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PRIMARY RESEARCH PAPER

Growth and stress responses of Nuttall's waterweed *Elodea nuttallii* (Planch) St. John to water movements

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Abstract Understanding the interactions between aquatic plants and environmental factors is important to clarify aquatic ecosystem functioning. The mechanisms governing the interactions between water flow and plants are not yet fully understood, and the responses of plants to main flow (without turbulence) compared to turbulence are largely unknown. Here, we compared the growth and stress responses of the aquatic macrophyte *Elodea nuttallii* to exposure to turbulence and main flow. Turbulence and main flow were generated using a vertically oscillating horizontal grid and a recirculating system, respectively and the experiment lasted for 3 weeks. A decrease in shoot elongation coupled with an increase in radial expansion was observed in plants exposed to water movements. These effects were further accompanied by significant increases in cellulose and lignin. 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. The turbulence triggered stress in *E. nuttallii* is more severe than that induced by main flow. Our findings can offer insights for explaining the habitat preferences of macrophytes and contribute to a better planning of the criteria that benefit in aquatic ecosystem management.

Keywords Turbulence · Main flow · *Elodea nuttallii* · Stress · Antioxidant

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Introduction

Aquatic macrophytes play a crucial role in maintaining the integrity of aquatic ecosystems. The main ecological functions of aquatic plants include the provisioning of habitats, refuge and food for fish and invertebrates, primary production and the regulation of sediment transportation and contributions to biogeochemical cycles (Bornette & Puijalon, 2011; Folkard, 2011; Nepf, 2012). Macrophytes are subjected to a wide range of fluctuations in biotic and abiotic factors such as water movement, water level, substrate characteristics, nutrient availability in both the sediment and water column, light penetration, UV irradiation, heavy metals, temperature, etc. (Barko et al., 1982; Dale, 1986; Madsen et al., 2001; Handley & Davy, 2002; Babu et al., 2003; Asaeda et al., 2005; Cao et al., 2007; Mony et al., 2007; Šraj-Kržič et al., 2007; Thomaz et al., 2007; Zhang et al., 2010; Olson et al., 2012). Plants are persistently challenged by environmental stresses due to the highly dynamic

conditions of aquatic systems and the non-motile nature of aquatic plants (Chehab et al., 2009). Water movement is a ubiquitous environmental factor for submersed macrophytes, and flow-driven drag and lift forces can have either a positive or negative impact on plants depending on their magnitude. For instance, improved photosynthesis activity has been observed under low flow velocities (Westlake, 1967), whereas long-term exposure causes alterations in the growth, physiology, and morphology of plants because of the associated mechanical stress. Furthermore, variations in the biomass allocation and growth performance of aquatic plants have been observed in response to flow-driven mechanical stresses (Asaeda et al., 2010a, b; Bornette & Puijalon, 2011).

In addition to morphology, biochemical evidence has been widely employed to evaluate plant stress responses. Phytohormones and antioxidants in particular are involved in diverse biochemical processes as well as in various biotic and abiotic stress response pathways (Bari & Jones, 2009), which play an important role in plant stress physiology. Furthermore, an elevated level of reactive oxygen species (ROS) appears to occur in plants under stress conditions. For example, turbulence-induced mechanical stimulation of *E. densa* causes an increase in the in vivo levels of H_2O_2 (Champika Ellawala et al., 2011). Zaman & Asaeda (2013) also observed an elevated level of ROS in *Elodea nuttallii* after exposure to an anoxic environment. Plants initially sense these stimuli and then launch appropriate responses such as avoiding obstacles, clinging to support structures or activating a biochemical defense system. However, plants are able to protect against oxidative damage from ROS immediately as well as over time by scavenging the ROS. In this context, enzymatic antioxidants such as catalase (CAT), peroxidases (POD), superoxide dismutase (SOD), ascorbic peroxidase (APX), glutathione, and non-enzymatic antioxidants including ascorbic acid, glutathione, proline, and carotenoids are important. SOD catalyzes the dismutation of two molecules of superoxide into oxygen and H_2O_2, while CAT and POD scavenge H_2O_2. However, overproduction of ROS causes damage to proteins, lipids, carbohydrates, and DNA (Gill & Tuteja, 2010).

During their lifetime, aquatic plants face a vast range of challenges that perturb the equilibrium between the scavenging and production of ROS. Among these, drought, salinity, extreme temperatures, nutrient availability, redox conditions, UV radiation, herbicides, and heavy metals have been studied in the literature (Lin & Kao, 2000; Babu et al., 2003; Panda & Khan, 2004; Zaman & Asaeda, 2013). Although most lake, river, and estuarine flow motions are turbulent (Horne & Goldman, 1994), macrophytes are challenged by flow and turbulence simultaneously. Temporal variability at any point in a stream leads to rapid fluctuations of the flow velocity with the passage of turbulent eddies (Sand-Jensen & Pedersen, 1999). 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 being commonly cited as abiotic determinants of aquatic macrophyte assemblages (Chambers et al., 1991; Green, 2005; Thomaz et al., 2007; Olson et al., 2012), little attention has been paid to evaluate the plant stress in response to water movement (Champika Ellawala et al., 2011; Ellawala et al., 2011, 2013). As a result, the mechanisms governing flow/plant interactions are not yet fully understood, and the responses of plants to main flow (without turbulence) compared to turbulence are largely unknown. *E. nuttallii* is one of the most common invasive submerged plants in lakes, ponds, rivers, and channels in Japan (Kadono, 1994). Acquiring knowledge and understanding the interactions between aquatic plants and environmental factors is important for aquatic ecosystem management. Therefore, the aim of the present study was to compare the growth and stress responses of the aquatic macrophyte *E. nuttallii* to exposure to turbulence and main flow.

Materials and methods

This research consists of two experiments followed by a field investigation. Presence of excess nutrients in the growing medium has a potential to create oxidative stress in plants (Cao et al., 2007; Xiong et al., 2010). In this context, application of the nutrient medium which provides better plant growth without stress is important for plant stress studies. Therefore, the aim of the first experiment was to select a suitable nutrition medium to be employed in the subsequent main experiments. The second experiment was designed to compare the stress responses of plants after exposure to turbulence and to main flow. Finally, a field

investigation was conducted along the Moto-Arakawa River (360.7′30.1″N, 1,390.24′20″E), a tributary of the Arakawa River in southern Saitama, Japan, to compare the experimental results with field conditions. All of the experimental procedures and analyses were conducted using the research facilities available at the Ecological Engineering Laboratory, Saitama University, Japan, from March to December 2013.

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 & Amon (1938). There were six treatments (T1–T6) according to the percentage of HNS in the culture medium. T1–T6 contained 0 (control), 5, 10, 20, 50, and 100% HNS, respectively, with T1 containing only distilled water. The six treatments, each with two replicates ($n = 2$), were randomly allocated into 12 (6×2) plastic beakers (2 l) in a complete randomized design (CRD). Approximately 500 g of commercial river sand (90% $<$ 1 mm) was used as the substrate. The commercial sand was purchased from the local market (DIY, Doite, Japan), washed three times using tap water followed by distilled water, and then soaked in distilled water for 24 h before being used.

Experimental plants were obtained from a laboratory-maintained culture tank. Six apical tips of *E. nuttallii* of similar size (initial length (IL) $\sim$5 cm) were planted in each beaker. The light intensity was maintained at $\sim$100 µmol m^{-2} s^{-1} using florescence lamps with a photoperiod of 12 h light and 12 h dark. 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)], plant biomass and stress response were measured. Plant stress response was measured using chlorophyll fluorescence, the concentration of H_2O_2 and peroxidase (POD) activity. The procedures for plant extraction and the determination of H_2O_2 concentration and POD activity are described in "Experiment 2" section.

Chlorophyll fluorescence was determined using a chlorophyll fluorescence imaging technique (FC 1000-H; Photon Systems Instruments, Czech Republic) that involves auto-image segmentation. Cuttings of *E. nuttallii* were dark-adapted for 20 min, and the maximum quantum efficiency of photo-system II photochemistry (F_v/F_m) was calculated using the following equation (DeEll & Toivonen, 2003):

$$\frac{F_v}{F_m} = \frac{(F_m - F_o)}{F_m},$$

where F_v, F_m, and F_o are the variable, maximum, and minimum fluorescence in dark-adapted states, respectively.

Experiment 2

Four treatments (TR1–TR4), each with three replicates ($n = 3$), were established as follows:

TR1 Plants were exposed to main flow (without turbulence) using a specially designed recirculating system.
TR2 Control treatment for TR1 in which plants were exposed to stagnant conditions using the same experimental setup as used in TR1.
TR3 Plants were exposed to turbulence (without main flow) using an oscillatory grid attached to a microcosm.
TR4 Control treatment for TR3 in which plants were grown in stagnant water using the same experimental setup as in TR3.

The turbulence velocity for TR3 was selected after considering the velocity fluctuations measured in macrophyte stands in natural environments, and the main flow velocity for TR1 was designed to create the same magnitude of turbulence employed in TR3. All treatments were subjected to a light regime of 12 h dark and 12 h light. Light intensity was kept at 100-110 µmol m^{-2} s^{-1}, and the temperature was maintained at 23–24°C. On the basis of Experiment 1, 5% HNS was used as the nutrient medium for culturing plants. Over the experimental period, the pH ranged from 7.7 to 8.2.

Experimental setup of TR1 (main flow treatment)

Figure 1 illustrates the experimental setup employed to create a flowing condition in TR1. The setup consists of a transparent opaque pipe (length: 40 cm and internal 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 the inlet and

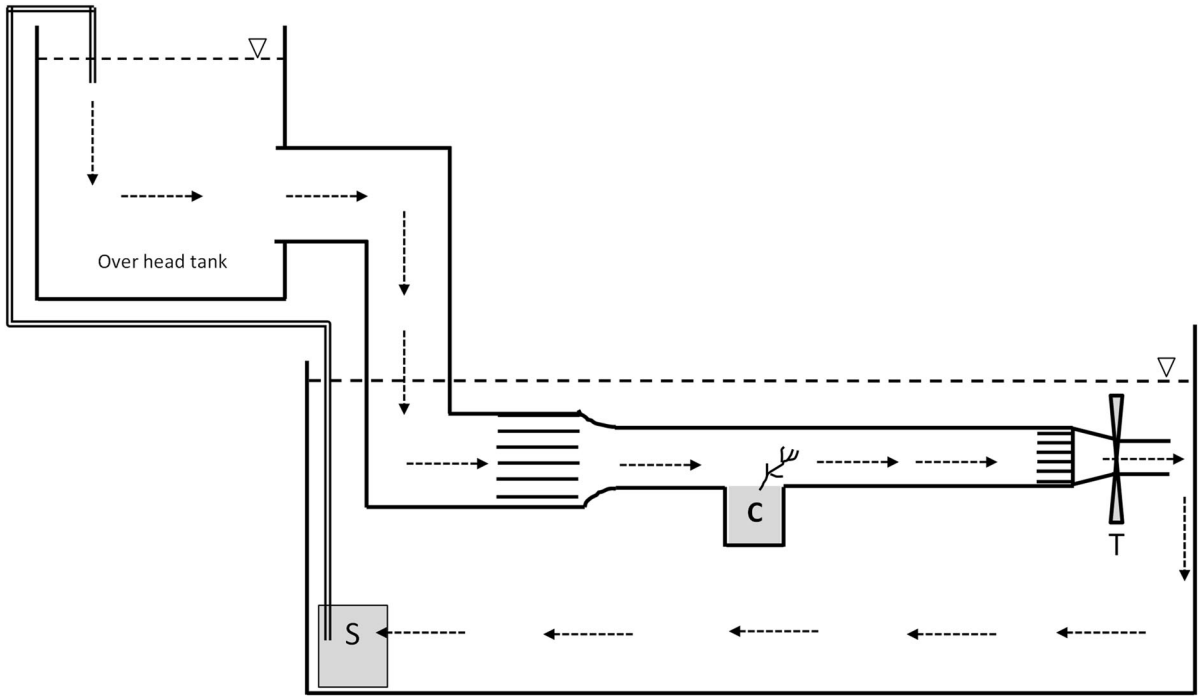


Fig. 1 Diagrammatic representation of the experimental set up used in TR1 (*arrows* indicate water flow)

outlet of the transparent pipe to minimize turbulence (Binzer et al., 2005). The whole setup was placed in a large glass tank (150 × 50 × 50 cm^3) and connected to an overhead tank (50 l). Water circulation was maintained by a submersible water pump (*S*), and flow velocity was adjusted using a valve (*T*) fixed at the outlet of the transparent tube. The water level inside the tank (45 cm) was kept above the transparent tubes. All replicate tubes of TR1 and TR2 (six tubes) were fixed in a CRD in the same glass tank. The tubes of the control treatment (TR2) were not connected to the overhead tank to maintain stagnant conditions.

The water flow velocity inside the transparent tube was measured every 7.5 cm from the PVC pot (*C*) using a two-dimensional electromagnetic current meter (EMCM) (SF-5712, Tokyo-Keiosoku Corporation, Tokyo, Japan). At each point, velocity fluctuations were recorded at every 0.5 cm depth interval from the surface of the pipe for 2 min. 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. The Reynolds number at each depth was also calculated using the following equation:

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

where *Re* is the Reynolds number (unit less), ρ is the density (g m^{-3}), *U* is the flow velocity (cm s^{-1}), *D* is the pipe diameter (cm), and μ is the dynamic viscosity (g m^{-1} s^{-1}).

Experimental setup of TR3 (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 with 5% HNS as the substrate. The water level was maintained at 17 cm above the substrate. The turbulence condition inside the microcosm was generated using a vertically oscillating horizontal grid (oscillating frequency was 2 Hz) according to published literature (Ellawala et al., 2011, 2013). The microcosm employed for generating turbulence was relatively small, but working at that particular scale was necessary to use the DC motors under these laboratory conditions. The

horizontal velocity profile of the microcosm was measured using the same EMCM as in TR1 at six different points, which were symmetrically distributed over the area. At 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 six measurements. Each of the three replicates of TR3 and TR4 was randomly located in a CRD.

Experimental plant and growing conditions

E. nuttallii plants were collected from the Moto-Arakawa River, Japan. Before culturing, plants were washed with de-chlorinated tap water to remove debris, and attached algae were removed with the aid of forceps. After that, plants were cultured in glass aquaria for nearly 1 month under laboratory conditions for use in the subsequent main experiments. After this growing period, the apical tips were clipped and used for the experiment. Before being transplanted into the experimental units, plants were observed for the presence of algae. Six similar-sized apical tips (3–5 cm) were then planted in PVC pots (for TR1 and TR2) or in microcosms (for TR3 and TR4). Before starting the experiments, plants were acclimatized to experimental conditions for 1 week. After being acclimatized, plants were exposed to either main flow (TR1), turbulence (TR3), or control conditions (TR2 and TR4) for 21 days. The experiment was stopped after 21 days because the apical tips of some plants in TR3 were close to touching the oscillating grid.

Growth measurements and sampling

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

Pigment extraction, chlorophyll fluorescence, and stress response assays

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

Sample preparation and stress assays

For the CAT, APX, POD, and H_2O_2 assays, fresh plant shoots were extracted ($\sim$500 mg) in an ice cold phosphate buffer (50 mM, pH 6.0) that contained polyvinylpyrrolidone (PVP). The IAA content of the apical tips of *E. nuttallii* ($\sim$1 g) was assayed after extracting the tips in distilled water. All extractions were centrifuged at 5,000 $\times$ g for 20 min at 4°C. The supernatant was separated and stored at −80°C until analysis. For every assay, each extract was analyzed in duplicate, and the results are presented on a fresh weight (FW) basis.

CAT, APX, POD, H_2O_2, and IAA assays

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. The CAT activity was calculated using the extinction coefficient of 40 mM^{-1} cm^{-1}.

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. The decrease in absorbance at 290 nm was recorded every 15 s. The APX activity was calculated using the extinction coefficient of 2.8 mM^{-1} cm^{-1}.

POD activity was measured based on guaiacol oxidation according to MacAdam et al. (1992). The reaction mixture contained 3.0 ml of 50 mM potassium phosphate buffer (pH 6), 40 µl of 30 mM H_2O_2 and 50 µl of 0.2 M guaiacol. The reaction was initiated by adding 100 µl of enzyme extract, and the absorbance was measured immediately and then every 15 s for 3 min. The rate of absorbance change was calculated, and the POD activity was determined using the extinction coefficient of 26.6 mM^{-1} cm^{-1}. CAT, APX, and POD activities are presented using the SI unit, nkat/g FW (Dybkaer, 2001).

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 sulfate in 20% H_2SO_4 (v/v). The mixture was centrifuged at 5,000×*g* for 15 min at room temperature, and the intensity of the resulting yellow color was measured at 410 nm. The H_2O_2 concentration was estimated using a standard curve prepared from known concentrations of H_2O_2. The concentrations of H_2O_2 are presented as µmol/g FW.

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

Cellulose and lignin

The cellulose content of the plant shoots was measured according to Updegraff (1969), and lignin was assayed following the improved acetyl bromide procedure (Morrison et al., 1995; Iiyama & Wallis, 1988). For each treatment, replicates were pooled together and analyzed in triplicate because they did not include enough dry weight to analyze individually.

Field investigation

Two locations (L1 and L2) along a stretch of the tributary were selected on the basis of two factors: the presence of *E. nuttallii* and the turbulence condition. At each location, two randomly selected quadrats (50 × 50 cm^2) were marked using poles and ropes. Surface velocity fluctuations within the plant stands (3–4 cm below the surface) were recorded for 5 min using the EMCM to calculate the turbulence velocity. After recording velocity fluctuations, the plants inside the quadrats were carefully removed to prepare plant extracts for the subsequent stress assays. All of the extractions were performed in the field on an ice bath using a chilled mortar and pestle. After extraction using the procedure described previously, all samples were immediately transported to the laboratory under freezing conditions.

Statistical analysis

Data were tested for normality using the Shapiro–Wilk test prior to beginning the statistical analysis. The results are presented as the mean ± SD ($n = 3$). Data were subjected to one-way analysis of variance (one-way ANOVA) followed by Duncan's multiple range test to evaluate the mean difference at a 0.05 significance level. The Student's *t* test was used to compare the two study sites investigated in the field survey. Statistical analyses were performed using SPSS version 16.

Results

Experiment 1

The highest biomass was found in plants grown with 5, 10, and 20% HNS (Table 1), and the total biomass values of the plants in these treatments were not significantly different. In contrast, the biomass of plants grown under both an excess (50 and 100%) and deficit (0%) of HNS exhibited lower biomass values that did not significantly different from each other (Table 1).

The shoot lengths of plants grown in HNS concentrations below 20% were statistically similar and significantly longer than those grown in high concentrations (Table 1). The shoot lengths of the plants in

Table 1 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	<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}	11.33	<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^{b}	5.3	<0.05
H_2O_2 concentration (μmol/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	<0.05
Peroxidase activity (nkat/g FW)	1.59 ± 0.19^{b}	1.35 ± 0.11^{b}	2.01 ± 0.58^{ab}	1.73 ± 0.47^{a}	2.90 ± 0.19^{a}	3.01 ± 0.65^{a}	5.87	<0.05

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

the latter two high-concentration treatments (50 and 100%) were not significantly different. The observed trend in the SER closely agrees with the variation in final shoot length. The lowest SER was observed in plants grown in 50% HNS, and it was statistically similar to that of control plants. However, the SER of control plants did not significantly differ from the SER of plants grown at HNS concentrations below 20%.

The H_2O_2 concentration gradually increased with increasing nutrient availability in the growth medium (Table 1). The highest H_2O_2 concentration was recorded in plants grown in 100% HNS medium, and the H_2O_2 concentration in plants grown in 50% HNS medium was not significantly different from the 100% group. Significantly lower H_2O_2 levels were observed when plants were grown in media that contained less than 20% HNS, and the H_2O_2 concentrations of plants in low-nutrient conditions (0–20%) were not significantly different from each other (Table 1).

Plants grown in 10, 20, 50, and 100% HNS exhibited the highest POD activity, and those activity levels were statistically similar (Table 1). The POD activity of plants in 10% HNS was similar to that of plants in the 0 and 5% treatments, which displayed the lowest POD activity.

The average maximum quantum efficiency values of photo-system II photochemistry (F_v/F_m) of the 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 F_v/F_m value, which was nearly 0.80, was observed for plants grown in 5 and 10% nutrition media compared to the control and other treatments. As there was no significant difference in the growth and stress responses, both 5 and 10% HNS are suitable for growing *E. nuttallii* for experimental purposes. The chemical requirement to prepare 10% HNS is twofold higher than to prepare 5% HNS. Therefore, we selected 5% HNS as the growing medium for the subsequent main experiment for two reasons: (i) its cost effectiveness and (ii) the use of a smaller amount of chemical is more environmentally friendly.

Experiment 2

Velocity profiles

In TR1, the highest flow velocity (2.5–2.7 cm s^{-1}) was observed in the middle region of the tube (Fig. 2A), and the turbulence inside the pipe was always less than 0.22 cm s^{-1} (Fig. 2B).

Calculated Reynold's numbers were less than 2,000 (Fig. 2C), and therefore, the flow in the tubes of TR1 could be considered laminar flow at all depths. The average turbulence velocity of the microcosm used in TR3 is presented in Fig. 2D.

Plant growth

Water movement significantly affected the shoot length ($F = 9.68$, $P < 0.05$), as the plants exposed to either turbulence or main flow had significantly shorter shoots compared to the control treatments (Fig. 3). The final shoot lengths of the control plants and stressed plants were approximately four and threefold longer than their initial length, respectively.

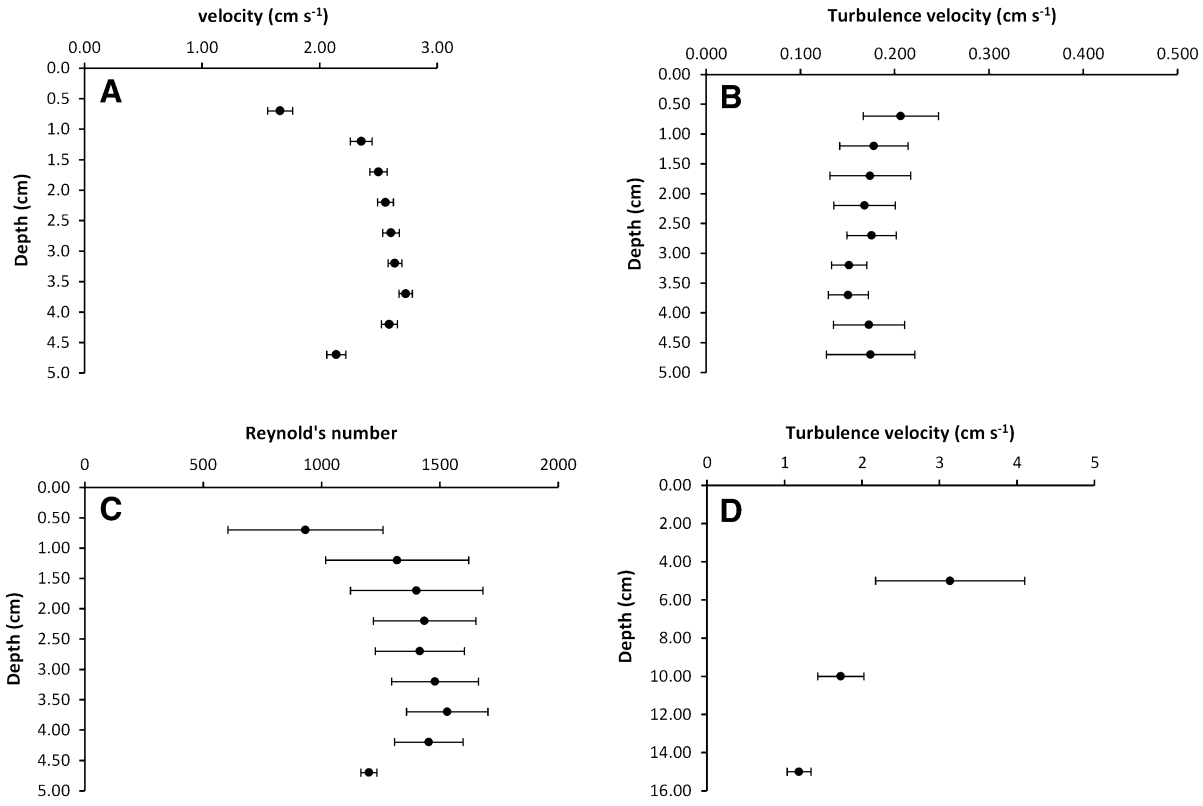


Fig. 2 Velocity profiles of experimental units (**A** main velocity of TR1, **B** turbulence velocity of TR1, **C** Reynolds's numbers of TR1, **D** turbulence velocity in microcosm used in TR3)

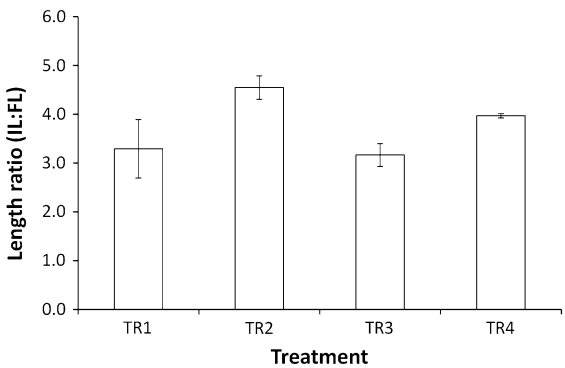


Fig. 3 Final length to initial length ratio (FL/IL) of *E. nuttallii* after exposure to turbulence and main flow

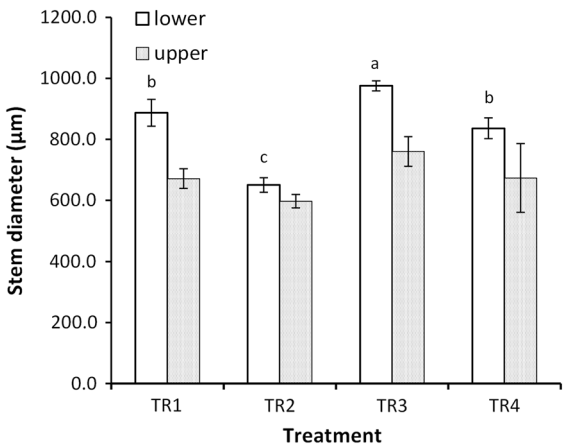


Fig. 4 Variation in the stem diameter of *E. nuttallii*

Even though the diameter of the apical stem was statistically similar among treatments (Fig. 4), the turbulence and main flow significantly increased the thickness of the basal stem ($F = 43.68$, $P < 0.001$).

The widest basal stem diameter was observed in TR3, where plants were exposed to turbulence, followed by TR1 (exposed to main flow), TR4, and TR2, respectively. The basal stem diameter of plants in TR1 was similar to that of TR4 and significantly wider than that of their control plants (TR2). The mean basal stem diameter of TR1 plants was approximately 300 µm wider than that of the control plants.

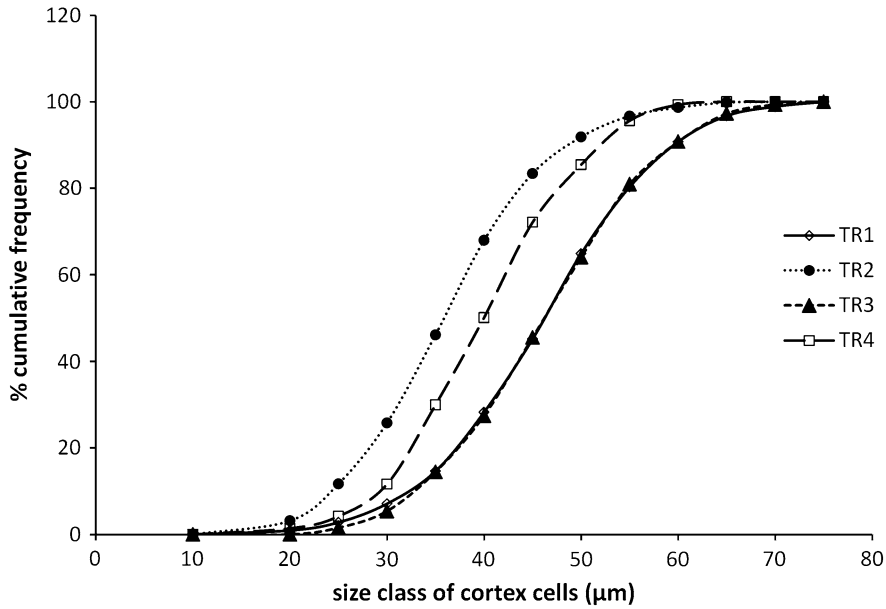


Fig. 5 Size class distribution of cortex cells

Similarly, the basal stem diameter of turbulence-induced plants (TR3) was also wider than that of their control plants (TR4). Plants developed shorter and wider stems when exposed to water movement, whereas the plants that grew in stagnant water were characterized by having a thinner and longer shoot. The wider stems observed in plants exposed to water movements (TR1 and TR3) were driven by wider cortex cells compared to those in control plants (Fig. 5).

Fifty percent of the cells in the cortex of plants exposed to main flow measured less than 40 µm in diameter, whereas the same percentage of cortex cells in TR2 control plants was less than 35 µm (Fig. 5). A similar trend was observed for TR3 and TR4, for which 50% of the cortex cells in TR4 were less than 40 µm and the same proportion was less than 45 µm in the plants exposed to turbulence.

Stress response

The highest H_2O_2 concentration was found in TR3 (turbulence stress) followed by TR1 (flow stress) and the control plants (TR2 and TR4) where plants were grown in stagnant waters (Table 2). The plants in the two control treatments (TR2 and TR4) contained statistically similar concentrations of H_2O_2.

Similar to H_2O_2 concentration, the highest CAT activity was found in the plants subjected to turbulence (TR3), whereas the plants grown in either flowing (TR1) or stagnant waters (TR2 and TR4) exhibited lower CAT activity (Table 2). The CAT activity of the flow-induced plants was not significantly different from that of the control plants.

In terms of APX, the mechanical stress caused by water movements (turbulence and main flow) significantly increased APX activity in *E. nuttallii* (Table 2). Significantly, the highest APX activity was recorded in plants exposed to turbulence (TR3), followed by TR1, TR2, and TR4.

IAA production in *E. nuttallii* was severely affected by turbulence, as the turbulence-stressed plants (TR3) presented significantly lower IAA content compared to the other three treatments (Table 2). The IAA contents of the plants in TR1 and TR2 were statistically similar.

It was clear that *E. nuttallii* strengthens its body to resist water movements by accumulating cellulose and lignin (Table 2). Significantly, the highest cellulose content was found in the plants grown under turbulence stress. Furthermore, the cellulose content of these plants was more than threefold higher than that of the control plants (TR4) and twofold higher than the plants exposed to main flow (Table 2). The cellulose contents

Table 2 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 (nkat/g FW)	65.11 ± 12.56^{b}	62.84 ± 21.14^{b}	114.08 ± 15.7^{a}	42.99 ± 10.01^{b}	9.79	*
APX activity (nkat/g FW)	0.81 ± 0.01^{b}	$0.57 \pm .08^{c}$	1.09 ± 0.07^{a}	0.25 ± 0.01^{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	**
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

of the plants grown in other treatments were not significantly different from each other. A similar trend was also observed for lignin, where the highest lignin content was observed in the plants exposed to turbulence followed by the plants in the main flow and control treatments (Table 2). The main flow-stressed plants contained approximately 10% lignin, which was not significantly different from their respective control, TR2. The lowest lignin content was found in TR4, and it was statistically similar to that of TR2 (Table 2).

Chlorophyll and chlorophyll fluorescence

There was a significant effect of turbulence on the chlorophyll content ($F = 25.41$, $P < 0.05$) (Fig. 6). Plants grown in both flowing (TR1) and stagnant conditions (TR2 and TR4) presented significantly higher concentrations of chlorophyll than the TR3 plants. Compared to TR3, the chlorophyll contents of the other plants were approximately 1.6-fold higher, i.e., the mechanical stress induced by turbulence reduced the chlorophyll concentration by approximately 40% compared to the control group (TR4).

The F_v/F_m ratios closely agreed with the above pattern (Fig. 6), as the lowest chlorophyll fluorescence was found in turbulence-induced plants ($F = 46.56$, $P < 0.001$). The F_v/F_m ratios of plants grown in main flow and stagnant conditions were statistically similar, indicating that turbulence created severe stress on *E. nuttallii* compared to main flow.

The average carotenoid contents of TR1–TR4 were 15.84 ± 0.39, 10.07 ± 0.08, 17.83 ± 0.72, and 12.82 ± 6.88 μg/g FW, respectively, and this content was independent of the water movement treatments ($F = 1.93$, $P > 0.05$).

Field observations

The L1 field site is a pool-like environment where water flows smoothly, whereas an elevated level of turbulence was observed in L2. The average turbulence velocities in L1 and L2 were 1.55 ± 0.68 and 4.07 ± 0.00 cm s^{-1}, respectively, where L2 presented a significantly higher magnitude of turbulence compared to L1 ($t = 5.17$, $P < 0.05$). The plants in L2 exhibited a significantly elevated level of stress response compared to the plants growing in L1 (Fig. 7).

The CAT activity of plants exposed to elevated levels of turbulence was significantly higher than that of plants (Fig. 7A) grown in a low turbulence environment ($t = 11.89$, $P < 0.05$). A similar trend was also observed for H_2O_2 (Fig. 7B) ($t = 60.56$, $P < 0.05$) and for APX (Fig. 7 D) ($t = 10.09$, $P < 0.05$). Although the IAA contents of *E. nuttallii* (Fig. 7C) were significantly different ($t = 4$, $P < 0.05$), the observed trend contradicted the experimental results. Similarly to the experimental results, a significantly higher cellulose content ($t = 15.68$, $P < 0.05$) was found in the plants exposed to high turbulence under field conditions (Fig. 7E). Plant stress responses observed under experimental conditions and field conditions were within the same range, and the trends observed under experimental conditions were supported by the field investigations.

Discussion

Experiment 1

High nutrient concentrations have been reported as one of the main factors that induce oxidative stress

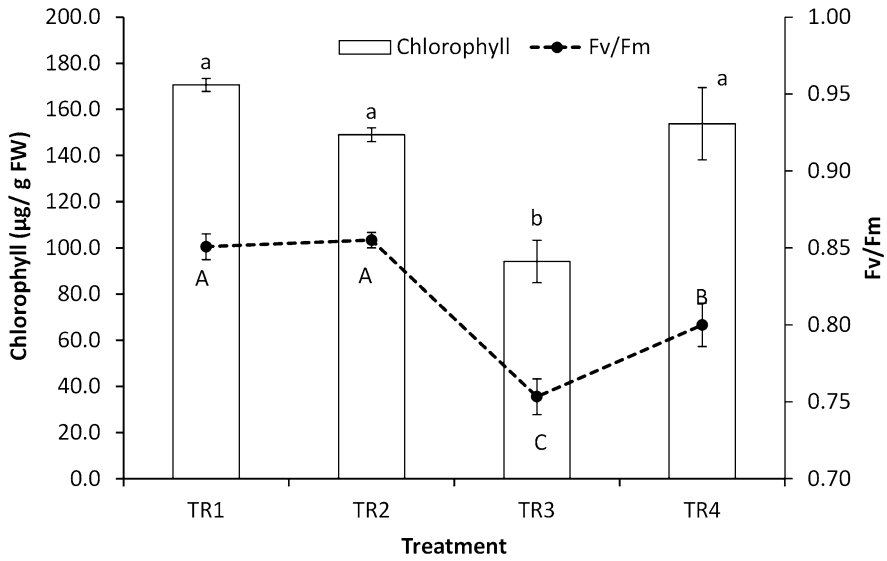
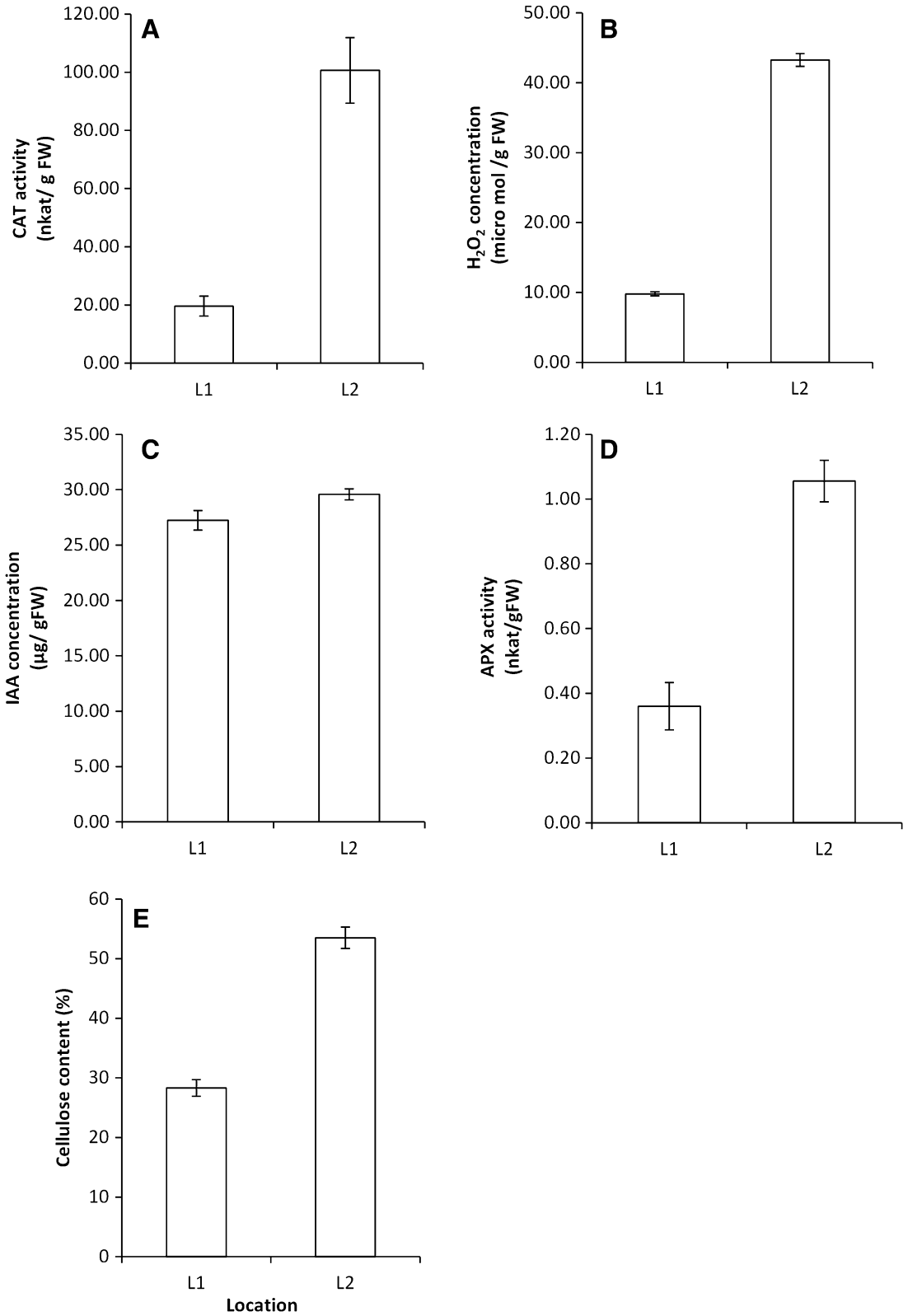


Fig. 6 Total chlorophyll and chlorophyll fluorescence of *E. nuttallii*. *Different superscripts* in the same category indicate the significant difference at 0.05 significant level (*lower case* and *upper case letters* represent total chlorophyll and chlorophyll fluorescence, respectively)

in submerged macrophytes in eutrophic waters (Cao et al., 2007). POD, SOD, CAT, and APX prevent oxidative damage in plants by scavenging reactive oxygen species. Therefore, an elevated level of POD in plants exposed to a high nutrient concentration is evidence of oxidative stress. Similarly to the present study, Zhang et al. (2010) observed an elevated level of POD activity in *P. crispus* after growing under high nutrient concentrations. Furthermore, the observed trend in plant growth responses agrees with the previous literature, as the inhibition of macrophyte growth has been reported under high nutrient concentrations (Smolders et al., 1996; Best, 1980; Zhang et al., 2010). However, the accumulation of H_2O_2 has been observed in different macrophyte species under various stress conditions (Wang et al., 2008; Ellawala et al., 2013; Zaman & Asaeda, 2013). As there was no stress in the control treatment, the stress responses of control plants were similar to those of plants exposed to low-nutrient media. The observed stress in *E. nuttallii* under high nutrient concentrations was further confirmed by the chlorophyll fluorescence analysis. Moreover, visual observation also confirmed that algae growth was prominent in high nutrient conditions, and excessive growth of algae hampers the growth of macrophytes. Therefore, low-nutrient concentrations of HNS (<10%) are suitable for growing *E. nuttallii* in experimental culture systems due to their better growth and lower oxidative stress.

Experiment 2

The mechanical stresses induced by turbulence and main flow reduced shoot length (Fig. 3); this trend agrees with previous literature (Ellawala et al., 2013). The growth response to mechanical stimuli is termed thigmomorphogenesis, and the retardation of tissue elongation coupled with increased radial expansion is one of the common responses to mechanical stress in terrestrial plants (Biddington, 1986). The radial expansion of stems observed in plants exposed to water movement could be a morphological adaptation to withstand the hydrodynamic forces exerted on plants by either turbulence or main flow. Similar observations have been reported for terrestrial plants in response to mechanical stress (Biddington, 1986; Braam, 2005). Increased stem thickness could accompany the morphology of stem cortex cells (Fig. 5), as the cortex cells of stressed plants were significantly larger than those of control plants. Furthermore, lignification has been identified as an adaptive 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 2) could be considered an adaptive mechanism that minimizes the mechanical damage caused by turbulence. Overall, elevated levels of cellulose and lignin in plant shoots and strengthened local cell walls lead to increased flow resistance in *E. nuttallii*. Our findings agree with previous observations reported for


A
CAT activity (nkat/ g FW)
120.00
100.00
80.00
60.00
40.00
20.00
0.00
L1
L2
B
H_2O_2 concentration (micro mol /g FW)
50.00
40.00
30.00
20.00
10.00
0.00
L1
L2
C
IAA concentration (µg/ gFW)
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
L1
L2
D
APX activity (nkat/gFW)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
L1
L2
E
Cellulose content (%)
60
50
40
30
20
10
0
L1
L2
Location

◀**Fig. 7** Stress response of *E. nuttallii* tested in the 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)

young *V. spiralis* tested under laboratory conditions with turbulence (Ellawala et al., 2013).

The ROS levels in plants increase in response to most biotic and abiotic stresses and consequently induce the activity of antioxidant enzymes. In this context, phytohormones and antioxidants play an important role in 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 to either suppress or activate the antioxidant enzymes in plants (Delledonne et al., 2001; Apel and Hirt, 2004; Jayakumar et al., 2006). Mechanical stress has an ability to induce the activity of POD, CAT, APX, SOD, etc. For instance, Ellawala et al. (2011) observed an elevated level of antioxidant activity in some aquatic macrophytes after being subjected to turbulence, and changes in antioxidant enzymes in mechanically induced tomato plants were observed by Saidi et al. (2009). Compared to control plants, the accumulation of H_2O_2 and the elevated levels of CAT and APX activity in TR3 indicate that turbulence triggered oxidative stress in *E. nuttallii*.

Auxins are considered a prime candidate for controlling stress-induced morphogenic responses in plants (Kawano et al., 2003; Potters et al., 2007), and a decrease in IAA following mechanical stress has been reported for both terrestrial and aquatic plants under experimental conditions (Saidi et al., 2009; Champika Ellawala et al., 2011). Therefore, the lowest IAA concentration observed in plants exposed to turbulence agrees with the former findings. Furthermore, the low concentration of IAA in turbulence-stressed plants could be responsible for their impaired growth compared to the control and flowing treatment. A significant negative correlation between IAA and shoot elongation has been reported for *V. spiralis* and *E. nuttallii* (Ellawala et al., 2011). Saidi et al. (2010) reported that the concentration and distribution of IAA could be controlled 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. However, the field observations in this study contradicted the experimental results of IAA, so additional data are required to explore this speculation. In the laboratory, plants were subjected to either turbulence alone, main flow alone, or stagnant conditions. Apart from water movements, plants in natural environments face different mechanical stress vectors including wind, rain, hail, and animal movements. Furthermore, plant stress response is a dynamic process, and the influences of other factors on plant often mask, negate, or modify the influence of mechanical stress (Mitchell, 1996). That is one possible explanation for the opposite trend found in IAA in plants studied in situ compared to the experimental results. Moreover, the biosynthesis of IAA takes place at the apical tips of plant shoots and roots, and it is then transported downward to regulate plant growth and development. However, the polar transport of IAA via stem is reported to be hampered by mechanical stress, and consequently synthesized products accumulate in apical tissues (Erner et al., 1980; Erner & Jaffe, 1982). As plants in natural environments are persistently challenged by a high magnitude of turbulence and face different mechanical stress vectors, it would be possible for IAA to accumulate in stressed plants.

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 monodehydroascorbate (Apel & Hirt, 2004). Consequently, the elevated levels of APX in response to turbulence might influence 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,* as observed from the lower chlorophyll concentration observed in the latter plants. Accumulation of ROS might be one possible explanation behind this reduction, as the chloroplast is one of the main organelles that produce ROS in plant cells. Furthermore, environmental stress has the ability to down regulate photosynthetic genes and cause photosynthetic decline, leading to the expression of leaf senescence-related genes and senescence induction (Sillanpää et al., 2005; Saibo et al., 2009). On the other hand, leaf orientation might lead to less efficient light absorption under turbulence. It was visually observed that the leaves of plants

exposed to turbulence quickly became yellowish. The reduction of photosynthesis ability in plants exposed to turbulence compared to those exposed to main flow was further explained by the observed trend in F_v/F_m values. Similar trends in chlorophyll and chlorophyll fluorescence have been observed in previous studies for *E. densa* and *V. spiralis*, after exposure to turbulence (Ellawala et al., 2011). Although Westlake (1967) observed rapid photosynthesis under low flow (0.02–0.5 cm s^{-1}), the flow tested in this study (TR1) was higher than that tested in the former study.

Carotenoids play an important role in protecting plant cells against the oxidative damage induced by excess light (Krinsky, 1978; Young, 1991; Demmig-Adams et al., 1996; Strzałka et al., 2003), and consequently, elevated levels of carotenoids have been observed in plants in response to excess light (Grumbach & Lichtenthaler, 1982). However, all treatments in the present study (TR1–TR4) received similar light conditions throughout the experimental period (100–110 μmol m^{-2} s^{-1}). As a consequence, we observed similar concentrations of carotenoids among the four treatments. Similar results have been observed for *M. spicatum* (Atapaththu & Asaeda, 2014). The ROS scavenging activity of carotenoids strongly depends on the nature of the oxidizing agent (Strzałka et al., 2003). Therefore, carotenoid-driven scavenging activity might not have been activated in *E. nuttallii*. On the other hand, carotenoid-mediated ROS detoxification in plants is not well documented (Apel & Hirt, 2004).

The generation of turbulence in both lakes and rivers depends on a combination of factors including the force applied to the water, water circulation, tidal movement or the effect of wind acting on the surface, and the resistance to free movement of water due to the coherence of its molecules (Reynolds, 1994). Therefore, wave disturbances are particularly important to generate turbulence in aquatic systems. For instance, wind-induced currents and waves generate forces on the submerged plants, and the magnitude of these forces depends on depth and fetch (Schutten et al., 2004). Moreover, Pujol et al. (2010) reported that the wind-induced turbulence is the major factor that affects the boundary layer of submerged plants in wetlands and shallow lakes. Gravity on the downstream slope of the water surface causes unidirectional flow in streams, and the flow pattern has implications for the community structure, metabolism, physical resistance, and the development of aquatic macrophytes (Madsen et al., 1993; Sand-Jensen and Pedersen, 1999). In reality, the mechanical stress induced by turbulence might have an impact on plants in any direction, as turbulent fluctuations are independent of 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 strong defense mechanisms that work against the mechanical stress caused by water movements, particularly turbulence.

In response to turbulence, the increasing trends in antioxidant enzyme activities and the concentration of H_2O_2 in both the experimental and field observations indicates the activation of an antioxidant defense mechanism against oxidative stress. The turbulence velocity employed in the experimental treatment (TR3) was within the same range observed in the natural environment. Therefore, we observed approximately similar responses in CAT and APX under both experimental and field conditions. However, the observed H_2O_2 concentration of plants in L2 was approximately twofold higher than that of the experimental condition (TR3). Plants in experimental conditions were stressed by a constant turbulence over time, whereas macrophytes in the natural environment are subjected to wide fluctuations in flow velocity and turbulence. Furthermore, several other factors such as water level fluctuations and other mechanical disturbances (Mitchell, 1996; Thomaz et al., 2006) might also cause mechanical stress to plants in natural environments.

Conclusion

The results of the present study suggest that the mechanical stress cause by turbulence has a more significant impact on the physiology of *E. nuttallii* than main flow that has a similar magnitude. Therefore, mechanical stress induced by turbulence could be considered one of the main abiotic determinants that affect the physiology of macrophytes in aquatic systems. Our findings are particularly helpful for understanding the interaction between macrophytes and their surrounding environment and explaining the habitat preferences of macrophytes. Such information is particularly important for aquatic ecosystem management.

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