DOI: https://doi.org/10.17921/1415-6938.2022v26n3p344-352

The Use of Chlorophyll *a* fluorescence as a Marker for the Stress Detection *in situ* Bioassays with *Limnobium laevigatum*

O uso da Fluorescência da Clorofila *a* como Marcador para Detecção de Estresse em Bioensaios *in situ* com *Limnobium laevigatum*

Rhuann Carlo Viero Taques*[a]; Vanderlei Aparecido de Lima[b]; Gabriela Pupo Hagemeyer[a]; Patricia Carla Giloni Lima[a]

[a]Universidade Estadual do Centro-Oeste. PR, Brasil.
[b]Universidade Tecnológica Federal do Paraná. PR, Brasil.
*E-mail: rhuanntaques@gmail.com

Abstract

The aim of this study was to evaluate the use of the Fluorescence of chlorophyll *a* (FChla) as a marker for stress detection in bioassays with *Limnobium laevigatum*. Therefore, three tests were carried out: 1) in the laboratory; 2) in situ in a polluted aquatic environment; 3) in situ in a preserved aquatic environment. To diagnose the environmental status of aquatic environments, physical and chemical parameters were measured using a Multiparameter probe (Hanna, Hi 9828). FChla was measured and calculated using the PAM-2500 Fluorometer (Pulse Amplitud Modulated, Heinz Walz, GmB) and the JIP test. The chlorophyll *a* extraction and analysis was carried out according to the methodology proposed by Porra (2002). The data set was submitted to statistical analysis by generalized linear models (GLM) with normal and gamma distribution with functions of Identity and Log binding. The chlorophyll *a* fluorescence using the JIP test parameters was able to detect changes in the physiological conditions of the macrophyte *Limnobium laevigatum* after in situ ecotoxicological assays in preserved and anthropized aquatic environments. This type of spectroscopy can be used as a marker to indicate degrees of pollution in aquatic environments. It was that *L. laevigatum*, a species not conventionally used yet in bioassays in the field of aquatic ecotoxicology, is sensitive to changes in environmental conditions.

Keywords: OJIP Test. Energy Dissipation. Photosynthesis. Macrophyte.

Resumo

O objetivo deste estudo foi avaliar o uso da Fluorescência da clorofila (FChla) a como marcador para detecção de estresse em bioensaios com Limnobium laevigatum. Para tanto, foram realizados três ensaios: 1) em laboratório; 2) in situ em um ambiente aquático poluído; 3) in situ em um ambiente aquático preservado. Para diagnosticar o status ambiental dos meios aquáticos foram mensurados parâmetros físicos e químicos por meio de sonda Multiparâmetros (Hanna, Hi 9828). A FChla foi mensurada por meio do Fluorômetro PAM-2500 (Pulse Amplitud Modulated, Heinz Walz, GmB) e do JIP teste. A extração e análise de clorofila a foi realizada segundo a metodologia proposta por Porra (2002). O conjunto de dados foi submetido a análise estatística pelos modelos lineares generalizados (MLG) com distribuição normal e gama com funções de ligação Identidade e Log. A fluorescência da clorofila a por meio dos parâmetros do JIP teste foi capaz de detectar mudanças nas condições fisiológicas da macrófita Limnobium laevigatum após os ensaios ecotoxicológicos in situ em ambientes aquáticos preservados e antropizados. Esse tipo de espectroscopia pode ser utilizado como marcador para indicar graus de poluição em ambientes aquáticos. Concluímos que L. laevigatum, espécie ainda não convencionalmente utilizada em bioensaios na área da ecotoxicologia aquática, se apresenta sensível às mudanças em condições ambientais.

Palavras-chave: *Teste OJIP. Dissipação Energética. Fotossíntese. Macrófita.*

1 Introduction

The ecotoxicological evaluation of rivers and streams with aquatic plants constitutes an approach that highlights the environmental status and the effect caused in these organisms by waters that commonly receive poisoning effluents along their drainage basin (BARSZCZ *et al*., 2019). In ecotoxicology, it is common to follow guidelines and methodologies that propose analyzes and evaluations of aquatic plants submitted to different conditions and contamination of water bodies in a simulated way from laboratory tests (DOGAN; KARATAS; AASIM, 2018).

However, recent studies indicate that ecotoxicological assays in laboratories do not simulate the complexity of physical, chemical, and biological aspects of natural aquatic environments, in which the test organisms are being submitted (MOTA; SANTANA, 2016; FAJARDO *et al*., 2019; HOLMES; PATHIRATHNA; HASHEMI, 2019; ILLUMINATI *et al*., 2019). Thus, in situ macrophyte tests are increasingly being recommended to reflect the natural dynamic processes in aquatic ecosystems (HOLMES; PATHIRATHNA; HASHEMI, 2019; ILLUMINATI *et al*., 2019; YANG *et al*., 2021).

In this context, the Fluorescence of chlorophyll *a* (FChla) is one of the emerging techniques for terrestrial and aquatic plant stress assessment and monitoring. FChla is the energetic dissipation of luminous energy by plants, algae, and cyanobacteria due to radioactive deactivations of its chlorophyll *a* molecules (KHAN *et al*., 2020). Its analysis allows the identification of structural and functional damage in the plants' photosynthetic complexes from their non-photochemical performances (CARVALHO *et al*., 2020). Thus, FChla parameters show great sensitivity when detecting the effects of biotic and/or abiotic stressors in plants (STIRBET *et al*., 2019). The emission kinetics of the FChla, information about events that occur in the plants' photosynthetic complexes is obtained. The FChla kinetic emission standard is established through the JIP test, proposed by Strasser and Strasser (1995) and improved by other researchers (ZHANG *et al*., 2018; STIRBET *et al*., 2019; KHAN *et al*., 2020).

Bioassays involving FChla kinetics are already well established in several types of research in Europe, Asia, and the United States (PATEL; TIWARI; PRASAD, 2018; SIMKÓ *et al*., 2020; TSAI *et al*., 2019). However, the scarcity of data and research on bioassays with the macrophyte *Limnobium laevigatum* motivated us to carry out this research. Given the above, this study aimed to evaluate the photosynthetic activity through the spectroscopic analysis of the chlorophyll *a* fluorescence in *L. laevigatum*, allocated in three different environments. The biotests were carried out in the laboratory, and *in situ*, that is, in bodies of water. Bioassays took place in an anthropized lotic environment and a stream inside a Conservation Unit (CU) in natural environments.

2 Material and Methods

2.1 Cultivation of *Limnobium laevigatum* and experimental design

Macrophytes of the species *Limnobium laevigatum* Humb. & Bonpl. Ex Willd. were bought in online trading of aquatic plants. Their individuals were cultivated in the Laboratory of Plant Physiology and Ecotoxicology of the State University of the Midwest (Unicentro) in water tanks with a capacity of 12 liters. In each tank, 3 cm of vegetable soil and 30 g of fertilizer were added (N:P:K, in a ratio of 4:14:8) (GILONI-LIMA, 2010).

The experiments were separated into three batches with five individuals of *L. laevigatum*. One batch was intended for *in situ* tests in the inland stream of the Conservation Unit of the Municipal Natural Park of Araucárias (MNPA), Guarapuava, Paraná, Brasil (25°35'02.2" N 51°46'72.5" E). Another batch was kept *in situ* tests in a lotic environment, in the Carro Quebrado River in the CEDETEG campus of Unicentro, Guarapuava, Paraná, Brasil (25°38'28.1" N 51°48'76.5" E). A third batch was placed in a laboratory growth medium. Macrophytes remained conditioned in experimental environments for 15 days.

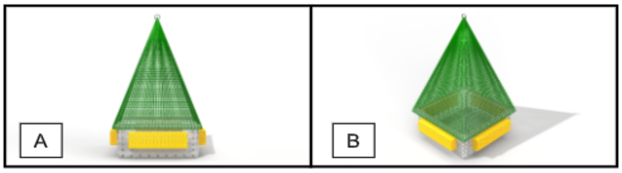
2.2 Physical-chemical analyzes of aquatic environments

The measurements of the physical-chemical parameters were performed employing the Multiparameter Probe (Hanna, Hi 9828). The measurements took place in all the treatments at time zero (beginning of the biotest) and after 15 days of maintenance of macrophytes under the bioassay conditions.

2.3 Apparatus for *in situ* bioassay with the macrophyte *L. laevigatum*

In order to keep macrophytes floating in continuous contact with the waters of the lotic environment and the stream in the CU, plastic containers were used with holes and side buoys. A multiflex nylon mesh was used, which covered the plastic structures, so that maintenance could be done to the macrophytes in its interior (Figure 1). The structures with *L. laevigatum* were kept in aquatic environments fixed to river banks by steel cables.

Figure 1 - Apparatus to support macrophytes in situ biotests. (A) front view; (B) perpendicular view



Source: Data research.

2.4 Analysis of chlorophyll *a* fluorescence in *L. laevigatum* bioassays

Fluorescence analyzes of chlorophyll *a* in L. laevigatum were performed in triplicate in all the experiments. FChla was measured with the PAM-2500 Fluorometer (Pulse Amplitude Modulated, Heinz Walz, GB). The macrophyte leaves were dark-adapted with a leaf clip for 10 minutes, and the assessment of rapid kinetics was used to obtain the OJIP curve (Fast Kinect, Poly300ms protocol). The relative values of chlorophyll fluorescence kinetics were submitted to the JIP test (STRASSER; STRASSER, 1995; GOLTSEV *et al*., 2016). The parameters of quantum yield with their respective formulas can be visualized in Table 1.

Table 1 - Quantum yields of photosynthesis obtained by means of JIP Test

Parameter	Formula	Description
δRo	$= (FM - FI)/(FM - FJ)$	Probability of eletron being transferred from PQ to acceptor of photosystem I
ΨEo	$= 1 - VJ$	Probability of energy of excitation captured by RC II to move an eletron after QA
φPo	$= Fv/FM = 1 - (F50\mu s/FM)$	Maximum quantum efficiency of photosystem II
φDo	$= DIo/ABS = 1 - \varphi Po = (F50\mu s/FM)$	Maximum quantum efficiency of non-photochemical excitation
φEo	$= \varphi Po.\ \Psi Eo = [1 - (F50\mu s/FM)] (1 - VJ)$	Maximum quantum efficiency of eletron transportation
φRo	$= [1 - (Fo/FM)] (1 - VI) = 1 - FI/FM$	Maximum quantum efficiency of eletrons that reach their respective receptors
ABS/RC	$= [(TRo/RC) / (TRo/ABS)]$	Effective size of antenna complex per active RC

Parameter	Formula	Description
TRo/RC	= Mo/VJ	Maximum catch per RC
ETo/RC	= [(TRo/RC) (ETo/ TRo)]	Electron transportation rate per RC
DIo/RC	= [(ABS/RC) - (TRo/RC)]	Active RC dissipation

Source: Adapted from Goltsev *et al.* (2016).

2.5 Chlorophyll *a* measurements in *Limnobium laevigatum* bioassays

Thechlorophyll α extraction was carried out according to the proposed methodology by Porra (2002) and Lichtenthaler and Buschmann (2005). The plant's raw mass was measured on an AAKER brand analytical scale (model JA3003N). The mass was macerated and homogenized in graal and pistil with 2 ml of 80% acetone with 0.01 g of calcium carbonate ($CaCO_{3)}$. Then, the volume was completed to 8 mL of extract, which was subsequently centrifuged at 3000 rpm for 30 minutes. The supernatant was analyzed in a spectrophotometer for absorbance 663 nm and 646 nm, as shown in equation 1.

$$Clorofila\ a = 12.21.A_{663} - 2.81.A_{646} \tag{1}$$

2.6 Statistical analysis of the data set

The data set was subjected to statistical analysis by the generalized linear model (GLM) with normal and gamma distribution with identity and Log functions. Bonferroni performed the mean contrast test at 5% significance level in SPSS software. The correlation analysis was performed in the PAST 4.03 Software.

3 Results and discussion

3.1 Analysis of physical-chemical parameters to *L. laevigatum* bioassays

From the comparative analysis among the three bioassays, the highest values for pH and temperature were for the bioassay in the growth medium in the laboratory. The other physicochemical parameters presented intermediate values in this environment (Table 1). The highest value of the Oxide Reduction Potential (ORP) was observed for the stream waters of the CU from The Municipal Natural Park of Araucárias (Table 1). Studies show ORP is associated with good chemical quality of water in rivers and streams, as this parameter measures the ability of water to cleanse itself or break down products and contaminants. When the ORP value is high, there is a lot of oxygen present in the water (HUSSAR; BASTOS, 2008). This means that bacteria that break down contaminants can work more efficiently. In general, the higher the ORP value, the healthier the water (SIQUEIRA *et al.*, 2012). In addition to the values ranging from 98.33 mV to 221.18 mV revealing the good quality of water in the stream, it also indicates the presence of salts (nitrate and nitrite), as detected in this work.

The waters of the Carro Quebrado River were acidic compared to the waters of the spring and the cultivation medium. In this research pH values below 6.0 were recorded, therefore, in disagreement with Resolution 357/05 of the National Council for the Environment (CONAMA) to bodies of water from Class II. The low pH of the lotic environment (Carro Quebrado Stream) may reflect the intense decomposition process of organic matter of allochthonous origin in its waters. Waters with acidic pH can affect metabolic reactions and result in the mortality of sensitive aquatic organisms (MENDES; FAGUNDES; PEREIRA, 2016). Waters from the UC stream had a neutral pH, while the growth medium had a slightly alkaline pH. Resources show that benthic macroinvertebrates, fish, algae, and macrophytes are generally adapted to neutral pH. (FARIA *et al.*, 2006; PEREIRA *et al.*, 2012; PRESTES; VINCENCI, 2018).

Table 2 - Physicochemical parameters evaluated for the *Limnobium laevigatum* bioassays (Stream of the Conservation Unit of The Municipal Natural Park of Araucárias, in the lotic environment (Carro Quebrado Stream) and the growth medium)

Parameters	Unit	CU Stream	Lotic environment	Growth medium
pH	-	7.28 ± 0.3^{a}	$5.34 \pm 0.2^{b*}$	7.94 ± 0.2^{a}
ORP	mV	221.18 ± 4.5^{a}	98.33 ± 5.0^{c}	188.27 ± 4.3^{b}
DO	$mg.L^{-1}$	6.38 ± 0.80^{b}	14.59 ± 0.7^{a}	3.37 ± 0.6^{c}
Conductivity	$(\mu S.cm^{-1})$	27.00 ± 2.0^{c}	170.33 ± 3.0^{a}	96.33 ± 2.0^{b}
TDS	$(mg.L^{-1})$	14.67 ± 1.2^{c}	291.67 ± 4.9^{a}	90.67 ± 2.3^{b}
Turbidity	(NTU)	0.013 ± 0.0^{c}	0.08 ± 0.001^{a}	0.026 ± 0.0^{b}
Salinity	PSU	5.30 ± 0.4^{b}	7.30 ± 0.2^{a}	1.30 ± 0.4^{c}
Temperature	ºC	18.93 ± 0.3^{c}	22.44 ± 0.2^{b}	24.36 ± 0.2^{a}
Total phosphorous	$(mg.L^{-1})$	0.03 ± 0.0^{b}	$0.21 \pm 0.0^{a*}$	0.08 ± 0.0^{b}
Nitrite	$(mg.L^{-1})$	0.07 ± 0.0^{b}	$1.18 \pm 0.0^{a*}$	0.01 ± 0.0^{c}
Nitrate	$(mg.L^{-1})$	6.84 ± 0.0^{b}	$12.71 \pm 0.0^{a*}$	2.41 ± 0.0^{c}
Ammonia	$(mg.L^{-1})$	$1.68\ 0.0^{b}$	$4.95 \pm 0.0^{a*}$	0.06 ± 0.0^{c}

Caption: pH - potential of hydrogen, ORP- oxidation reduction potential, DO – Dissolved Oxygen; TDS – Total Dissolved Solids. Note: Mean ± standard deviation. Different letters indicate statistically significant differences ($p<0.05$). Asterisks (*) indicate values outside the limits established by Conama Resolution 357/05 for Class II bodies of water.

Source: Research data.

The lotic environment presented high contents of Total Dissolved Solids (TDS) (Table 2), probably due to the large amount of decomposing material carried to these environments from the drainage basin. Titers more significant than 100 µS/cm for conductivity and 200 µg/ml for TDS, as in the Carro Quebrado Stream (Table 2), may indicate effluents from residential and urban drainage water from irrigation systems, and runoff from agricultural areas. Meyer and Franceschinelli (2011) argue that the greater the pollution of an aquatic body, the greater its conductivity and TDS contents. This fact may be due to the high content of mineral and organic content dissolved in this environment. Furthermore, according to Hussar and Bastos (2008), bodies of water rich in humic compounds and with low pH values, as observed in the Carro Quebrado Stream, can present high conductivity values due to the allochthonous material decomposition. These types of effluents can lead to the salts accumulation (cations and anions), in addition to decomposition residues in lotic systems (PIRATOBA *et al.*, 2017).

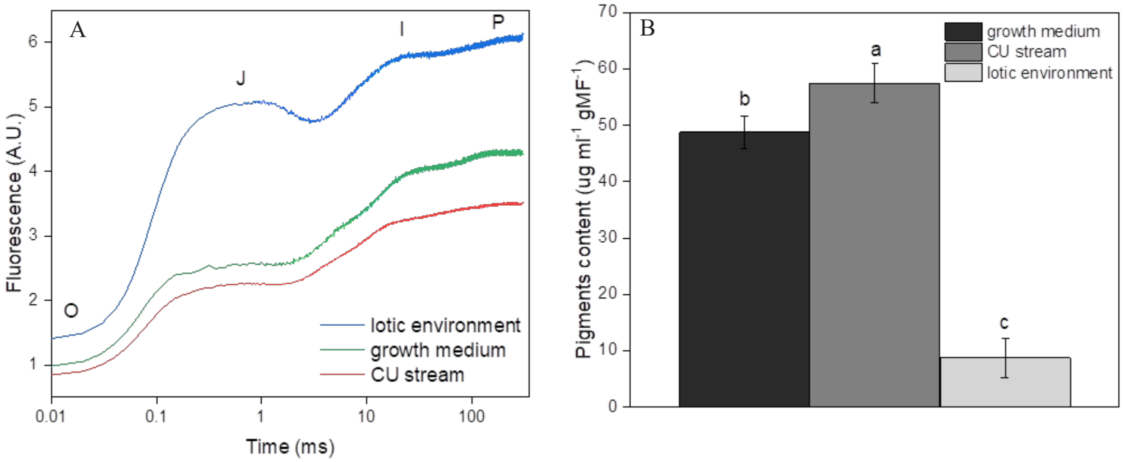
The contents of total phosphorus, nitrite, nitrate and ammonia in the Carro Quebrado Stream were in disagreement with the limits established by Conama Resolution 357/05: 0.21 mg.L -1 (0.1 mg.L^{-1} Phosphorus total); 1.18 mg.L^{-1} (0.1 mg.L^{-1} Nitrite); 12.71 mg.L^{-1} (10.0 mg.L^{-1} Nitrate) and 4.95 mg.L^{-1} (3.7 mg.L^{-1} Ammonia). These results confirm the hypothesis that in this body of water there was allochthonous origin contribution, as pointed out by the study by Carvalho and Monteiro (2020). Ammonia, in particular, is quite restrictive to the life of aquatic organisms. Benthic macroinvertebrates, fish, and macrophytes aquatic species do not support ammonia concentrations above 0.5 mg.L^{-1} (MARCHETTO; NOQUELLI; ALVES, 2019). The average value of the ammonia content recorded in the lotic environment (Table 2) is at the limit of this value, indicating bad environmental conditions in this body of water. There are several other studies carried out in this lotic environment, including with bioindicator organisms. In these studies, pollutants were found (organic and industrial), including heavy metals present in the river, loaded from their urban drainage basin (PUSSININI, 2011; OLIVEIRA; VESTENA, 2012; LIMA, 2018).

3.2 Fluorescence of chlorophyll *a* in *Limnobium laevigatum* bioassay

Distinct patterns of chlorophyll fluorescence were observed in the leaves of *L. laevigatum*. However, the three bioassays analyzed in this study presented typical polyphase Fchla emission curves. It was possible to identify four main points of inflection, representing the O, J, I and P phases (Figure 2).

Individuals of *L. laevigatum* placed in a lotic environment had the highest fluorescence emission and the lowest content of chlorophyll *a* (9.62 mg.L^{-1}) (Figure 2). It is essential to highlight that the three main processes of light energy dissipation by chlorophyll molecules in vegetables are magnetic resonance, heat, and fluorescence emission (GOLTSEV *et al.*, 2016). These processes are competitive (GISRIEL *et al.*, 2020) and, therefore, changes in photosynthetic rates and heat dissipation will cause changes in chlorophyll fluorescence emission (ARSLAN *et al.*, 2020). Thus, the higher values of fluorescence emission by *L. laevigatum* after the *in situ* bioassays in the lotic environment demonstrates that the poor physical and chemical conditions of the waters at this location may have jeopardized and caused structural and/or functional damage in the species' photosynthetic processes (KÜPPER *et al.*, 2019). Plants that emit more fluorescence (non-photochemical dissipation) tend to do less photosynthesis (GOLTSEV *et al.*, 2016). In the present research, this hypothesis was proved, because in the anthropized lotic environment, the lowest chlorophyll *a* content in *L. laevigatum* leaves could be attested.

Figure 2 - (A) Kinetic curves of chlorophyll *a* fluorescence emission in the Stream of the Conservation Unit of The Municipal Park of Araucárias, in the lotic environment (Carro Quebrado Stream), and in the growth medium of the macrophyte *Limnobium laevigatum* bioassays; (B) Chlorophyll *a* content of *Limnobium laevigatum* bioassay after a 15-day stay in the Stream of the Conservation Unit of the Municipal Park of Araucárias, in the lotic environment (Carro Quebrado Stream), and growing medium



Source: Research data.

The macrophyte *L. laevigatum* allocated to the CU stream presented the lowest fluorescence and highest chlorophyll content (57.37 mg.L^{-1}) (Figure 2B). These results demonstrate that after the macrophyte remained in this environment, light energy was better. According to Yang *et al*. (2021) this enables the conduction of photochemical reactions in the membranes of its thylakoids. It can be suggested that the *in situ* plants benefited from the better condition of the aquatic environment since the stream located within the CU has a better preserved ecological status. Results that corroborate what was observed in the plants in the growth medium presenting intermediate chlorophyll values (49.09 mg.L^{-1}) (Figure 2B) and intermediate Fchla emission curves (Figure 2A).

There is a consensus in the scientific literature that phase O of the OJIP curve, parallel to the emission of the initial fluorescence, indicates the moment when all the reaction centers (CR) of photosystems I (FSI) and II (FSII) are open (GOLTSEV *et al*., 2016; FRANKLIN *et al*., 2018; KUMAR *et al*., 2020). In phase O, all the electron acceptors of plastoquinone molecules (QA) are oxidized, able to conduct electrons to the transport chain (TÓTH; SCHANSKER; STRASSER, 2007). After testing in a lotic environment, the high O-phase shift in *L. laevigatum*, indicated as polluted by the physicochemical parameters analyzed in this research, may be related to irreversible damage to the FSII antenna complexes (AC) in FSII. Damage to the FSII blocks electron transfers on the reducing side of this photosystem and dissociates the ACs by high non-photochemical energy dissipation (KUMAR *et al*., 2020). This effect has already been observed in other studies in which aquatic and terrestrial plants were subjected to biotic and abiotic stresses (ARSLAN *et al*., 2020; GOLTSEV *et al*., 2016; KHAN *et al*., 2020; YANG *et al*., 2021).

Generally, values smaller than F50µs reflect a high number of open reaction centers in the plants' photosynthetic apparatus. This phenomenon occurs due to the adequate transport of electrons through the FSII donor sites until the pool of reduced QA plastoquinones. Due to this, the photosynthetic pigment molecules can receive light energy (GOLTSEV *et al*., 2016). Fluorescence initial value smaller than 50 indicates healthy photosynthetic processes (YANG *et al*., 2021). Therefore, in the bioassay stored in a CU stream, the L. laevigatum was in healthier physiological conditions than in other bioassays.

The other phase of the OJIP curve (J phase) denotes the chlorophyll fluorescence emission in 2 ms. This phase occurs in the oxi-reduction process between the plastoquinone molecules QA and the plastoquinone molecules QB (GOLTSEV *et al*., 2016). The most significant displacement of this phase in *L. laevigatum* after the lotic environment experiments reflects the most significant accumulation of the pool fraction of reduced QA in the plant's electron transport chain (Figure 2A). The values of the quantum yield ΨEo (Figure 3) confirm this phenomenon since in a lotic environment, the lowest value for this parameter was recorded, implying the lowest probability that the energy of excitation captured by the CR of the FSII is moved after the plastoquinone QA. This fact also demonstrates that the lotic environment seems polluted by the physicochemical parameters measured in this research (Table 2). In addition to suffering structural and functional damage to its AC, the species also suffered damage to its photosynthetic system (GONÇALVES *et al*., 2007).

Nevertheless, the low J-phase shift (OJIP curve) in *L. laevigatum* after the test in the UC stream (Figure 2A) indicated an increase in the fraction of oxidized plastoquinones QA in plant tissues (YANG *et al*., 2021). These results corroborate their high values of ΨEo measured in the macrophyte in the same biotest (Figure 3).

Phase I of chlorophyll fluorescence (FChla at 30 ms) establishes the balance between the reduction of the plastoquinone pool and its reoxidation by the FSI (GOLTSEV *et al*., 2016). After the bioassay in the lotic environment, a high displacement at this point (FChla at 30 ms) was observed in *L. laevigatum* (Figure 2A). This displcement reflects a low efficiency in reducing final electron acceptors (Ferredoxin and NADP^{+}) on the acceptor side of the FSI (GOLTSEV *et al*., 2016; KHAN *et al*., 2020).

This phenomenon can be confirmed by the significant difference (Table 3) in the δRo values (Figure 3) compared to the bioassay conditions in a growth medium, similar to laboratory experiments. This parameter (δRo) demonstrates that the probability of electrons being transported from the PQ to the FSI acceptors after testing in the lotic environment was smaller than other bioassays.

Table 3 - Average parameters of chlorophyll *a* fluorescence in bioassays with *Limnobium laevigatum* in its growth medium and after *in situ* tests in the lotic environment and in the Conservation Unit stream. Different letters denote statistically significant differences ($p<0.05$)

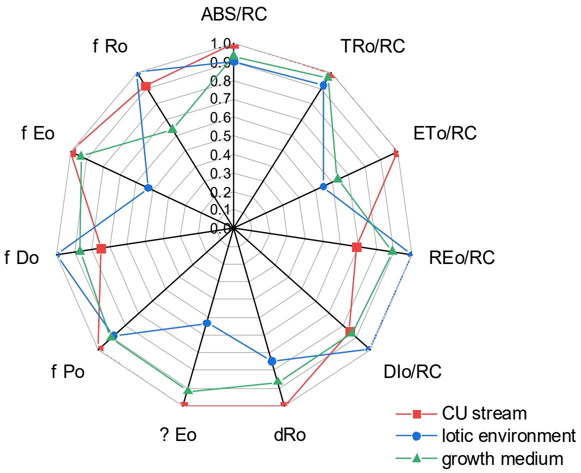
Parameter	CU Stream	Lotic Environment	Growth Medium
ABS/RC	5.90800^{a}	5.32430^{c}	5.49190^{b}
TRo/RC	3.96860^{a}	3.64560^{c}	3.84180^{b}
ETo/RC	1.98030^{a}	1.08130^{c}	1.25710^{b}
REo/RC	0.31250^{c}	0.45350^{a}	0.40440^{b}
DIo/RC	1.65020^{c}	1.93930^{a}	1.67870^{b}
δRo	0.28580^{a}	0.21380^{c}	0.24770^{b}
ΨEo	0.52120^{a}	0.27760^{c}	0.47820^{b}
ϕPo	0.77370^{a}	0.68480^{c}	0.70030^{b}
ϕDo	0.21380^{c}	0.28580^{a}	0.24770^{b}
ϕEo	0.35630^{a}	0.18760^{c}	0.33450^{b}
ϕRo	0.07640^{b}	0.08320^{a}	0.05290^{c}

Source: Research Data

The FChla kinetics from phase I to phase P reflects the gradual FSI electron acceptors reduction and the plastoquinone pool reduction in the electron transport chain (PERBONI *et al*., 2015). For this reason, the I–P phase transition represents the pool of final electron acceptors on the acceptor side of the FSI. The P phase (maximum fluorescence) is involved in the reduction of all-electron acceptors in FSII and FSI (GOLTSEV *et al*., 2016; ZHANG *et al*., 2018).

Studies indicate a positive correlation between FChla P phase and the number of RC temporarily closed to receive light energy (KUMAR *et al*., 2020; NELSON; JUNGE, 2015; WANG *et al*., 2011). Therefore, it can be observed that *L. laevigatum* after the in situ biotest in a lotic environment, had the highest number of PQ molecules in the reduced state and the high of reaction centers temporarily closed in FSII.. Given these facts, *L. laevigatum* in a polluted lotic environment showed high maximum quantum efficiency of non-photochemical excitation (φDo) and low maximum quantum efficiency in FSII (φPo) (Figure 3).

Figure 3 - Normalized values of the quantum yields of the photosynthesis of *Limnobium laevigatum* after the tests in the Conservation Unit Stream of the Municipal Park of Araucárias, in the lotic environment (Carro Quebrado Stream) and its cultivation environment (Growth medium)



Source: Research data.

The low displacement of phases I and P in *L. laevigatum* bioassay after the experiment in the CU interior stream represents a high rate of reaction centers available for its photochemical processes. As a result, in the plants that remained in this location, the highest values for the maximum quantum efficiency of FSII (φPo) and the lowest values for the maximum quantum efficiency of non-photochemical excitation (φDo) were recorded. According to the studies carried out by Gonçalves *et al*. (2007), low values of φDo can be considered adaptations to the species' physiological state due to good environmental conditions.

On the other hand, low non-photochemical excitation generates a much lower quantum yield in FSII, which does not sufficiently reduce QA for electron transport (KÜPPER *et al*., 2019). The highest values of the maximum quantum efficiency of electron transport (φEo) and the maximum quantum efficiency of the electrons that reach the FSI and FSII receptors (φRo) confirm the good physiological conditions of the aquatic plant in the CU stream.The maximum quantum efficiency (φRo) of the macrophyte in the CU stream presented values higher than those recorded in the culture medium as an ideal condition for the development of *L. laevigatum*.

In the literature, it is well established that the value for maximum quantum efficiency in FSII (φPo) should be close to 0.80 to reflect physiologically healthy conditions in plants (GONÇALVES; SANTOS JUNIOR, 2005; OUKARROUM *et al*., 2007). The furthest value from this (0.68480) was recorded in the biotest with *L. laevigatum* after the test in the lotic environment (Carro Quebrado Stream) (Table 3), indicating that the macrophyte faced stress conditions when kept in possibly polluted waters in the local. A highler value close to 0.80 was observed in the bioassay in the CU stream (Table 3). This result corroborates the other analyzes in this study that point to the good physiological conditions of the species kept in this environment. This parameter represents the total number of photons absorbed by chlorophyll α molecules in the reaction centers, divided by the total number of inactive reaction centers (GOLTSEV *et al*., 2016). Thus, in the biotests analyzed after the experiment in a lotic environment, the low number of FSII active centers due to the increase in the P phase (Figure 2A) resulted in a low ABS/RC ratio (Figure 3). After the test in the CU stream, the low displacement of the P phase (Figure 2A) resulted in the highest ABS/RC values recorded (Figure 3), demonstrating that there was greater use after the macrophyte remained in this environment of light energy in its photochemical processes.

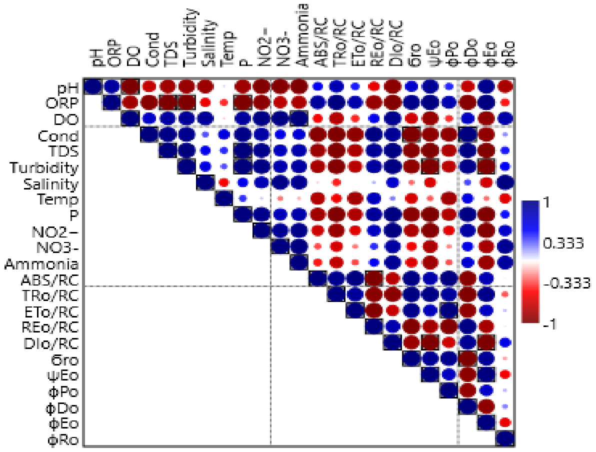
The TRo/RC parameter represents the maximum rate at which the reaction center captures electrons, reducing the plastoquinone pool (GOLTSEV *et al*., 2016). ETo/RC, in turn, represents the rate of these electrons that were transferred to the transport chain for photosynthetic processes. The lowest values for ETo/RC were observed in *L. laevigatum* after testing in the lotic environment (Figure 3). This fact indicates that this local seems polluted. The macrophyte presented the lowest reduction rates and reoxidation in its QA pool. The highest values for ETo/RC were recorded after the test in the CU stream (Figure 3), demonstrating that in this location, *L. laevigatum* showed gains in its photochemical yields due to better water quality.

The high values for REo/RC to the *in situ bioassay* in the lotic environment (Figure 3) reflect the decreased capacity of its reaction centers to reduce plastoquinone molecules. High values to REo/RC indicate stressful environment conditions (TÓTH; SCHANSKER; STRASSER, 2007). The increase in the titers of this parameter has already been registered in plants subjected to light (GONÇALVES *et al*., 2007) and water stress (GOLTSEV *et al*., 2016).

In this research, the chlorophyll fluorescence parameters ABS/RC, TRo/RC, and ETo/RC showed negative correlations with the values of the physical-chemical parameters of TDS, turbidity, total phosphorus, nitrite, nitrate, and ammonia. Such physicochemical parameters indicate possible water pollution of the lotic environment analyzed (Figure 4). These pollutants can affect *L. laevigatum* photosynthetic apparatus due to structural damage to their photosynthetic complexes.

In addition, it found positive correlations of ABS/RC, TRo/RC, and ETo/RC with pH and ORP. It demonstrates that pH and the oxi-reduction capacity of substances in the water are factors that favor the *L. laevigatum* photosynthesis.

Figure 4 - Analysis of linear correlation between the aquatic physicochemical parameters of the experimental environments and the estimated fluorescence parameters in the macrophyte *Limnobium laevigatum*



Caption: pH - Hydrogen Potential; ORP - Oxide Reduction Potential; DO - Dissolved Oxygen; TDS - Total Dissolved Solids; Temp – Temperature; P – Phosphor; NO2- – nitrite; NO3- – Nitrate.
Source: Research data.

The highest values for REo/RC and DIo/RC were observed after the *in situ* test in a lotic environment. The lowest values for these parameters were observed for the experiments allocated in the stream inside the CU (Table 3). The DIo/RC parameter represents the proportion of the total dissipation of uncaptured excitation energy from all the reaction centers concerning the number of active reaction centers (KUMAR *et al*., 2020) that is dissipated as fluorescence (MARTINAZZO *et al*., 2012).

Therefore, this parameter (DIo/RC) corroborates the same φDo results, demonstrating that after the test in the lotic environment, there was an increase in non-photochemical energy dissipation.

REo/RC represents the rate of electron transportation per reaction center (GOLTSEV *et al*., 2016). The lowest values for REo/RC recorded for this parameter occurred after the bioassay in the CU stream. This effect may have been due to the good bed conditions at this location. These types of effects were also observed by Tóth, Schansker and Strasser (2007) in their research.

A positive correlation was observed (Figure 4) between REo/RC and DIo/RC with some measured physicochemical parameters (TDS, total phosphorus, nitrite, nitrate, and ammonia). High concentrations of these chemical species and TDS indicate organic and industrial pollution in bodies of water (MARMONTEL; RODRIGUES, 2015). This polluting load in the lotic environment increases the non-photochemical energy dissipation yields per *L. laevigatum*. Consequently, this event decreases the macrophyte species' photosynthetic capacity. Furthermore, negative correlations of REo/RC and DIo/RC with pH and ORP were observed, indicating acidic waters, such as those from the analyzed lotic environment, and with little capacity for oxidation and water reduction, increase non-photochemical energy dissipation by the species.

4 Conclusion

The chlorophyll *a* fluorescence and the JIP-test detected changes in the physiological patterns of the macrophyte *Limnobium laevigatum, in both bioassays.* This type of spectroscopy can be used as a marker to indicate degrees of pollution in aquatic environments. Thus, *L. laevigatum*, a species not conventionally used yet in bioassays in the area of aquatic Ecotoxicology, is sensitive to changes in environmental conditions. Given these results, it is recommended the macrophyte species *L. laevigatum* as a possible test organism in bioassay studies in the field of Ecotoxicology.

Another relevant aspect found in this research was the physiological responses of this macrophyte species in bioassays maintained in the Conservation Unit (CU) stream. In this place, *L. laevigatum* presented the best results from chlorophyll fluorescence parameters. Thus, the CU of the Araucárias Municipal Natural Park reflects healthy conditions to shelter aquatic life. This CU could be used as a control point in the future in situ bioassays and aquatic ecotoxicology experiments.

References

ARSLAN, O. *et al*. Special issue in honour of Prof. Reto J. Strasser: analysis of drought response of sunflower inbred lines by chlorophyll a fluorescence induction kinetics. *Photosynthetica*, v.58, n.1, p.348-357, 2020. doi: 10.32615/ps.2019.171.

BARSZCZ, L.B. *et al*. Ecotoxicological evaluation of effluents treated by constructed wetlands. *Eng. Sanitaria Amb.*, v.24, n.6, p.1147–1156, 2019. doi: 10.1590/S1413-41522019184120.

CARVALHO, D.A.F.; MONTEIRO, C.A.B. Avaliação da qualidade da água para consumo humano na zona urbana de Campo Maior – PI. *Rev. Bras. Gestão Amb.*, v.14, n.1, p.43-61, 2020.

CARVALHO, R.C. *et al*. Using chlorophyll a fluorescence imaging to select desiccation-tolerant native moss species for water-sustainable green roofs. *Water*, v.12, n.6, p.1-11, 2020. doi: 10.3390/w12061748.

DOGAN, M.; KARATAS, M.; AASIM, M. Cadmium and lead bioaccumulation potentials of an aquatic macrophyte *Ceratophyllum demersum* L.: a laboratory study. *Ecotoxicology and Environ. Safety*, v.148, n.1, p.43-440, 2018. doi: 10.1016/j.ecoenv.2017.10.058.

FAJARDO, C. *et al*. Heavy metals immobilization capability of two iron-based nanoparticles (nZVI and Fe_3O_4): Soil and freshwater bioassays to assess ecotoxicological impact. *Scie. Total Environ.*, v.656, n.1, p.421-432, 2019. doi: 10.1016/j.scitotenv.2018.11.323.

FARIA, M.S. *et al*. Biological and functional responses of *in situ* bioassays with Chironomus riparius larvae to assess river water quality and contamination. *Scie. Total Environ.*, v.371, n.1, p.125-

137, 2006. doi: 10.1016/j.scitotenv.2006.08.036.

FRANKLIN, H.M. *et al*. A novel bioassay to assess phytoplankton responses to soil-derived particulate nutrients. *Scie. Total Environ.*, v.636, n.1, p.1470-1479, 2018. doi: 10.1016/j.scitotenv.2018.04.195.

GILONI-LIMA, P.C. *Estudo dos mecanismos de detoxificaçao e tolerância aos metais cromo e cobre em Pseudokirchneriella subcapitata e Pistia stratiotes e o uso das macrófitas Typha sp. e Phragmites sp. na remoção de nutrientes em wetlands construídos*. São Paulo: USP, 2010.

GISRIEL, C. *et al*. The structure of Photosystem I acclimated to far-red light illuminates an ecologically important acclimation process in photosynthesis. *Scie. Adv.*, v.6, n.6, p.1-12, 2020. doi: 10.1126/sciadv.aay6415.

GOLTSEV, V.N. *et al*. Variable chlorophyll fluorescence and its use for assessing physiological condition of plant photosynthetic apparatus. *Russian J. Plant Physiol.*, v.63, n.6, p.869-893, 2016. doi: 10.1134/S1021443716050058.

GONÇALVES, J.F.C. *et al*. Energetic flux and performance index in copaiba (*Copaifera multijuga* Hayne) and mahogany (*Swietenia macrophylla* King) seedlings grown under two irradiance environments. *Braz. J. Plant Physiol.*, v.19, n.3, p.171-184, 2007. doi: 10.1590/S1677-04202007000300001.

GONÇALVES, J.F.C.; SANTOS JUNIOR, U.M. Utilization of the chlorophyll a fluorescence technique as a tool for selecting tolerant species to environments of high erradiance. *Plant Physiology*, v.17, n.3, p.307–313, 2005. doi: 10.1590/S1677-04202005000300005.

HOLMES, J.; PATHIRATHNA, P.; HASHEMI, P. Novel frontiers in voltammetric trace metal analysis: Towards real time, on-site, *in situ* measurements. *Anal. Chem.*, v.111, n.1, p.206-219, 2019. doi: 10.1016/j.trac.2018.11.003.

HUSSAR, G.J.; BASTOS, M.C. Treatment of effluent of psiculture with aquatic *eichornia crassipes* floating. *Eng. Amb.*, v.5, n.3, p.274–285, 2008.

ILLUMINATI, S. *et al*. In-situ trace metal (Cd, Pb, Cu) speciation along the Po River plume (Northern Adriatic Sea) using submersible systems. *Marine Chem.*, v.212, n.1, p.47-63, 2019. doi: 10.1016/j.marchem.2019.04.001.

KHAN, N. *et al*. Natural variation in the fast phase of chlorophyll a fluorescence induction curve (OJIP) in a global rice minicore panel. *Photos. Res.*, v.3, n.2, p.23-41, 2020. doi: 10.1007/s11120-020-00794-z.

KUMAR, D. *et al*. Chlorophyll a fluorescence kinetics of mung bean (*Vigna radiata* L.) grown under artificial continuous light. *Biochem. Biophys. Rep.*, v.24, n.1, p.100813-100826, 2020. doi: 10.1016/j.bbrep.2020.100813.

KÜPPER, H. *et al*. Analysis of OJIP chlorophyll fluorescence kinetics and QA reoxidation kinetics by direct fast imaging. *Plant Physiol.*, v.179, n.2, p.369-381, 2019. doi: 10.1104/pp.18.00953.

LICHTENTHALER, H.K.; BUSCHMANN, C. Chlorophylls and Carotenoids: Measurement and Characterization by UV-VIS Spectroscopy. *Handbook Food Anal. Chem.*, v.2, n.2, p.171–178, 2005.

LIMA, E.S. *Otimização de metodologia para extração e determinação de metais em sedimentos e aplicação de analises multivariadas e índices geoquímicos na avaliação da contaminação da bacia hidrográfica do rio Cascavel.* Guarapuava: Universidade Estadual do Centro-Oeste, 2018.

MARCHETTO, M.; NOQUELLI, L.H.; ALVES, M.F. Qualidade e enquadramento dos recursos hídricos na bacia do rio Cuiabá na região metropolitana de Cuiabá. *Eng. Scie.*, v.8, n.3, p.2-20, 2019. doi: 10.18607/ES201988029.

MARMONTEL, C.V.F.; RODRIGUES, V.A. Parâmetros indicativos para qualidade da água em nascentes com diferentes coberturas de terra e conservação da vegetação ciliar. *Floresta e Amb.*, v.22, n.2, p.171–181, 2015. doi: 10.1590/2179-8087.082014.

MARTINAZZO, E.G. *et al*. Fluorescência da clorofila a em plantas de pessegueiro da cultivar maciel enxertada sobre diferentes porta-enxertos. *Rev. Bras.Fruticul.*, v.34, n.3, p.678-685, 2012. doi: 10.1590/S0100-29452012000300005.

MENDES, T.A.; FAGUNDES, A.K.B.; PEREIRA, T.S.R. Classificação preliminar de corpos d'água com base na resolução Conama 357/2005: caso do rio Meia Ponte - GO. *Ciênc. Nat.*, v.38, n.3, p.1382-1396, 2016. doi: 10.5902/2179460X21832.

MEYER, S.T.; FRANCESCHINELLI, E.V. Influência de variáveis limnológicas sobre a comunidade das macrófitas aquáticas em rios e lagoas da Cadeia do Espinhaço, Minas Gerais, Brasil. *Rodriguésia*, v.62, n.4, p.743-758, 2011. doi: 10.1590/S2175-78602011000400004.

MOTA, A.C.F.; SANTANA, G.P. Plantas e metais potencialmente tóxicos-estudos de fitorremediação no Brasil. *Scie. Amaz.*, v.5, n.1, p.22–36, 2016.

NELSON, N.; JUNGE, W. Structure and energy transfer in photosystems of oxygenic photosynthesis. *Ann. Rev. Biochem.*, v.84, n.1, p.659-683, 2015. doi: 10.1146/annurev-biochem-092914-041942.

OLIVEIRA, E.; VESTENA, L.R. Morphological changes of river channels in the urban area of Guarapuava (PR), Brazil. *Rev. Amb.*, v.8, n.1, p.757–773, 2012.

OUKARROUM, A. *et al*. Probing the responses of barley cultivars (*Hordeum vulgare* L.) by chlorophyll a fluorescence OLKJIP under drought stress and re-watering. *Environ. Experim. Bot.*, v.60, n.3, p.438–446, 2007. doi: 10.1016/j.envexpbot.2007.01.002.

PATEL, A.; TIWARI, S.; PRASAD, S.M. Toxicity assessment of arsenate and arsenite on growth, chlorophyll a fluorescence and antioxidant machinery in *Nostoc muscorum*. *Ecotoxicol. Environ. Safety*, v.157, n.1, p.369-379, 2018. doi: 10.1016/j.ecoenv.2018.03.056.

PERBONI, A.T. *et al*. Baixas temperaturas sobre a fluorescência da clorofila a em plantas de diferentes híbridos de canola. *Cienc. Rural*, v.45, n.2, p.215-222, 2015. doi: 10.1590/0103-8478cr20131427.

PEREIRA, S.A. *et al*. Aquatic macrophytes as indicators of water quality in subtropical shallow lakes, Southern Brazil. *Acta Limnol. Bras.*, v.24, n.1, p.52-63, 2012. doi: 10.1590/S2179-975X2012005000026.

PIRATOBA, A.R.A. *et al*. A tecnologia de remoção de fósforo: Gerenciamento do elemento em resíduos industriais. *Rev. Amb. Agua*, v.9, n.3, p.445-458, 2017. doi: 10.4136/ambi-agua.1403.

PORRA, R.J. The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls *a* and *b*. *Photosynthesis Res.*, v.73, n.1, p.149-156, 2002. doi: 10.1023/A:1020470224740.

PRESTES, R.M.; VINCENCI, K.L. Bioindicadores como avaliação de impacto ambiental. *Braz. J. Anim. Environ. Res.*, v.2, n.3, p.168-178, 2018.

PUSSININI, N. A gestão urbana e ocupação em áreas de preservação permanente na cidade de Guarapuava (PR): o caso do arroio do Carro Quebrado. *Rev Amb.*, v.7, n.1, p.133-153, 2011.

SIMKÓ, A. *et al*. Evaluation of nitrogen nutrition in diminishing water deficiency at different growth stages of maize by chlorophyll fluorescence parameters. *Plants*, v.9, n.6, p.1–13, 2020. doi :10.3390/plants9060676.

SIQUEIRA, H.E. *et al*. Diagnóstico da qualidade da água de conflito de uso do solo na microbacia Mangabeira, Uberaba-MG. *Enciclop. Biosfera*, v.8, n.14, p.1164–1178, 2012.

STIRBET, A. *et al*. Photosynthesis: basics, history, and modeling. *Ann. Bot.*, v.14, n.126, p.1-27, 2019. doi: 10.1093/aob/mcz171.

STRASSER, B.J.; STRASSER, R.J. Measuring fast fluorescence transients to address environmental questions: the JIP-Test. In: Photosynthesis: from light to biosphere Montpellier: France, 1995.

TÓTH, S.Z.; SCHANSKER, G.; STRASSER, R.J. Photosynthetic electron transport activity in heat-treated barley leaves: The role of internal alternative electron donors to photosystem II. *Scie. Direct*, v.1767, n.1, p.295–305, 2007. doi: 10.1016/j.bbabio.2007.02.019.

TSAI, Y.C. *et al*. Chlorophyll fluorescence analysis in diverse rice varieties reveals the positive correlation between the seedlings salt tolerance and photosynthetic efficiency. *BMC Plant Biol.*, v.19, n.1, p.403-415, 2019. doi: 10.1186/s12870-019-1983-8.

WANG, G. *et al*. Effects of salinity stress on the photosynthesis of *Wolffia arrhiza* as probed by the ojip test. *Fresenius Environ. Bull.*, v.20, n.2, p.432–438, 2011.

YANG, J. *et al*. Simulating chlorophyll-a fluorescence changing rate and phycocyanin fluorescence by using a multi-sensor system in Lake Taihu, China. *Chemosphere*, v.264, p.128482-128495, 2021. doi: 10.1016/j.chemosphere.2020.128482.

ZHANG, Q. *et al*. Composition of photosynthetic pigments and photosynthetic characteristics in green and yellow sectors of the variegated *Aucuba japonica* 'Variegata' leaves. *Flora,* v.240, n.1, p.25–33, 2018. doi: 10.1016/j.flora.2017.12.010.