Tissue Hydrogen Peroxide Concentration Can Explain the Invasiveness of Aquatic Macrophytes: A Modeling Perspective

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In recent years, an invasive macrophyte, Egeria densa, has overwhelmingly colonized some midstream reaches of Japanese rivers. This study was designed to determine how E. densa has been able to colonize these areas and to assess the environmental conditions that limit or even prevent colonization. Invasive species (E. densa and Elodea nuttallii), and Japanese native species (Myriophyllum spicatum, Ceratophyllum demersum, and Potamogeton crispus) were kept in experimental tanks and a flume with different environmental conditions. Tissue hydrogen peroxide (H$_2$O$_2$) concentrations were measured responding to either individual or multiple environmental factors of light intensity, water temperature, and water flow velocity. In addition, plants were sampled in rivers across Japan, and environmental conditions were measured. The H$_2$O$_2$ concentration increased in parallel to the increment of unpreferable levels of each abiotic factor, and the trend was independent of other factors. The total H$_2$O$_2$ concentration is provided by the sum of contribution of each factor. Under increased total H$_2$O$_2$ concentration, plants first started to decrease in chlorophyll concentration, then reduce their growth rate, and subsequently reduce their biomass. The H$_2$O$_2$ concentration threshold, beyond which degradation is initiated, was between 15 and 20 µmol/gFW regardless of the environmental factors. These results highlight the potential efficacy of total H$_2$O$_2$ concentration as a proxy for the overall environmental condition. In Japanese rivers, major environmental factors limiting macrophyte colonization were identified as water temperature, high solar radiation, and flow velocity. The relationship between the unpreferable levels of these factors and H$_2$O$_2$ concentration was empirically obtained for these species. Then a mathematical model was developed to predict the colonization area of these species with environmental conditions. The tissue H$_2$O$_2$ concentration decreases with increasing temperature for E. densa and increases for other species, including native species. Therefore, native species grow intensively in spring; however, they often deteriorate in summer. For E. densa, on the other hand, H$_2$O$_2$ concentration decreases with high water temperature in summer, allowing intensive growth. High solar radiation increases the H$_2$O$_2$ concentration, deteriorating the plant. Although the H$_2$O$_2$ concentration of E. densa increases with low water temperature in winter, it can survive in deep water with low H$_2$O$_2$ concentration due to diffused solar...
INTRODUCTION

Macrophyte responses to environmental conditions are species specific, and invasive plants tend to exhibit more tolerance than native species (Zerebecki and Sorte, 2011; Bates et al., 2013). Therefore, invasive species are able to dominate or distribute in areas where native species fail to survive. Among different invasive aquatic macrophytes, Egeria densa is a well-known worldwide species that causes significant ecological issues in freshwater ecosystems. In Japan, E. densa was used as an ornamental aquarium plant in the early 19th century. However, it has escaped into natural freshwater bodies and became naturalized in the 1940s. Although E. densa mainly invaded lakes during the initial spreading stage in the 1970s (Kadono, 2004), this species has been recorded increasingly in many western Japanese rivers over the last two decades (MLIT, 2019). These rivers were originally nearly free of macrophytes and consisted of gravel beds and hyporheic flow (Tanida, 1984; Hauer et al., 2016). Though native species (e.g., Myriophyllum spicatum, Potamogeton crispus, and Ceratophyllum demersum) were colonized patchily, no large colonies were found in major rivers (Kuni, 1982; Kadono, 2004). Another alien species, Elodea nuttallii, also invaded at nearly the same time in 1961. However, it did not produce large colonies except for lakes and small streams. In contrast, E. densa spread to cover the entire river channel of major rivers. The widespread colonization of E. densa has led to extreme changes in these river ecosystems. After establishment, E. densa behaved as ecological engineers, changing the environment to their benefit (Schoelynck et al., 2012; Schoelynck et al., 2014). They reduced water flow velocity and attenuated wave energy, leading to particle settlement and, consequently, hyporheic flow capacity reduction (Madsen et al., 2001; Boano et al., 2014). It has also caused economic losses. For example, the presence of macrophytes substantially decreases the yield of Ayu (Plecoglossus altivelis altivelis), a grazer of benthic algae (Kawanabe, 1970). Casual monitoring between present-day abiotic conditions and plant traits, such as growth rate and biomass, is the method commonly used to evaluate the preferable habitat for macrophyte species (Barko et al., 1991; Riis et al., 2012; O’Hare et al., 2018). However, environmental conditions frequently change, and there are various types of effective factors in the natural rivers. Thus, it is difficult to apply the monitoring system in the field, particularly to derive the most influential factor.

Aquatic macrophytes growing in their natural environment often face an array of unfavorable environmental conditions, for example, too low or too high water temperatures, high flow velocity (Atapaththu and Asaeda, 2015), pollution, or substrate alteration (Asaeda et al., 2013). They can survive and propagate if the conditions remain within the plants’ tolerance levels. When the environmental conditions exceed the tolerance thresholds for a considerable period of time, macrophytes become stressed, lose their colonization capacity, and ultimately decay. However, following a short-term exposure to such conditions, they can recover, depending on the extent of the damage caused and the characteristics of the species (Weerakoon et al., 2018). Thus, the presence of a specific macrophyte species in an area depends on whether environmental factors are within their tolerance levels as well as on the duration of the exposure. When plants are subjected to unfavorable environmental conditions, reactive oxygen species (ROS) are generated in different organelles (Zaman and Asaeda, 2013; Das and Roychoudhury, 2014; Asaeda et al., 2017; Choudhury et al., 2017; Helaly et al., 2017; Parveen et al., 2017; Asaeda et al., 2018; Elsheery et al., 2020a; Elsheery et al., 2020b), which damages the plant body by the oxidative stress. Some ROS are scavenged relatively quickly by antioxidants (Omar et al., 2012), and the homogeneity of ROS in tissues is maintained by balancing the ROS and antioxidants. The balance flips over when oxidative stress surpasses the scavenging capacity of the antioxidants (Naser et al., 2016; El-Sheery, 2017; Dumont and Rivoal, 2019). Among ROS, hydrogen peroxide (H2O2) is widely generated (Asada, 2006; Sharma et al., 2012), relatively stable, and can be easily measured (Satterfield and Bonnell, 1955; Zhou et al., 2006; Asaeda et al., 2020). The concentration of H2O2 in plant tissues does not depend on a particular stress but is subjected to sum magnitude of unfavorable environmental conditions (Suzuki et al., 2014; Asaeda et al., 2020). Thus, H2O2 concentration in the plant tissue can be used as an indicator of the physiological status of a particular macrophyte species (Smirnoff and Arnaud, 2019). The system has been used for E. densa, which has successfully identified the channel slope that it can colonize (Asaeda et al., 2020).

The trend of H2O2 concentration is likely as a result of a long history of acclimatization to the natural condition of a particular area; thus, it may vary widely between native and invasive species. To apply tissue H2O2 concentration as an indicator to elucidate the intensive growth of invasive species, it is necessary to determine the relationship between H2O2 concentration and environmental factors both for native and invasive species. The main objective of the present study is to 1) empirically determine the H2O2 concentration generated by unfavorable conditions of abiotic environmental factors for both native and invasive species, 2) develop the model to predict the environment where these species can colonize, and 3) elucidate the reason for the overwhelming growth of E. densa in rivers of particular areas.

METHODOLOGY

Experimental Methodology

In the experiment, invasive macrophyte species (E. densa and E. nuttallii) and major Japanese species (C. demersum, P. crispus, and M. spicatum) were tested (MLIT, 2019). They were exposed
to different types of physical conditions, temperature, irradiance, and water flow velocity, following the range of the rivers where these species were colonized from ~8°C in winter to 30°C in summer for water temperature, 0–1,200 µmol/m²/s for the irradiance in water, and 0–50 cm/s for flow velocity (MLIT, 2019). For the laboratory experiments, healthy macrophyte stocks were collected from the Saba River (E. densa) and the Moto-Arakawa River near Tokyo (E. nuttallii, C. demersum, P. crispus, and M. spicatum). Collected plants were cleaned with water to remove debris, and any attached macro-algae were carefully separated with tweezers. The plants were then cultured in a glass tank at 25 ± 2°C under a 12/12 h photoperiod with photosynthetically active radiation (PAR) (~125 µmol/m²/s using fluorescent lamps) for over 2 months. Commercial sand (D₅₀ < 0.1 mm) was used as a substrate, and 5% Hoagland solution was provided as the nutrient medium (Atapaththu and Asaeda, 2015). Algae were removed weekly, and algae-free plants were used in the experiments. Three types of experiments (triplicate) were conducted in total, each focusing on different combinations of environmental factors.

Experiment 1: Water Temperature and Irradiance
A number of studies have reported that water temperature can significantly affect the abundance of different aquatic plant species (Pip, 1989; Barko et al., 1991; Lougheed et al., 2001; Pandit, 2002). An experiment was conducted to identify the increment of H₂O₂ concentration of the plant tissue under different water temperatures and irradiance levels, and, thereby, to make empirical relations between these factors. Several light levels (0–1,300 µmol/m²/s of PAR) were tested in small aquaria (dimensions: 50.0 cm × 35.0 cm × 35.0 cm). Temperature level was maintained at 10 ± 2 (E. densa), 15 ± 2 (E. densa), 20 ± 2, 25 ± 2 (E. densa), and 30 ± 2, 35 ± 2°C using a temperature controlling system (Aquarium cooler ZC-100a, Zensui Corporation, Tokyo, Japan). PAR intensity was irradiated under natural solar radiation or using LED lights (Model LT-NLD85L-HN, OHM Electric Inc., Japan) with a 12 h light:12 h dark photoperiod for 3 weeks.

Experiment 2 and 3: Flow Velocity and Irradiance
This experiment was designed to test the effect of water flow velocity on the H₂O₂ concentration of the plant tissues and the interaction with irradiance (Atapaththu and Asaeda, 2015; Asaeda et al., 2017). Two sets of experiments were conducted. In the first experiment, experimental plants (E. nuttallii, P. crispus, C. demersum) were exposed to two water flow levels (16 and 25 cm/s) using custom-made recirculating flumes (dimensions: 240 cm long × 25 cm width × 28 cm depth) exposed to artificial light intensity by the LED lights, or dark conditions. Pre-aerated tap water was circulated by centrifugal electric motor pumps. Pre-acclimatized potted plants were allocated to a section in the flume where water was introduced though a gradually shrinking entrance section to reduce turbulence. Plants were continuously exposed to low or high mean flow velocities for up to 4 days. During the experiment, mean water flow velocity was detected using an ultrasonic velocimeter (Tokyo Keisoku Co. Ltd., Japan) directly above the plant leaf surface and recorded daily to minimize flow variation. Temperature level was maintained at 15 ± 2°C using an aquarium water temperature controlling system (Aquarium cooler ZC-100a, Zensui Corporation, Tokyo, Japan). Stress assays by means of H₂O₂ measurements were performed every 3 h from 6:00 to 18:00 after 4 days’ exposure, and each treatment contained three replicate flumes. For another experiment, a flume channel 2.4 m long, 25 cm wide and 22 cm depth was constructed outdoors. Eighteen flat pots with more than three E. densa plants were carefully and randomly installed. Water temperature was kept at 25 ± 2°C throughout the experiment. Flow velocities from stagnant to 40 cm/s were employed under different solar radiation, and after 3 h, three plants were sampled at each time and a stress assay was conducted immediately. PAR intensity in the water was measured with a portable quantum flux meter (Apogee, MQ-200, United States).

Field Observations
Several rivers that are highly colonized by E. densa were selected from the species distribution database in Japan (MLIT, 2019). The selected rivers were assessed to obtain detailed location information pertaining to the colonization of E. densa. Sampling was conducted in the Eno River and its tributary Tajibi River (April, May, and September 2016; April and June 2017); in the Saba River and its tributary Shimaji River (May, June, and September 2016; April and June 2017; August 2018), and in the Hii River (October 2016). At each sampling point, water flow velocity was measured with an ultrasonic velocimeter (Tokyo Keisoku Co. Ltd., Japan) at 20% (reference velocity) and 80% (depth of the colony) of the total water depth (Chow, 2009). PAR intensity in the water was measured with a portable quantum flux meter (Apogee, MQ-200, United States) at 10 cm depth intervals. M. spicatum was sampled in the Moto-Arakawa River near Tokyo in April 2015. The river was approximately 5 m wide and 40 cm deep, and the channel slope was approximately 1/1,000. The bottom surface was patchily covered with M. spicatum, E. nuttallii, C. demersum, and Sparganium spp. PAR, and velocity distributions were measured with a portable quantum flux meter (Apogee, MQ-200, United States) and an ultra-sonic velocimeter, respectively. E. nuttallii was sampled in July and September 2018 from the same river. Sampling was conducted approximately every 3 h in the light-exposed and dark-adapted conditions to remove the effect of solar radiation. The dark treatment involved placing a black plastic sheet (3 m × 3 m) floating over part of the plant colony for 30 min. The 30 min pre-dark period was determined by laboratory experiments, which were specifically conducted to determine the optimum pre-darkness duration (data not shown). In August 2017, a sampling of M. spicatum was conducted in the Sakuradabori of the Imperial Palace Moat, at the center of Tokyo, where M. spicatum made a mono-specific stand. The depth of the sampling site was 0.3 m–2.5 m. Both solar-exposed and dark-adapted samples were taken. Plant biomass was sampled from 50 cm × 50 cm quadrats in all sampling sites. The
plant samples were placed in plastic bags and immediately stored in a cooling box containing dry ice for transfer to the laboratory where it was stored at ~80°C until an H2O2 assay and chlorophyll estimation were conducted.

**Determination of Shoot Growth Rate, H2O2 and Chl-a Concentrations**

The length of the plants grown in the experimental units was measured using a millimeter scale at 5–7 day intervals. The shoot growth rate (SGR) was calculated as the difference in shoot length between two observations divided by the duration, and it was expressed in cm/day. At the end of each experiment, fresh plant shoots were extracted (~500 mg) in an ice-cold phosphate buffer (50 mM, pH 6.0) that contained polyvinylpyrrolidone (PVP), and the extractions were centrifuged at 5,000 × g for 20 min at 4°C. This extraction was used to analyze the H2O2 content spectrophotometrically following the TiSO4 method (Satterfield and Bonnell, 1955) with modifications. The reaction mixture contained 750 µl of enzyme extract and 2.5 ml of 1% TiSO4 in 20% H2SO4 (v/v), which was centrifuged at 5,000 × g for 15 min at 20°C. The optical absorption of the developed yellow color was measured spectrophotometrically at a wavelength of 410 nm. The H2O2 concentration in samples was determined using the prepared standard curve for known concentration series and was expressed in µmol per gram fresh weight (µmol/gFW).

Chlorophyll a (Chl-a) concentrations of experimental plants were determined spectrophotometrically (UV Mini 1210, Shimadzu, Japan) by extracting pigments with N,N-dimethylformamide after keeping them in darkness for 24 h, and they were expressed in terms of fresh weight (FW) (Wellburn, 1994).

**Statistical Analysis**

Data were tested for normality with the Shapiro–Wilk test before statistical analyses. All results were presented as the mean ± SD of more than three replicates. Data were subjected to a one-way analysis of variance (ANOVA) with Tukey’s post-hoc test for mean separation. The t-test was performed where necessary. Bivariate analysis was used and followed by Pearson’s correlation to evaluate the relationship among parameters. Statistical analyses were performed in IBM SPSS V25.

**Development of the Species-Specific Model to Identify the Colonization Zones**

Asaeda et al. (2020) proposed the total H2O2 concentration formed in plant tissues for a particular temperature (Temp) by the sum of H2O2 generated by metabolism (H2O2met), flow velocity (H2O2vel), and solar radiation (H2O2rad). If the value is between 15 and 20 µmol/gFW, then *E. densa* growth deteriorates.

\[
H_{2O2_{total}}(\text{Temp}) = H_{2O2_{merged}}(\text{Temp}) + H_{2O2_{met}}(\text{Temp}) + H_{2O2_{rad}}(\text{Temp})
\]

\[
< H_{2O2_{vel}}(r = 15 – 20 \text{ } \mu \text{mol/gFW for } E. densa)
\]

For other species, empirical formulas obtained by experiments and field observation were introduced to H2O2 concentrations generated by each environmental component, solar radiation, H2O2rad(Temp), temperature increment, H2O2met(Temp), the basal level of the metabolism, the H2O2vel (Temp) (Apel and Hirt, 2004), and the threshold level to deteriorate, H2O2velT.

In rivers flowing with moderate velocity, water is fully mixed. Therefore, the light attenuation coefficient is nearly uniform at all depths, and the light intensity is given by

\[
I_0 \exp(-kr)
\]

where \(I_0\) is the light intensity just below the water surface, \(k(=0.083 \text{ cm}^{-1})\) is the attenuation constant of light in water, and \(z\) is the canopy depth. The intensity of solar radiation, \(I_s (\mu \text{mol/m}^2/\text{s})\), and water temperature (°C) at the Ena and Saba rivers are empirically given as a function of month, month:

\[
I_0 = 0.93 \text{month}^4 - 22.3 \text{month}^3 + 134.5 \text{month}^2 - 22.2 \text{month} + 868
\]

\[
\text{Temp} = 0.022 \text{month}^4 - 0.66 \text{month}^3 + 6.16 \text{month}^2 - 17.5 \text{month} + 20.4
\]

Flow velocity in a river channel “Vel” (cm/s) is estimated by the Manning’s equation, assuming the channel is sufficiently wide compared to the depth and is longitudinally uniform, such that:

\[
Vel = \frac{4.63}{n}R^{2/3}S^{1/2}
\]

where \(R\) is the hydraulic radius, approximately given by the depth \(H\) (cm), \(S\) is the channel bed slope, and \(n\) is the Manning’s roughness coefficient, where \(n\) is ~0.090 in the river zones considered in the present study (personal information).

**RESULTS**

**Empirical Relationships of H2O2 Concentration With Abiotic Factors**

Combined effects of temperature and light intensity on H2O2 formation in macrophytes tissues showed a species-specific response (Figure 1). The basal H2O2 concentrations were 4.6 µmol/gFW at 20°C for *E. densa* and *E. nuttallii*, and 3.0 µmol/gFW at 20°C for other species, respectively, after being exposed to dark conditions. The increment of H2O2 driven by the temperature change were ~0.32 µmol/gFW/C for *E. densa* (\(r = -0.985, p < 0.01\)), 0.39 µmol/gFW/C for *M. spicatum* (\(r = 0.800, p < 0.05\)), 0.41 µmol/gFW/C for *C. demersum* (\(r = 0.900, p < 0.01\)), 0.60 µmol/gFW/C for *P. crispus* (\(r = 0.974, p < 0.01\)), and 0.48 µmol/gFW/C for *E. nuttallii* (\(r = 0.956, p < 0.01\)), respectively. H2O2 concentrations of different light intensity groups were plotted nearly in parallel, higher with higher light intensity groups (\(p < 0.05\)).

Water flow velocity and light intensity had significant impacts on the H2O2 metabolism in macrophytes. The tissue H2O2 concentration linearly increased responding to increasing water flow velocity for all these species (Figure 2). The increasing rate of H2O2 concentration with respect to flow velocity showed no significant difference among species with
the gradient due to the velocity of 0.09 \( \text{H}_2\text{O}_2 / \text{velocity (µmol/gFW/cm/s)} \) \( (r = 0.921, p < 0.01 \text{ for E. densa}, 0.878, p < 0.01 \text{ for E. nuttallii}, r = 0.875, p < 0.01 \text{ for P. crispus}, r = 0.700, p < 0.01 \text{ for C. demersum and } r = 0.957, p < 0.01 \text{ for M. spicatum}). \) It was similar to the results of field samples, 0.072 \( \text{H}_2\text{O}_2 / \text{velocity (µmol/gFW/cm/s)} \) as shown in the figure. No significant difference was obtained among the sampling seasons. For \( \text{E. densa}, \) \( \text{H}_2\text{O}_2 \) concentrations for different light intensity groups were plotted nearly in parallel, higher with higher light intensity groups \( (p < 0.01). \) The increments of \( \text{H}_2\text{O}_2 \) concentrations for the light-exposed samples with respect to the dark-adapted ones are shown in Figure 3. Experimental samples of \( \text{E. densa} \) had a similar increasing trend with field observation in \( \text{H}_2\text{O}_2 \) concentration.

From the field data, Asaeda et al. (2020) derived the following relationship for \( \text{E. densa}: \)

\[
\text{H}_2\text{O}_2_{\text{rad}}(\text{Temp}) = \frac{[I_0e^{(-kz)} - 40]^2}{10} \text{ for } I_0e^{(-kz)} \geq 40 \text{ µmol/m}^2/\text{s}
\]

\[
\text{for } I_0e^{(-kz)} < 40 \text{ µmol/m}^2/\text{s}
\]

(5)
H₂O₂ and Chl-a concentrations for E. densa and SGR decreased with increasing H₂O₂ concentrations (\( r < 0.01 \) respectively). Regardless of environmental factors, both Chl-a concentrations and SGR decreased with respect to solar radiation was slightly lower, decreasing the effect of the solar radiation. Therefore, a different equation was derived for M. spicatum, and C. demersum (\( r = 0.98, p < 0.01 \) for M. spicatum, and \( r = 0.89, p < 0.01 \) for C. demersum, respectively).

\[
\begin{align*}
H₂O₂_{\text{rad}}(\text{Temp}) &= \frac{[I_0e^{(-4k)} - 40]^{\frac{3}{2}}}{55} \quad \text{for } I_0e^{(-4k)} \geq 40 \text{ µmol/m}^2/\text{s} \\
H₂O₂_{\text{rad}}(\text{Temp}) &= 0 \quad \text{for } I_0e^{(-4k)} < 40 \text{ µmol/m}^2/\text{s}
\end{align*}
\]

\[(6)\]

**The Threshold H₂O₂ Concentration for Growth Deterioration**

Chl-a concentrations and SGR as functions of H₂O₂ concentrations at different flow velocities, water temperatures, and light intensities of E. densa are shown in Figure 4A. Regardless of environmental factors, both Chl-a concentrations and SGR decreased with increasing H₂O₂ concentrations (flow velocity \( r = -0.944, p < 0.01 \) for Chl-a and \( r = -0.964, p < 0.01 \) for SGR; temperature \( r = -0.945, p < 0.01 \) for Chl-a and \( r = -0.980, p < 0.01 \) for SGR; light \( r = -0.924, p < 0.01 \) for Chl-a and \( r = -0.965, p < 0.01 \) for SGR). Figure 4B presents the relationships of H₂O₂ and Chl-a concentrations for M. spicatum, C. demersum, E. nuttallii, and P. crispuss. Chl-a concentration decreased with the H₂O₂ concentration (\( r = -0.896, p < 0.01 \) for M. spicatum, \( r = -0.752, p < 0.01 \) for C. demersum, \( r = -0.497, p < 0.01 \) for E. nuttallii, and \( r = -0.963, p < 0.01 \) for P. crispuss), and was eliminated at approximately 16–20 µmol/gFW. In the field observation, tissue deterioration occurred when similar H₂O₂ concentrations continued for a few days.

**Simulated Results**

The Comparison With the Observed Data

Figure 5 shows the comparison between the observed H₂O₂ concentration and simulated H₂O₂ concentration for experimental and observed results. Satisfactory agreement between the simulated and observed values were found in the simulation (\( r = 0.798, p < 0.01 \) for E. densa, \( r = 0.700, r < 0.05 \) for E. nuttallii, \( r = 0.919, p < 0.01 \) for M. spicatum, \( r = 0.976, p < 0.01 \) for C. demersum, and \( r = 0.974, p < 0.05 \) P. crispuss).

The Depth-Wise Distribution of H₂O₂ Concentration of Different Species

The H₂O₂ concentration of E. densa was simulated for channel slopes of 1/300 at 10, 20, and 30°C, which were close to the condition of the observed reaches of the Eno and the Saba rivers in March, May/June, and October as well as August to September, respectively. Figure 6 shows the simulated results with respect to the depth, observed H₂O₂, and macrophyte biomass. The threshold H₂O₂ concentration was assumed as 16 µmol/gFW. The H₂O₂ concentration was high at the water surface and gradually decreased. With deeper depth, increasing velocity increases the H₂O₂ concentration. The decreasing or increasing trend with respect to depth depends on the combination of these two factors. The H₂O₂ concentration of the stagnant water is lower than the sloped channel, as H₂O₂ generated by the velocity is zero. In the case of E. densa, the H₂O₂ concentration is higher with lower temperature, and...
mostly above the threshold value at 10 °C, indicating the colonization is limited. The observed H$_2$O$_2$ concentrations were plotted within 2 µmol/gFW from the simulated corresponding temperature line. All the biomass data were plotted in the depth where the H$_2$O$_2$ line of the corresponding temperature was below the threshold value. The simulated results agreed with the field sampling. The H$_2$O$_2$ concentration was simulated for M. spicatum; C. demersum, P. crispus, and E. nuttallii were compared to the observed H$_2$O$_2$ values and biomass in the field (Figures 7–9). Both the H$_2$O$_2$ concentration and the existing biomass range agreed well with observed data (H$_2$O$_2$ concentration: within 2.5 µmol/gFW, all positive biomass range was in the range where the H$_2$O$_2$ values were below the threshold). The H$_2$O$_2$ concentration of these species increases with increasing temperature. The H$_2$O$_2$ concentration is higher at the shallow zone; thus, the total H$_2$O$_2$ concentration exceeds the threshold value.

**FIGURE 4** (A) Chlorophyll a concentration and shoot growth rate (SGR) of E. geriadensa with respect to H$_2$O$_2$ concentration. Velocity (Ellawala et al., 2011), others: the present experiment. Vertical bars indicate standard deviation. (B) Chlorophyll is a concentration of M. spicatum, C. demersum, E. nuttallii, and P. crispus with respect to H$_2$O$_2$ concentration. Vertical bars indicate standard deviation.

**FIGURE 5** The comparison of simulated H$_2$O$_2$ concentration with observed H$_2$O$_2$ concentration. The dashed line indicates the perfect agreement. The shaded band (green) represents the 95% confidence interval on the fitted values.
The Composition of the H₂O₂ Component for Different Types of Rivers

Figure 10 presents the simulated results of H₂O₂ fractions generated by environmental conditions: temperature-dependent metabolism (H₂O₂<sub>met</sub>(Temp)), solar radiation (H₂O₂<sub>rad</sub>), velocity, H₂O₂<sub>vel</sub> fora 0.4 m deep (E. densa and M. spicatum), and colonized and non-colonized rivers. A 5 year average of monthly temperatures was used for the Eno and the Saba rivers, where E. densa are colonized, and for the Arakawa River and the Tone River in the Tokyo metropolitan area, where no E. densa colonies were recorded while M. spicatum was colonized (MLIT, 2019). The former groups are slightly warmer than the latter. The total H₂O₂ concentration without a velocity component is available to estimate for stagnant water. At >1 m depth, the H₂O₂ fraction for the solar radiation was almost nil. The increment of H₂O₂ concentration due to increasing temperatures after spring has opposite trends between E. densa and M. spicatum; the H₂O₂ concentration decreased with E. densa and increased with...
M. spicatum. Temperature was the most effective component to differentiate the annual patterns of the total H2O2 concentration. The fraction of H2O2 due to solar radiation was higher for E. densa than for M. spicatum; thus, E. densa colonization was highly affected by solar radiation. For E. densa, the H2O2 concentration maintained higher than the threshold value until June in the colder group of rivers, but it becomes lower than the threshold from April/May in the warmer group. For M. spicatum, on the other hand, the total H2O2 concentration exceeded the threshold value from April in the warmer group while only in August in the colder group. In the stagnant water, the total H2O2 concentration of M. spicatum exceeded the threshold value in summer.

DISCUSSION

Tissue H2O2 Concentration as Affected by Environmental Conditions

Previous studies have shown that H2O2 concentration of the plant tissues increases in unpreferable environmental conditions.
and it is highly correlated with the intensity of a single environmental factor (Asaeda et al., 2017; Asaeda and Sanjaya, 2017; El-Sheery, 2017; Parveen et al., 2017; Chalanika De Silva and Asaeda, 2018). The present study elucidates that under a combination of different environmental factors, the total H$_2$O$_2$ concentration is provided as the sum of H$_2$O$_2$ generated by individual factors and the amount generated by metabolism (Apel and Hirt, 2004). In addition, the relationship between H$_2$O$_2$ concentration and the intensity of each environmental factor does not vary much between seasons and phenological stages of the plant (Asaeda et al., 2020). Therefore, the H$_2$O$_2$ concentration is considered an indicator of the degree of the unpreferable condition. The Chl-a concentration and the growth parameter decreased with increasing intensity of the total H$_2$O$_2$ concentration (Coleman et al., 1989; French and Moore, 2003; Boustany et al., 2010). Interestingly, when the tissue H$_2$O$_2$ concentration exceeded 16–20 µmol/gFW, the plants became brownish and deteriorated. Therefore, the environmental conditions reflected by H$_2$O$_2$ concentrations below this threshold allows macrophytes to form a large and healthy colony. This system can be applied to elucidate the growth area of macrophyte species, by formulating the H$_2$O$_2$ concentrations and abiotic conditions in the environment.

Environmental Conditions Influencing Macrophyte Colonization in Japanese Rivers

In Japanese rivers, the water quality is relatively good and there is no salinity in the midstream (Luo et al., 2011). Organic matter accumulates on the bottom in stagnant zones, which creates an anoxic zone in the sediment layer. There are such areas in the lowland zones; however, anoxia of the bottom sediment contributed only ~3 µmol/gFW of H$_2$O$_2$ (Parveen et al., 2017). Chalanika De Silva and Asaeda (2018) showed that the H$_2$O$_2$ concentration differs between mono- and mixed-cultures of species in stagnant water, indicating the effect of species competition. However, the difference was only ~2 µmol/gFW. In field sampling, Japanese native species, P. crispus and C. demersum, were often found in thick E. densa colonies due to the reduction of flow velocity inside the colony (20 cm/s inside compared to 50 cm/s outside, according to our own observation). This indicates that the increment of H$_2$O$_2$ concentration due to competition is less than the reduction of velocity-induced H$_2$O$_2$ (~3 µmol/gFW). In this study, the H$_2$O$_2$ concentrations attributed to water temperature, high solar radiation, and high flow velocities of natural conditions are ~10, ~10, and ~5 µmol/gFW, respectively. Therefore, water temperature, solar radiation and flow velocity are the major dominant environmental factors determining the colonization patterns of macrophytes in the midstream of Japanese rivers.

Species-Specific Trait of H$_2$O$_2$ Concentration in Response to Environmental Conditions

Laboratory and field experiments showed a typical species-specific relationship between H$_2$O$_2$ concentration and the environmental factors of temperature, flow velocity, and solar radiation. With increasing flow velocity, the similar increasing trend of H$_2$O$_2$ concentration was obtained for all tested species. Although flow velocity generates a large amount of H$_2$O$_2$, it does not affect the dominance of a particular species. High solar radiation
radiation intensively generates H$_2$O$_2$ (Asada, 2006). Specifically, the H$_2$O$_2$ concentrations of *E. densa* and *E. nuttallii* were ~10 µmol/gFW under the daily highest solar radiation, which were much higher than those of Japanese native species, *M. spicatum* and *C. demersum*, which were ~6 µmol/gFW. This impacts the ability of *E. densa* to colonize in the shallow zone, where the solar radiation is high; thus, *E. densa* was found to colonize in relatively deep zones (<30 cm deep). Japanese native species were, on the other hand, often found at shoreline or close to the water surface. There was an opposite trend in H$_2$O$_2$ concentrations for water temperature between *E. densa* and other species. With *E. densa*, H$_2$O$_2$ concentration decreased with increasing temperature, while major Japanese native species, *M. spicatum*, *C. demersum*, and *P. spicatum* as well as another invasive species, *E. nuttallii*, showed an increasing trend of H$_2$O$_2$ concentration with temperature. The different trends between Japanese native species and *E. densa* are reflected to their phenology and colonization area.

### Possible Reason for the Overproduction of *E. densa* in Japanese Rivers

Low water temperature increases *E. densa* H$_2$O$_2$ concentration. In rivers where *E. densa* colonized overwhelmingly, water temperature decreases below 8°C in winter. However, even at that time, H$_2$O$_2$ concentration remains below the threshold value at around 1 m deep in stagnant water. Small patchy colonies were found in the upstream of weirs. With increasing temperatures in spring, they started to grow and form a large summer colony, expanding to a shallow zone in the downstream. The channels were originally covered with gravel bed; the bed morphology easily changed under high flow and pools disappeared. However, river rehabilitation for the flood control has been intensively conducted in the last five decades. The shallow zone of the channels was excavated to deepen the channel, and *E. densa* can now colonize with low H$_2$O$_2$ concentration. Weirs were constructed frequently, which created deep stagnant water in the upstream. Thus, the H$_2$O$_2$ concentration of *E. densa* due to velocity and solar radiation may decrease. Gravel mining was conducted, substantially reducing the amount of gravel in the river channel, and there is no longer any sediment transport even at flood time (Asaeda and Sanjaya, 2017). Thus, the modified river morphology does not change even during floods. The artificially created deep zone became a trigger for the overproduction of *E. densa* in the river channel. In the last three decades, river water temperature has significantly increased due to global warming at approximately 0.1°C/year, often reaching 30°C, particularly in western Japanese rivers (Ministry of Environment, 2013). It seems difficult for Japanese native species and *E. nuttallii* to grow in these rivers. Particularly, *E. densa* and *E. nuttallii* are closely related species and came to Japan at nearly the same time. However, the overwhelming colonization of *E. densa* and the limitations of *E. nuttallii* seem to be attributed to the different temperature traits of these species. Local people emphasize the recent reduction of flow rate (personal communication). During the day, in addition to high solar radiation, water temperature is approximately 2°C higher than at night, particularly under a low summer flow rate. Thus, during this time, both solar radiation and temperature increases the H$_2$O$_2$ concentration for Japanese native species. However, their effects are reciprocal and do not affect *E. densa* very much. It is likely another reason for the overwhelming presence of *E. densa*.

### CONCLUSION

Under unpreferable environmental conditions, H$_2$O$_2$ concentrations increase in plant tissues and reflect the macrophyte condition fairly accurately. Potentially, this could be a good indicator of submerged macrophyte colonization. This approach will save time by not requiring casual observations and biomass monitoring of macrophytes in ecosystem monitoring. The experimental and field observations indicated a clear positive relationship between the level of unpreferable conditions and H$_2$O$_2$ concentrations, regardless of abiotic factors. The total H$_2$O$_2$ concentration is provided by the sum of H$_2$O$_2$ generated by each environmental factor, and 16–20 µmol/gFW is required for colonization. The relationships of H$_2$O$_2$ concentrations and the contribution of each abiotic factor were obtained for invasive species (*E. densa* and *E. nuttallii*) and three major Japanese native species (*M. spicatum*, *C. demersum*, and *P. crispus*). The system was applied to develop a mathematical model to simulate the colonization area of these species. The tissue H$_2$O$_2$ concentration decreases with increasing temperature for *E. densa* and increases for other species, including native species. Therefore, native species grow intensively in spring; however, they often deteriorate in summer. For *E. densa*, on the other hand, H$_2$O$_2$ concentration decreases with high water temperatures in summer, allowing intensive growth. High solar radiation increases the H$_2$O$_2$ concentration, deteriorating the plant. Although the H$_2$O$_2$ concentration of *E. densa* increases with low water temperatures in winter, it can survive in deep water with low H$_2$O$_2$ concentration due to solar radiation. Currently, river rehabilitation has created a deep zone in the channel, which has supported the growth and spread of *E. densa*.

### DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

### AUTHOR CONTRIBUTIONS

TA: contributed the conceptualization and field work, and wrote the manuscript together with other members; MR: contributed to field sampling, laboratory and data analyses, and helped write the manuscript; JS: reviewed and commented on the manuscript.
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Conflict of Interest: Author TA was employed by Hydro Technology Institute of Japan. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor is currently organizing a Research Topic with one of the authors JS, and confirms the absence of any other collaboration.

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