROS and stiffening cell wall by forming complex structural components such as lignin. That could explain the elevated level of lignin in plants grown in turbulence environments compared to control site. Particularly, high concentration of cellulose and lignin in *M. spicatum* shoots is thickening and local cell wall strengthening, led to increased flow-resistance which was similarly observed in experimental condition for *E. nuttallii*. Further, lignification has been identified as an adoptive defense mechanism against mechanical stress (Jaegher et al., 1985; Saidi et al., 2009). Therefore the increased lignin and cellulose levels in plants exposed to turbulence can be considered as an adaptation to minimize the mechanical damage caused by water movements and the field findings in consistent with the laboratory observations.

We have shown that the *M. spicatum* exposed to elevated level of turbulence led to a decreased content of total chlorophyll (Figure 15) and parallel increase in the levels of H_2O_2 (Table 5). Despite the chlorophyll content of L3-plant was statistically similar to that of L1-plants; the results suggested that the photosynthetic system of *M. spicatum* might affect in response to turbulence. Compared to the less stress control site (L1), minimum chlorophyll content was observed in plants in L4, L5 and L6 ($p<0.05$). Therefore loss of chlorophyll upon exposure to mechanical stress might cause by the elevated level of ROS via damaging either chloroplast structure or chlorophyll membrane. However, the effects of mechanical stress on chlorophyll have been clearly observed for *E. nuttallii* together with plasmolysis in apical leaf cells. Generally, stressed plants increase their carotenoid concentration to provide protection against the formation of free radicals and a decrease in total chlorophyll and in the ratio of chlorophyll: carotenoids is often observed (Ferrat et al., 2003). The former observation has been reported for some aquatic plants in response to stress caused by trace metals and herbicides (Nichols et al., 2000) while no information available for the stress induced by turbulence. Even though the relationship between chlorophyll and turbulence was not significant, the correlation was negative ($r=-0.370$, $p>0.05$).

Except for the PO_4^{3-} concentration, other water quality parameters remained in the same range for all study sites. It could be possible to observe similar conditions in all locations as study sites located within a short distance of a lotic system. Even though the PO_4^{3-}concentration was

significantly different among study sites, we did not observe any correlation between stress response and PO_4^{3-} concentration. Therefore it can be considered that the water quality may not significantly influence for the observed stress responses.

Gravity on the downstream slope of the water surface makes unidirectional flow in streams and the flow pattern has implications on the community structure, metabolism, physical resistance and developments of aquatic macrophytes (Madsen et al., 1993; Sand-Jensen and Pedersen, 1999). In reality, mechanical stress induced by turbulence might impact on plant in any direction as these fluctuations are independent from the direction. Therefore, the stress induced by turbulence might severe than that of main flow as latter force is unidirectional. As a consequence, plants are in need of strong adoptive mechanism to withstand in such circumstances, particularly against the water turbulence. Present study observed increasing trends in antioxidant enzyme activities, H_2O_2, cellulose and lignin in response to main flow and turbulence while significant relationships were only observed for turbulence. Although few biochemical responses of aquatic plants such as photosynthesis and respiration have been reported, information on stress response of plants to water flow is scare to our knowledge. However, further studies are needed for better clarifications as present study was only focused on *M. spicatum*. Taking together, our results suggest that mechanical stress induced by turbulence makes severe impact in macrophytes compared to main flow. These observation supported to the laboratory findings observed for *E. nuttalli* .

5.3. Experiment 2

5.3.1. **Effect of turbulence on plant growth and ultra-structure of plant cells**

Environmental stress is a potential factor that disrupts cellular structures and consequently impairs key physiological functions in plants (Larcher, 2003). These changes in plants result in various functional disorders such as the inhibition of photosynthesis, metabolic dysfunction, and damage to cellular structures, which lead to impaired growth, reduced fertility, and premature senescence (Krasensky and Jonak, 2012). We observed growth reductions in *E. nuttallii* (Figure 16) responding to turbulence, and the SER of the control plants was more than twofold higher than that of plants exposed to turbulence. This reduction in growth was consistent with the observations in the literature for the same species and for some other aquatic plants (Champika Ellawala et al., 2011; Ellawala et al., 2011; Ellawala et al., 2012; Ellawala et al., 2013). The complete plasmolysis that was observed in leaves after exposure to high turbulence might responsible for the impaired growth in the turbulence-stressed plants because the total chlorophyll content of *E. nuttallii* was reduced by approximately 40% with exposure to the mechanical stress of turbulence (Figure 11). Furthermore, an impaired photosystem-II was reported for same species together with the formation of ROS, and chloroplasts are very sensitive to injury resulting from ROS (Dinakar et al., 2012). Therefore, the chlorosis combined with the plasmolysis could explain the reduction in growth of *E. nuttallii* during exposure to turbulence.

The *Elodea* leaf has two cell layers, and the upper layer has comparatively larger cells than the lower cell layer (Luttge, 1983), and our findings were consistent with this observation. Plasmolysis is the separation of the living protoplasmic envelope from the cell wall, and different forms of plasmolysis occur in plant cells depending on two factors, i.e., the type of cell under investigation and the nature of the plasmolyticum that is used (Oparka, 1994). With exposure to high turbulence, the cytoplasm of the leaf cells accumulated locally as a ball (Figure 17 and Figure 18), which is the systrophe type of plasmolysis (Oparka, 1994). Furthermore, the systrophe type of plasmolysis occurs with the separation of the plasma membrane from the cell wall rather than osmotic withdrawal of water. The mechanical stress of turbulence continuously shook the cytoplasm of *E. nuttallii* and consequently detached the plasma membrane from the cell wall, which formed the systrophe type of plasmolysis in the leaf cells (Figure 18C). As reported by McNeil and Steinhardt (2003), plasma membranes are frequently disrupted by mechanical disturbances in the environment of the plants. Although plasmolysis in plants is a

known response to osmotic changes (Durand-Smet et al., 2014), there are no literature reports on the effects of mechanical stress on plasmolysis in aquatic macrophytes, in particular.

Additionally, the continuous shaking of the cytoplasm might reduce the growth and functional properties of chloroplasts, and comparatively larger chloroplasts were found in control plants than in plants exposed to turbulence. As the entire plant is continuously shaking under the turbulence, plants require structures that can withstand such circumstances. Thus, the plant body was strengthened (leaves and stem) with an increased epidermal cell wall thickness against the turbulence , and the remarkable increase in the cell wall thickness in both leaves and stems of turbulence-stressed plants suggested an adaptive mechanism to withstand the mechanical stress of turbulence. This observation was supported by the findings of experiment-I in which plants accumulated cellulose and lignin together with radial expansion in response to water movements.

The most remarkable difference between the control and turbulence affected plants was the amount of starch granules present in the chloroplasts of the stem (
Table 9 and Figure 18). The number and size of the starch granules increased in the stem chloroplasts with exposure to turbulence. The stems might assume the primary role of photosynthesis because the photosynthetic mechanism in apical leaves was severely damaged by the turbulence (Figure 17). However, stem cells might also synthesize large amounts of starch to synthesize structural components such as cellulose to strengthen the cell walls. Similar to the present study, Wample and Davis (1983) observed the accumulation of starch in sunflower (*Helianthus annuus* L) leaves responding to flooding stress. Moreover, Ariovich and Cresswell (1983) observed high levels of starch in the bundle sheath of chloroplasts with ammonium nitrogen as the source for nitrogen. Furthermore, salinity stress enhanced the accumulation of starch in the chloroplast of *Thellungiella halophila* (Wang et al., 2013). However, Asn caused an increase in the number and size of starch granules in the developing cotyledons of yellow and white lupine (Borek et al., 2013), similar to the observations of the present study. Therefore, the significant increase in the content of Asn (Figure 21) could support the accumulation of starch in stressed plants. The connection between the content of Asn and the accumulation of starch is not clear, however, and further studies are recommended for better clarification. The amino acids and other organic metabolites accumulated in response to stress and consequently, stressed plants

required large amounts of organic acids obtained from photosynthesis to incorporate inorganic nitrogen into the amino acids (Wang et al., 2013). On the other hand, starch is considered as a ROS scavenging substance (Parida and Das, 2005) and therefore, starch might also play a role in defense mechanism against turbulence driven oxidative stress.

5.3.2. **Effect of turbulence on major metabolites**

The synthesis of amino acids is of considerable interest as amino acids are essential for the synthesis of proteins and as precursors for a large array of metabolites with multiple functions in plant growth and various responses to stress (Less and Galili, 2008). Similar to present study, as a consequence of mechanical stress, the accumulation of amino acids and the reductions in plant growth coupled with the accumulation of solutes were also reported for terrestrial plants (Maggio et al., 2002; Wallace et al., 1984). However, information on the accumulation of amino acids and other metabolites in aquatic macrophytes in response to the mechanical stress is not available in the literature. Asn was the dominant amino acid in *E. nuttallii*. Similarly, for *E. canadensis*, a high content of Asn (76%) was reported, and the plant was identified as an asparagine accumulating plant (Janauer, 1977). Therefore, the high content of Asn in *E. nuttallii* was consistent with the concentration in a sister species, and thus *E. nuttallii* is a potential asparagine accumulating plant. Plants accumulate asparagine in response to environmental stress (Brouquisse et al., 1992; Lea et al., 2007; Martinelli et al., 2007), and the accumulation of Asn in *E. nuttallii* was therefore consistent with the literature on plant response to stress.

Either an increase in citrate production or a reduction in citrate catabolism (Neumann et al., 2000) was responsible for the accumulation of citrate in *E. nuttallii* (

Figure 21citrate). The content of isocitrate was a trend in decline (

Figure 21, isocitrate) compared with the content of citrate, and isocitrate dehydrogenase might be activated in stressed plants. Anoop et al. (2003) reported that the turnover of citrate was regulated by the enzymes aconitase and isocitrate dehydrogenase. Ascorbate is the precursor of oxalic acid in several oxalate-calcium accumulating plants and there are several pathways for the biosynthesis of oxalate in plants. The ascorbate-oxalate pathway (ascorbate pathway) is recognized as the primary route to form oxalate in some aquatic plants such as *Lemna minor* and

Pistia stratiotes (Franceschi and Frank, 1995; Keates et al., 2000; Kostman et al., 2001). Moreover, ascorbate is one of the major metabolites that scavenge ROS under oxidative stress (Gill and Tuteja, 2010; Mittler et al., 2004). Therefore, the elevated levels of ascorbate in plants exposed to turbulence (low and high) could indicate oxidative stress caused by the turbulence. The contents of both ascorbate and oxalate in the plants exposed to turbulence were significantly higher than those of control plants (

Figure 21), and a significant positive correlation between contents of oxalate and ascorbate in *E. nuttallii* was found (R=0.868, $p < 0.01$). Therefore, the excess ascorbate could induce oxalate biosynthesis in plants grew in turbulence. The content of the dominant amino acid, asparagine, of *E. nuttallii* was significantly positively correlated with the oxalate content (R=0.641, $p < 0.05$) and with the total amino acid content (R= 0.715, $p< 0.05$). Additionally, there were strong correlations between oxalate and amino acids because the synthesis of oxalate requires nitrogen assimilation (Miyagi et al., 2010; Miyagi et al., 2013).

Glutamate is a precursor of Gln, GABA, Pro and His. Therefore, the efflux of GABA and His could explain the declining trends in Glu and Gln contents. Similar to the present study, decreases in Glu were reported for wheat seedlings after exposure to cold stress (Naidu et al., 1991). The GABA and proline play important roles in protecting plants during environmental stress (Liu et al., 2011), and the role of GABA is emphasized as a protectant against ROS in stressed plants (Rhodes et al., 1999). Therefore, the increasing trend in the accumulation of GABA that was observed could be an adaptive mechanism to scavenge the excess ROS in stressed plants because the turbulence causes oxidative stress in aquatic plants. The increase in GABA content together with impaired growth as a consequence of turbulence is consistent with the previous literature (Braam, 1992). Moreover, Wallace et al. (1984) observed a significant increase in GABA concentration in soybean leaves after exposure to mechanical stimulation. However, GABA is also synthesized from Glu through a biochemical pathway catalyzed by glutamate decarboxylase (GAD). The activity of GAD is highly dependent upon the intracellular Ca^{2+}calmodulin activity (Ling et al., 1994), and the plant intracellular Ca^{2+} concentration is highly affected by different types of mechanical stresses (Braam, 2005). In particular, the mechanical stress induced by the turbulence might result in rapid increases in intracellular Ca^{2+} concentrations and consequently result in the accumulation of GAD. However, much more work

is required to clarify the mechanism responsible for the intracellular Ca^{2+} concentrations and the GABA activity in *E. nuttallii* as this phenomenon was not explored in the present study. Although Pro is the dominant amino acid involved in the scavenging of ROS in many stressful conditions, GABA is the prominent scavenger of ROS under some circumstances (Liu et al., 2011). In response to the turbulence in the present study, declining and increasing trends for Pro and GABA, respectively were observed. Similarly, Zaman and Asaeda (2013) reported a declining trend in Pro content for the same plant after exposure to ammonium stress. However, plant species differ considerably in the amount of Pro that accumulates with stress, and a clear relationship between the ability to accumulate Pro with the imposition of stress and stress tolerance among species has not been identified Maggio et al. (2002).

5.4. Experiment-III

5.4.1. **Effect of mean flow on plant growth, ultrastructure and metabolism**

Although the main direct effects of water movements are the hydrodynamic force it exerts on rooted macrophytes, their impact depends on two factors; (i) the direction and (ii) the variability of the force (Bornette and Puijalon, 2001). The flowing condition employed in this step provides flow directional stimulation for growing *E. nuttallii*. On the other hand *E. nuttallii* failed to grow against the water flow, and all *Elodea* stands grew in parallel to the water flow in the central part of the tubes (Figure 22). Compared to control, visual observation revealed that, the plants in flowing tubes were in a tension due to water flow. Even though the total shoots length of plant strands were not significantly different among control and flowing treatment, the shoot elongation has been affected by the mean flow. Former observation explained by the variations observed for the intermodal lengths. Puijalon et al. (2005) proposed that the hydrodynamic performance of a plant is its ability to withstand hydrodynamic forces induced by water movement when it exposed to flow stress. Similar to other sessile organisms, aquatic macrophytes growing in flowing environments experience two mechanical forces; (i) a drag force (parallel to flow) and (ii) a lift force (perpendicular to flow) (Koehl, 1982). Therefore, the alteration plant growth by elongating their internode could be a morphological adjustment that leads to decreased plant drag. On the other hand, former observation could also be a reduction of hydrodynamic resistance through increased flexibility. The studied macrophyte; *E.*

nuttallii is a flexible aquatic plant and therefore, it might have lower drag because of their flexibility. In the present study, we did not observe significant difference in biomass allocation between above ground and below ground portion responding to water flow.

Even though the chlorophyll contents were not significantly different among two treatments, there was a declining trend in chlorophyll content in response to increased mean flow. When compared with experiment-I, the low flow (~3 cms^{-1}) has beneficial to *E. nuttallii* in terms of chlorophyll production. Despite the total chlorophyll content in low flow plants (170.5 µg/ gFW) was not significantly different from the control plants (149.01µg/ gFW), the total chlorophyll in plants exposed to high magnitude of mean flow (~10 cms^{-1}) was significantly lower than former two conditions (99.0 µg/ gFW). The trend observed for chlorophyll production was in consistent with the published literature. At low velocity as opposed to stagnant water, the reduction in thickness of the diffusive boundary layer (DBL) surrounding the macrophyte leaves and an increase in aeration of the water with atmospheric carbon dioxide improves photosynthetic rates. However, this positive relationship between photosynthesis and current velocity applies up to a saturating velocity (i.e. above a critical DBL thickness) (Fonseca and Kenworthy, 1987; Koch, 1994; Wheeler, 1980). The critical current velocity value changes for plant species, even for varieties, depending on their physiology and/or morphology. None of these previous studies linked the photosynthetic limitation at higher flow or turbulent velocities to the plant physiological factors, more especially enzymatic stress and impaired function of photosynthetic apparatus. However, the present study showed some evidences for the effect of main flow and turbulence on the chloroplast apparatus, chlorophyll production and the plant stress responses against the mechanical stress induced by those forces.

According to metabolome analysis, present study revealed that the interaction between water flowing and *E. nuttallii*, changes the contents of some metabolites in the glycolysis. For further, F-6-P, G-1-P, G-6-P and PEP, of plants exposed to water flow was increased compared to plants grew in stagnant waters (control). Almost all metabolite contents (Table 10 and Table 11) in the TCA cycle were not significantly different between two treatments (control and flow). However, significant increment in the content of cinnamate in plants exposed to water flow indicates the cause of water flow on the shikimate pathway of *E. nuttallii*. Compared to control plants which

were in the stagnant condition, an increased peroxidase (APX) activity together with high content of lignin was observed in plants exposed to flowing in the experiment-1. As peroxidases stimulate the lignin production, plants might strengthen their plant body against water movements via lignification as noticed in previous experiment 1. Except amino acids, there was an increasing trend in all most all other major metabolite contents when exposed to water flow. However, the differences between two treatments in this regard were not statistically significant. Even though the TCA cycle was identified as one of the main stress response pathways responding to various stresses, flow driven mechanical stress was not significant in this regard compared to turbulence. Therefore, flowing might influence to plant metabolism via shikimate pathway. Further studies are recommended for more information.

Conclusions and future research needs

Laboratory findings together with field observations suggest that the mechanical stress driven by the turbulence make significant impact to the physiology of aquatic macrophytesand the oxidative stress highly correlate to the magnitude of turbulence. Therefore, mechanical stress induced by turbulence could be considered as one of the main abiotic determinant which decides the macrophytes assemblages in lotic systems. Plant responses to mechanical stress induced by water movements depends upon the plant species and therefore, he tolerance limit of plant either to main flow and turbulence vary with species. The elevated level of antioxidant enzymes in particular, catalase, peroxidase, indole acetic acid oxidase and ascorbic peroxidase together with increased hydrogen peroxide content reflect the water movements driven (turbulence and mean flow) oxidative stress in *E. nuttallii* under experimental condition and some other species in natural waters. Growth reduction together with radial expansion of plant stem with increased lignin and cellulose was an adaptive mechanism to withstand in turbulent waters.

The mechanical stress triggered by turbulence led to accumulate several metabolites in plant body. Especially, ascorbate, GABA and Asn, accumulate in *E. nuttallii*altering several metabolic pathways of plant, in particular the isocitrate–oxalate, the Glu-His, the Glu-GABA, ascorbate-oxalate and the Asp-Asn pathways. Further, plant accumulated starch granules in stem and leaf chloroplasts in stress conditions whereas these accumulations were prominent in stems under elevated magnitude of turbulence.

Beside the biochemical properties, water movements make significant impact to the structural alteration in aquatic plants. Under this context, turbulence makes severe damages to plant ultrastructure such as plasmolysis and thickening cell wall. The stems and leaves thickened the cell walls as an adaptive mechanism against the turbulence. Present study revealed that, water movements (turbulence and main flow) significantly contributed to the alteration in the plant metabolism, oxidative stress and defense mechanisms and the architecture of plant cells. Therefore, present findings offer insights to explain the importance of water movements on aquatic plants, in particular on their function and the cell structure.

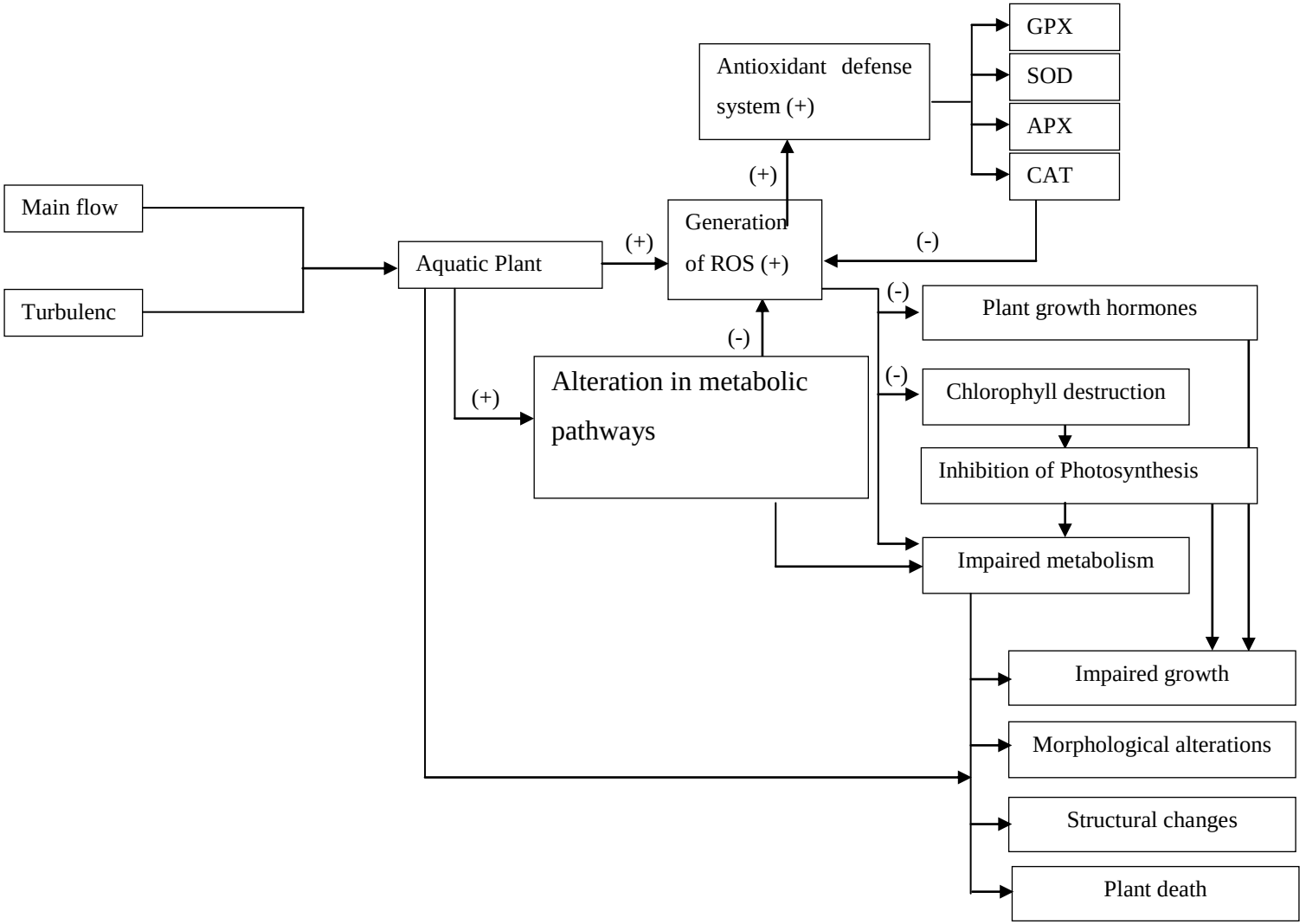

Figure 27.Cause and effect relationship between abiotic stress vectors and plant responses (+ and – marks indicates positive and negative impact)

Recommendations

1. Genomic studies are needed for further clarification for the stress responses against water movements as the plant response to a particular stress vector depends upon genetically determined reaction norms of plants.

2. As Ca^{2+} activated channels are important in plant stress responses against mechanical stresses, further explorations would suggest to study the Ca^{2+} sensory proteins such as calmodulin in response to turbulence and mean flow.

3. Even though the starch accumulation was reported in the present study, further studies are recommended to clarify the underline mechanism behind this phenomenon. On the other hand, present study merely focused on *E. nuttallii* and therefore observations in this regard in other macrophytes are recommended.

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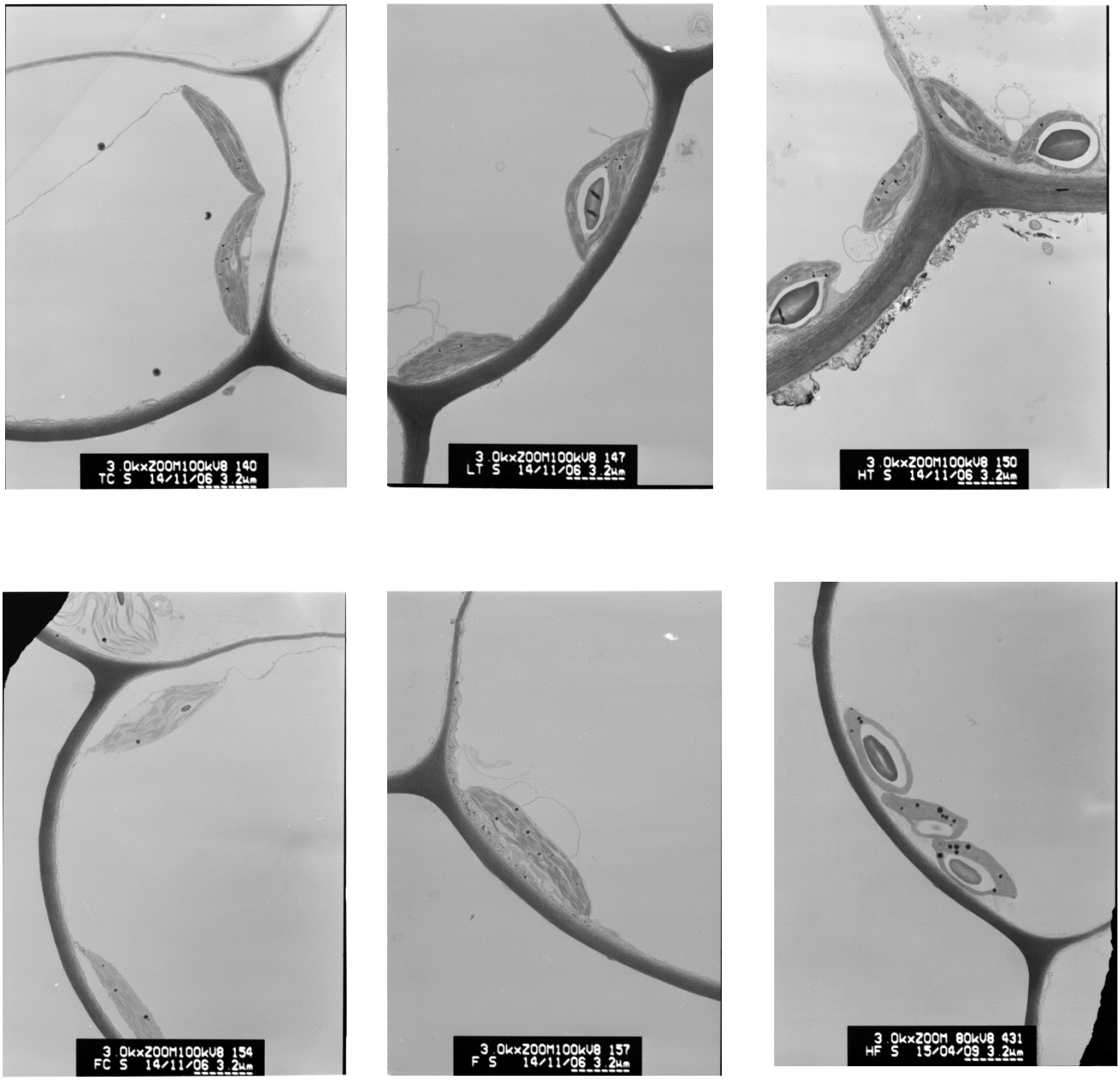
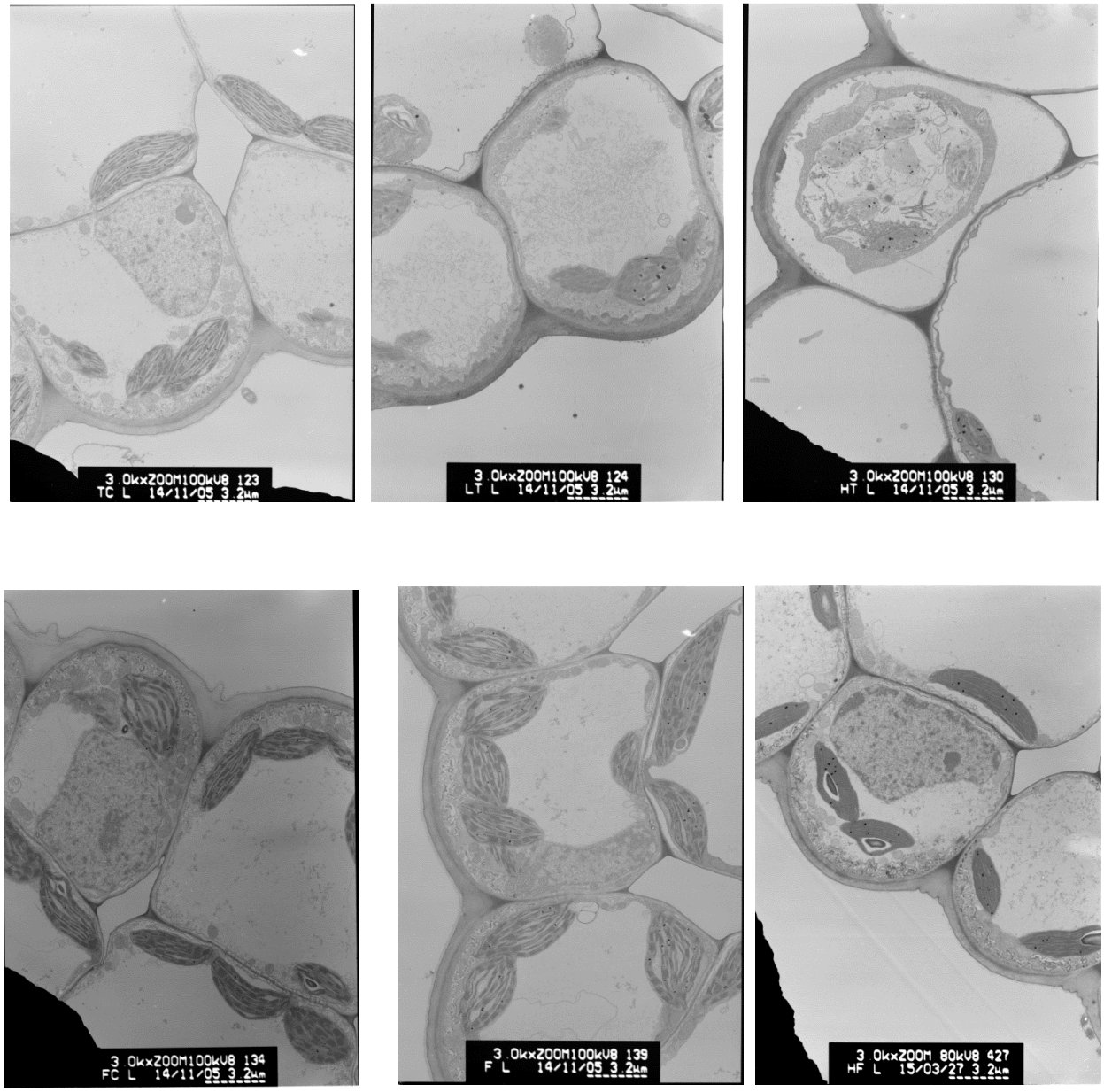
Appendix



Electron microscopic pictures of *E. nuttalli* stem (TC S: turbulence control, LT S: low turbulence stem, HT S: high turbulence stem, FC S: flow control stem, FS: low flow stem, HF S: high flow stem)



Electron microscopic pictures of *E. nuttalli* leaves (TC L: turbulence control leaf, LT L: low turbulence leaf, HT L: high turbulence leaf, FC S: flow control leaf, FL: low flow leaf, HF L: high flow Leaf)