Chlorophyll fluorescence changes, as plant early state indicator under different water salinity regimes on the invasive macrophyte *Elodea canadensis* (Michx., 1803)

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Academic editor: Evangelia Drakou

Abstract

Analysis of the photosynthetic apparatus provides information on the physiological state of plants. The changes of metabolites in plant cells analysed with the pulsed chlorophyll fluorometer make it possible to determine these changes in plant cells even in the presence of insignificant cell damage. The possible effects of different salinity levels, 0.584, 1.461, 2.922 and 5.844 PSU (denoting Practical Salinity Unit) on the fluorescence properties of the pigment complexes of the aquatic invasive *E. canadensis* photosynthetic apparatus were investigated. Information about *E. canadensis* macrophyte photosynthetic systems (PSI and PSII) was obtained. After a prolonged impact, the results indicate that high salinity levels in substrates 2.922 and 5.844 PSU seriously affect plant photosynthetic apparatus inhibition. The decrease in $\Delta F_v/F_m´$ values at 2.922 and 5.844 PSU indicates general deterioration in macrophytes' physiological state. In the post-stress period, photosynthesis intensified. An interesting feature was noted: a low water salinity level (0.584) stimulates chlorophyll formation and increases the F_vF_m parameter. The research revealed the influence of salinity levels in the substrate on the photosynthesis processes in plants. The PSII system of submerged macrophytes responds rapidly to high salinity levels, probably due to the inhibition of protein synthesis. These data provide information for further bio-diagnosis of overall plant health and prediction of exposure levels, as well as the ability to make predictions of invasive plant growth and spread. The invasion of this plant macrophyte causes the most serious concern in Europe nowadays.

Keywords

invasive species, Canadian waterweed, salinity, freshwater macrophyte, photosynthesis, bio-testing

Introduction

After analysing the latest data from the various international studies, it can be concluded that *E. canadensis* (Michx., 1803) has been established in more than 26 European countries, while its congeners, the non-native *E. nuttallii* (Planch.) H. St. John and *E. callitrichoides* (Rich.) Casp. are very rare or absent in most of Europe. Due to their morphological similarities, similar habitat preferences and weedy growth, *Elodea* sp. are often misidentified, particularly in the early invasion phases (Nichols and Shaw 1986, Lambdon et al. 2008).

The effects of dissolved sodium chloride stress on freshwater plants have been little studied. However, it is expected to present different levels of salt sensitivity or salt resistance on the plant species. It is noted that one of the generally accepted indicators for the practical study of the overall condition of terrestrial and aquatic plants is the assessment of changes in the primary processes of photosynthesis and the formation of photosynthetic pigments in response to environmental factors (Grinberga and Priede 2010). This indicator's general value for the plant's overall state analysis is the high sensitivity to processes in the photosynthetic apparatus under the earliest stages' adverse factors.

When exposed to even low to moderate salinities, salt-sensitive plants have reduced survival, growth and development. In contrast, salt-tolerant species can grow and reproduce even at oceanic salinities. High salt concentrations impose both osmotic and ionic stresses on the plants, leading to several morphological and physiological changes, such as interruption of pigment synthesis and an overall decrease in photosynthetic activities. However, different species of plants inherently possess various measures and other capacities to cope with exposure to high salinity and salt stress responses and tolerance vary between species (Misra et al. 1998, Jampeetong and Brix 2009).

The F_v/F_m parameter that indicates Maximum Quantum Efficiency (MQE) is the most used chlorophyll fluorescence measuring parameter. F_v/F_0 - while it does not directly correlate with carbon assimilation, it is a very sensitive stress detector and allows comparison of samples in the dark-adapted state. This protocol for measuring the MQE of PSII in plants has withstood time and was developed by Butler and Kitajima 1975. Disturbances in the primary functions of photosynthesis are directly reflected in chlorophyll fluorescence changes and appear long before the visible deterioration of the plant's physiological state. It was also noticed that the measurement of chlorophyll fluorescence is the fastest, most informative and most convenient compared with other experimental methods that also apply to the ecological monitoring of plants (Murata et al. 1966, Jiang et al. 2018). The photosynthesis process is susceptible to high salinity levels, which affect many aspects of this process. The electron transfer along the photosynthetic electron transport chain (ETC) suggests the sequential participation of two photosystems: PSII and PSI. However, the carriers reduced in PSII serve as electron donors for PSI. The activity of photosynthetic systems contemporaneously affects the redox state in plants in another photosystem. This relationship between PSII and PSI manifested in the fluorescence of chlorophyll, the level

of which depends on the redox state of the quinone acceptor (QA). The photoreaction of PSII restores QA, increasing the level of fluorescence and the activity of PSI leads to the oxidation of QA and a decrease in fluorescence (Falkowski et al. 1986, Trissl et al. 1993, Herlory et al. 2007).

Induction changes include primary nitrogen reduction and subsequent reduction due to electrons' appearance in PSII and resulting in changes of the acceptor part of PSI during the photo-activation of ferredoxin-NADP reductase (PNR) (Jassby and Platt 1976, Brack and Frank 1998, Chekalyuk and Hafez 2008, Heidbuchel et al. 2019). An essential advantage of fluorescence methods is their speed and high sensitivity, making it possible to quickly diagnose *in situ* the state of aquatic macrophytes cells under the influence of different adverse factors directly in their environment, non-destructive and in real-time.

The measurement and analysis of F_vF_m parameters were chosen to research the efficiency of the photosynthetic apparatus of the aquatic plant. Changes in chlorophyll fluorescence make it possible to measure the level of plant stress, which is one of the most important criteria for assessing the physiological state of plants; this method is reliable and has proven itself to be accurate and informative (Murata et al. 1966, Jiang et al. 2018). F_V/F_m is a normalised parameter that tests whether or not plant stress affects PSII in a dark-adapted state. F_V/F_m is the most used chlorophyll fluorescence measuring parameter in the world. “The majority of fluorescence measurements are now made using modulated fluorometers with the leaf poised in a known state” (Baker 2008).

The measured levels of F_vF_m in plants make it possible to assess the degree of impact of various abiotic stresses on the plant. Stress factors, such as extreme temperatures, salinity, excessive lighting or vice versa, insufficient lighting and drought, can negatively affect plant metabolism. Such changes can lead to a significant imbalance between the light energy absorption by chlorophyll in plant chloroplasts and, from another side, the efficiency of using this absorbed energy in the process of photosynthesis. Based on changes in the dynamics of the F_vF_m parameter, one can conclude about the ability or inability of the plant for further effective development and growth in general.

Thus, our study aimed to investigate the effect of different salt concentrations (close to those in the sea) on the invasive aquatic plant. This paper presents data about the salinity impact on photosynthetic activity changes of *E. canadensis* leaves. We obtained insights about understanding the possible effect on the invasive freshwater plant after the salinity level increases and subsequent saline water intrusion into the freshwater.

Material and methods

Study site and Sampling

The experiment's plant: Canadian waterweed or pondweed *Elodea canadensis* (Michx., 1803), has been transferred from the natural environment to a laboratory aquarium for further experiment (fresh plants with water were short-time stored in 5-litre polyethylene containers during transportation to the laboratory). Sampling coordinates:

55.92123079059287, 26.58441262054083, Lake Lielais Stropu: Daugavpils, Latvia, northern Europe. *Elodea canadensis* plant sampling was carried out in June 2021. The Lake Stropu's average depth is 3.6 metres, the maximum depth is 6.3 metres and the Lake's area is 417.9 hectares. The Lake is located at an altitude of 110 m above sea level. The Lake is 2.1 km long and 1.7 km wide. Lake Stropu is connected by a small channel to Lake Mazais-Stropu, (a small Strope River), flowing out of the Lake in the northeast and taking out excess water to the Liksna River (right tributary of the Western Dvina River). For this experiment, 15 visually homogeneous green sprouts were selected, each approximately 45 cm long; the depth of macrophyte growth during sampling underwater did not exceed 1.6 m. This Lake is not unique or specific and does not affect the course of this study and all further studies were carried out in the laboratory with fully simulated conditions.

Plant material and experimental conditions.

The plant for the experimental part propagated in the laboratory aquarium tank. For the analysis, we used twice-distilled water mixed with 1.6 g/l (Merck, Hoagland's No. 2 Basal Salt Mixture) solution as a nutrient and various salt concentrations: 0.584, 1.461, 2.922 , 5.844 PSU and control. The growth solution pH level varied within pH 6.0–6.4 ± 0.2 (Mettler Toledo *Five Easy* pH Meter) and was replaced every seventh day. For controlling the salinity level of water in lab tanks during the experimental part, a portable digital refractometer ATAGO (Japan) was used. Measurements were carried out per the refractometer routine procedure: sample size as small as two metric drops (≈ 100 µl) and all readings were automatically compensated for temperature variations. Changes in pigment content and plant photosynthetic activity were carried out every week throughout the experiment (three weeks in total) in June. The plants were grown under the optimal conditions in a Versatile Environmental climate test chamber (Japan), with controlled photoperiodicity: 16 hours light and 8 hours dark, relative illumination 30 $\mu E/m^2$, temperature 18 ± 1 °C and ambient relative humidity (RH) in the climate chamber ≈ 80%.

Measurement of Chl fluorescence, photoinhibition and recovery under controlled conditions.

All measurements were performed at room temperature (20 ± 1°C) and carried out by a hand-held pulse-modulated chlorophyll fluorometer OS–30p (Opti-Sciences, US) with a leaf-clip holder for plant leaves. It allowed simultaneous recording of fluorescence and measured the pigment's redox state of the PSI reaction centre, monitoring the reactions of PSII and PSI simultaneously and recording the induction of changes in delayed fluorescence. Measurements of *Chl* fluorescence and determination of the redox state by the change in absorbance were accomplished at 700–750 nm wavelengths and beam saturation intensity was exposed at 6000 µM as a saturation light source: an array of red LEDs 660 nm. Detection method: pulse-modulated, test duration: 0.1–1.5 seconds and automatic light calibration set to 3500 µM. The default saturation pulse duration was set at 2.0 seconds; however, the software takes a rolling eight-point 25 ms average to determine F_0 and F_m.

Measurements of photoinhibition and the process of natural recovery of chlorophyll fluorescence were carried out on the 7th, 14th and 21st day of exposure by various concentrations of sodium chloride (0.584, 1.461, 2.922, 5.844 PSU) on the plant. The F_m F_0 and F_v/F_m were measured every seven days by OS-30p pulse fluorimeter. After that, the plant was exposed to bright light for 60 seconds (fluorescent lamp used as a light source: photosynthetically active photon flux: 150 µmol $m^{-2}S^{-1}$, Brightness: 12400 ± 1Lx (Testo 545 light meter)). The F_v/F_m Dark-adapted test was carried out in the next stage. This measurement ratio would represent the maximum potential quantum efficiency of Photosystem II if all capable reaction centres were open (Maxwell and Johnson 2000, Baker 2008). For the F_v/F_m Dark-adapted test, the following sampling intervals were used: after 30 minutes; 60 minutes; 90 minutes; 120 minutes and 150 minutes after the exposure to bright light for each concentration (recovery period). In this study, the following time intervals were chosen as the most informative and based on other scientists' experience studying abiotic stresses on plants. It has been experimentally established that similar time intervals might give the complete state of the dynamics of changes in photosynthetic processes in chloroplasts at early stages. Recommended times can vary by chlorophyll fluorescence test type and environmental conditions. Dark adaptation is used in some chlorophyll fluorescence measurements to fix a common known reference point relative to various measurements (Maxwell and Johnson 2000). The data obtained during fluorimetry were used for further analysis.

The ratio of variable to maximal fluorescence or photochemical efficiency (Fv/Fm) of Dark-adapted plants was calculated automatically according to F_o and F_m and measured according to the formula: $[F_v/F_m = (F_m-F_o)/F_m]$ (Hader et al. 1997, Franklin and Badger 2001, Antal et al. 2011, Murchie and Lawson 2013).

Statistical analysis

All measurements were analysed statistically through statistical variance analysis and presented as means and standard deviations (SD). Statistical variance analysis of the independent data with three replicates (n = 3) for the *Chl* fluorescence parameter (F_v/F_m) was analysed using *Statistica* ver.13.3 (StatSoft, Palo Alto, California, USA) and compared with the minor significant differences at $P \leq 0.05$ and $P \leq 0.01$, respectively.

Results and Discussion

Measurements are made by employing a weak modulated light, which is too low to drive photosynthesis, but sufficient to excite pre-photosynthetic antenna fluorescence. After dark adaptation, the fluorometer allows the accurate measurement of minimum fluorescence (F_0). In this state, photosystem II maximally oxidised. The xanthophyll cycle, ∆pH of the thylakoid lumen, state transitions and chloroplast migration have relaxed in their inactive forms. The modulated fluorometer then irradiates the plant leave-plates with an intense saturation light that is high and long enough to reduce or close all available PSII reaction centres entirely (Van Kooten and Snel 1990, Misra et al. 1998). Our studies have shown that high salinity levels of 2.922 and 5.844 PSU after a prolonged time at the third week of

influence substantially reduce the growth of *E. canadensis* (data not shown). The F_v/F_m ratio reflects the maximum quantum yield of charge separation in PSII. Towards the donor side, it partially inhibits the oxygen-releasing complex, while on the acceptor side, it inhibits electron transport. This process also manifests itself in a negative effect on the efficiency of non-cyclic electron transport. The combined parameters serve as indicators of the functional activity of PSII, referred to as the absorbed energy (ABS). These parameters showed the high sensitivity of plants to increasing salinity levels (Krause and Weis 1991, Kolber et al. 1998, Ilik et al. 2003).

The influence of salinity level (5.844 PSU) on *E. canadensis* was manifested in a decrease of chlorophyll F_v/F_m's fluorescence by more than 42% and, after the dark adaptation phase, on 86%, but in the substrate with a 2.922 PSU concentration, observed a decrease of 21% and after the dark adaptation phase, on 78% compared to control samples on the third week (Fig. 1). The observed reduction in the F_v/F_m value was mainly associated with a decrease in the maximum fluorescence ($F_{m)}$ value, which is characteristic of the process of photo-inhibition of PSII. A typical fluorescence trace on a dark-adapted leaf shows how minimum fluorescence intensity (F_0) and maximum fluorescence intensity (Fm) are formed. The measuring beam excites chlorophyll, but is not of sufficient intensity to induce electron transport through PSII. This reaction gives (F_0) a minimal level of fluorescence and reaction centres are said to be open. A brief saturating pulse of light results in the maximum possible yield of fluorescence (F_m). During this, pulse reaction centres are effectively closed. The data show how the values of the indicators F_0, F_m and F_v have changed over three weeks at different salt concentrations presented in the graphs (Fig. 2). A short-lag phase probably preceded the exponential reduction of F_v/F_m to the initiation level due to the accumulation of the active concentration of salt inside the cells. Increased salt levels also affect the donor/ acceptor part of PSII (Butler and Kitajima 1975, Genty et al. 1989, Kuster et al. 2007, Murchie and Lawson 2013). The F_m output during this saturating light radiation represents a sufficiently reduced PSII. It has been found that healthy aquatic macrophytes have an F_v/F_m value in the range of 0.7 to 0.75; lower and higher values possibly indicate plant stress and these indicators differ significantly from plants that grow in soil on the land surface (Maxwell and Johnson 2000).

The presence of photosynthetically active cells in the leaf may indicate their resistance to the effect of small salt concentrations (1.461 PSU) and give the possibility of participation in further plant development (data not shown). Indeed, the F_v/F_m ratio initially slowly decreased from 0.72 to 0.68, after which it returned to its initial state or slightly exceeded the initial value. The result obtained from microfluorimetry is consistent with the assumption that aquatic plants can adapt to the minor and short-lived negative effect of salt by preserving individuals' existing resistance and eliminating the unstable part of the plant population. Photo-inhibition of photosynthesis associated with the development of photo-oxidative stress in cells subjected to varying salinity levels may be enhanced by combining other adverse factors of different natures. The profound inhibition of photosynthesis is mainly associated with the photo-oxidation of the D_1 proteins in the PSII reaction centres (Kuster et al. 2007, Batjuka et al. 2016). The restoration of the activity of PSII is accompanied by resynthesising this protein in the chloroplast. The concentration of active

centres of PSII in plant cells mainly depends on the ratio of its photo-oxidative destruction and repair level. It can be determined by reducing the F_v/F_m value in the light conditions and subsequent relaxation in the dark phase, respectively (Aro et al. 1993, Baker 2008).

The low values of the F_v/F_m parameter for samples grown with 2.922 and 5.844 PSU salt concentrations indicate a decrease in the functional activity of PSII, mainly due to a reduction in the proportion of active reaction centres and an increase in the quenching of excited states in the antenna due to heat generation. A reduction in the efficiency of the excitation energy transfer from the light-harvesting complex to the reaction centre is accompanied by an increase in unused light energy dissipation. It also noted that the quantum efficiency of energy dissipation in cells exposed to high salt concentrations is high. Changes in the redox state of the reaction centre of PSI were less sensitive to elevated salt concentrations. However, a decrease in the recovery rate of PSII was observed. The appearance of delayed fluorescence is due to the accumulation of certain emitted redox states responsible for the reverse recombination of charges and emission of quanta (Massachiro and Takuo 1994, Riis and Hawes 2003, Schreiber 2004). Small concentrations of salt increase the photosynthesis rate and stimulate the growth and development of the aquatic plant. However, at the same time, increased concentrations of dissolved sodium chloride could lead to photo-inhibition and cause photo-oxidative destruction of plant photosynthetic pigments and even death of the organism after prolonged exposure (Sigaud-Kutner et al. 2005, Petjukevičs et al. 2015, Savicka et al. 2018).

F_v/F_m analysis could be confirmed as a method with high sensitivities, performance and accuracy. It allows non-destructive *in situ* measurements to be performed in real-time, which is important for solving various environmental tasks. The basis of fluorescence methods is that chlorophyll, located in photosynthetic membranes, serves as a natural indicator of plant cells' state when cells are disturbed under adverse conditions. Our data have proven its robust way to measure aquatic plant early stress that affects PSII and *Chl* fluorescence changes serve as a source of vital information. Probably high salt concentration also leads to the destruction of chlorophyll-*a* and chlorophyll-*b* while increasing the synthesis of carotenoids as plant protection mechanisms occur until the complete destruction of chloroplasts. These processes can be considered responses to ROS generation in the cell under adverse abiotic factors in the first stage in protecting plant chloroplasts (Loeblich 1982, Yang et al. 2008,Petjukevičs and Škute 2017, Skute et al. 2020).

The destructive effect of salt stress on chlorophyll biosynthesis may be due to the formation of proteolytic enzymes, such as chlorophyllase. However, chlorophyllase is responsible for photosynthetic apparatus damage and chlorophyll degradation (Lichtenthaler 1987,Tanaka et al. 2008). Reduction of photosynthesis rate under high salt levels is associated with decreased plant stomatal conductance and absorption of carbon dioxide occurs outside the stoma. As a result, carbon dioxide content in the intercellular space is reduced (Hasegawa et al. 2000). The photosynthesis reaction rate in the dark phase is reduced and absorbed light negatively affects chloroplasts' reaction centres. The impaired growth and development of plants under sodium chloride stress is a consequence

of physiological response reactions and involves changes in the cellular ionic balance, mineral nutrition, transfer of water through the plant's stomata conductance, photosynthetic rate and, ultimately, in the fixation and utilisation of carbon dioxide. The pigment-protein complex's instability and the disrupted photosynthetic electron transport chain increased chlorophyllase activity, which is the main reason for chlorophyll destruction under salinity (Brugnoli and Bjorkman 1992).

The effect of various salt levels on the aquatic, invasive plant *E. canadensis* was studied. Results suggest that the F_v/F_m distribution pattern in an individual plant may change according to the imposed stress factor. Analysing F_v/F_m parameter fluctuations may be an effective pattern for identifying stress factors. Our study results suggest that the long-term influence of salt in high concentrations suppressed the *E. canadensis* synthesis of chlorophyll and photosynthetic activity in general and disturbs physiological processes, apparently directly affecting the activity or metabolic enzyme synthesis of plant pigments. In turn, a slight increase in salinity level can cause stimulation of photosynthetic processes in this invasive species.

Nowadays, a rapidly changing climate, with an increase in the temperature of the environment, seas and oceans, under the influence of an increase in the anthropogenic factor in Europe and the World, can provoke an increase in salinity in water bodies, thereby changing the usual conditions for local endemic species and creating favourable conditions for the introduction and spread of invasive species. Such climatic changes can subsequently stimulate a negative impact on the local flora by replacing some species with others that were not characteristic of specific regions 10-20 years ago. Thus, it is essential to assess the degree of influence of different salinity levels in the habitat by modelling conditions close to possible scenarios for the development of climate change in the coming decades. The study showed that, for this invasive species distributed throughout Europe, a slight increase in salinity in the water could be a trigger factor for stimulating photosynthesis activity. These results allow us to conclude that a slight increase in salinity in water bodies can provoke more active plant growth and, thus, more productive distribution in new areas while influencing the local flora.

Acknowledgements

We thank the anonymous reviewer for providing helpful criticisms and sincerely appreciate all valuable comments and suggestions that helped us improve the the final manuscript's quality.

Funding program

This research was partly supported by the ESF Project No. 8.2.2.0/20/I/003 "Strengthening of Professional Competence of Daugavpils University Academic Personnel of Strategic Specialization Branches 3rd Call".

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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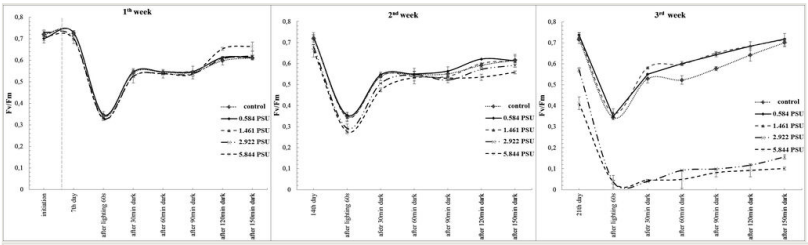

Figure 1.

Measurement of changes in maximum quantum efficiency of PSII photochemistry (F_V/F_m) at different salinities during different exposure days (7, 14 and 21). The error bar represents the standard deviation, n = 3 ($P \leq 0.01$).

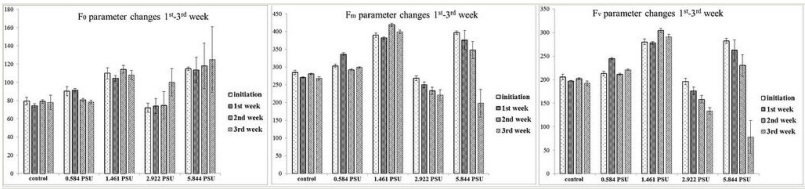

Figure 2.

Changes in maximum fluorescence intensity (F_m), minimum fluorescence intensity (F_0) and variable fluorescence (F_v) at different salinities during different exposure days (7, 14 and 21). The error bar represents the standard deviation, n = 3 ($P \leq 0.05$).