Short thesis for the degree of Doctor of Philosophy (PhD)

APPLICATION OF CHLOROPHYLL FLUORESCENCE IMAGING IN DUCKWEED ECOTOXICOLOGICAL TESTING

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ABBREVIATIONS

α	Maximal photon-use efficiency
ChlF	Chlorophyll Fluorescence
EC	Effective Concentration
$\mathbf{EC_{max}}$	Effective Concentration associated with maximum stimulatory response
ETR	Electron Transport Rate
$\mathbf{ETR_{max}}$	Maximum value of Electron Transport Rate
$\mathbf{F_m}$	Maximum fluorescence level under the dark-adapted photochemistry
$\mathbf{F'_m}$	Maximum fluorescence level under the stable photochemistry
$\mathbf{F_o}$	Ground state fluorescence under the dark-adapted photochemistry
$\mathbf{F'_o}$	Ground state fluorescence under the light-adapted photochemistry
$\mathbf{F_s}$	Steady state fluorescence
$\mathbf{F_v/F_m}$	Maximum quantum yield of PSII photochemistry
$\mathbf{F_v/F_o}$	Maximum ratio of quantum yields of photochemical and concurrent non-photochemical processes in PSII in dark-adapted state
ISO	International Standard Organization
OECD	Organization for Economic Cooperation and Development
PAM	Pulse Amplitude Modulated fluorometer
PSII	Photosystem complex II
Rfd	Relative fluorescence decrease
RGR	Relative Growth Rate
RLC	Rapid Light Curve
Y(II)	The effective quantum yield of photochemical energy conversion in PSII; a.k.a. Genty parameter
Y(NPQ)	Quantum yield of regulated non-photochemical energy loss in PSII
Y(NO)	Quantum yield of non-regulated heat dissipation and fluorescence emission

1. INTRODUCTION

A variety of pollutants including heavy metals, persistent organic pollutants (POPs), and emerging pollutants such as micro(nano)plastics and polyfluoroalkyl substances (PFAs) are released into the environment due to industrialization, modern agriculture and pharmaceutical advancements (Jafarzadeh et al., 2022; Eid et al., 2024). Freshwater bodies, being in the closest vicinity of industrial and municipal sources, are at the highest risk of getting contaminated (Reid et al., 2019; Ceschin et al., 2021). Therefore, it is important to continuously monitor the pollution levels in the waterbodies to maintain water quality and preserve an overall ecosystem health.

Bioindicators are the organisms that indicate the presence of an environmental stressor (e.g., pollutants, excess nutrient) by manifesting a physical, chemical, or behavioral response (Hee, 1993). In addition to *in situ* water quality monitoring, the toxicological effects of environmental pollutants can also be modeled using bioindicators to evaluate their overall impact on the ecosystem. Duckweeds are the most popular model organisms due to their fast vegetative growth, low maintenance needs and simple anatomy (Ziegler et al., 2016). Considering their practical advantages, internationally adopted growth inhibition-based test protocols have been developed by the International Standard Organization (ISO) and the Organization for Economic Cooperation and Development (OECD) for testing toxicity of environmental pollutants (ISO, 2005; OECD, 2006).

During these tests, the plants are exposed to the targeted pollutant for a defined duration and the changes in the specified endpoints are measured by comparing their initial and final values. These endpoints were initially limited to the biomass and biochemical contents of the test plants, however, the growing interest in predicting environmental impacts has led to the progressive adoption of further phytotoxicity endpoints promising higher sensitivity and a faster response. In phytotoxicological studies, amongst others, chlorophyll fluorescence (ChlF) based endpoints are also considered as promising alternatives or accessories to the traditional growth-based endpoints.

Measuring ChlF induction is non-destructive, and therefore can be jointly applied with other approaches. Several basic and derived endpoints have already been applied in a variety of ways to measure the response of plants to environmental stress factors (Bhagooli et al., 2021). Despite the increasing application of this technique in plant ecophysiological and toxicological studies, however, a comprehensive comparison of reliability and responsiveness has never been made between the classical growth-based methods and the ChlF-based methods using a wider set of toxicants in duckweeds.

2. AIMS AND OBJECTIVES

This thesis is based on two studies involving *Spirodela polyrhiza* L. Schleiden and *Lemna gibba* L. The first study with *S. polyrhiza* was aimed to identify the most suitable ChlF-based endpoints while the *L. gibba*-based study was focused to evaluate the suitability of these endpoints further under exposure to various environmentally relevant metals and metalloids. These latter results with *L. gibba* also formed the basis for comparison whether the findings of *S. polyrhiza*-based investigation were general or species specific. Overall, the objectives of the thesis were to address the following questions and hypotheses:

1. The first aim was to assess which endpoints are the most responsive in duckweed toxicological tests and thus can be recommended in practical applications. The first hypothesis of the study was that the light-adapted parameters are more sensitive than the dark-adapted ones when measured in standard kinetic curves.

2. The second aim was to extract the light- and dark-adapted parameters separately without recording a full induction curve and compare their sensitivities to the standard measurements. The second hypothesis of this study was that acquiring the light- and dark-adapted ChlF parameters in two separate steps still yields endpoints with a comparable sensitivity to the full ChlF induction curve.

3. The third aim was to test whether ChlF-based phytotoxicity endpoints were suitable to replace the growth-based ones. The third hypothesis of the study, therefore, was that ChlF-based endpoints have a comparable sensitivity to the growth-based ones.

4. The standard duckweed growth inhibition tests are conducted in such volumes of medium that are large enough to support growth of the cultures for seven days. The duration and medium requirements, however, can be significantly reduced by multi-well plate-based experimental setups. The fourth hypothesis of this study was that such multi-well plate-based setups can be adopted for duckweed toxicity testing without compromising the sensitivity.

3. MATERIALS AND METHODS

Axenic cultures of two duckweed species *Spirodela polyrhiza* (L.) Schleid. clone #UD0401 and *Lemna gibba* L. clone #UD0101 were picked from the main stocks and allowed to grow for 7 days under axenic conditions using 100 mL of Steinberg medium (Environment Canada, 2007). The sub-stocks were kept in the tissue culturing room of the Department of Botany, University of Debrecen under a continuous, white irradiation (60 ± 2 µE m^{-2} s^{-1}) and controlled temperature (24 ± 2 °C). These sub-stocks were then used as a starting inoculum for our toxicity experiments.

During these experiments, we exposed *S. polyrhiza* plants to nickel (Ni), chromate [Cr(VI)] and sodium chloride (NaCl), while *L. gibba* plants were exposed to 12 environmentally relevant metals and metalloids including silver (Ag), arsenite [As(III)], arsenate [As(V)], cadmium (Cd), chromite [Cr(III)], chromate [Cr(VI)], copper (Cu), mercury (Hg), nickel (Ni), selenite [Se(IV)], selenate [Se(VI)] and zinc (Zn). The exposures lasted for three days (72 ± 2 h) under identical ambient conditions to those of stock culturing.

3.1. *In vivo* chlorophyll fluorescence measurements

We used a Maxi Imaging-PAM chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany) operated via ImagingWin v2.47 software to measure the photosynthetic activity of the test plants. We used slightly different protocols for the two parts of the investigation as detailed in the following subsections.

3.1.1. Standard chlorophyll fluorescence measuring routine in S. polyrhiza

After dark-adapting the plants for 20 minutes, we measured dark-adapted ground fluorescence level (F_o) and maximum fluorescence level (F_m) of the test plants. Afterwards, light-adapted state of the plants was induced using a continuous actinic irradiation for 10 minutes to measure the light-adapted steady-state fluorescence level (F_s) and maximum fluorescence level (F'_m). We also calculated F'_o (i.e., ground state fluorescence under the light-adapted photochemistry) using the following formula (Heinz Walz GmbH, 2019):

$$F'_o = F_o/(F_v/F_m + F_o/F'_m))$$

We calculated six ChlF-based endpoints including F_v/F_m, F_v/F_o, Y(II), F'_v/F'_m, F'_v/F'_o and Rfd using the formulas given by Roháček (2002) and Lichtenthaler et al. (2005).

Additionally, a so-called rapid light curve (RLC) in the light-adapted plants characterizing the functional state of PSII photochemistry was also recorded along with 15 consecutively increasing illumination intensities ranging from 0–530 μE m^{-2} s^{-1}, with each light step lasting for 30 s. At the end of each light step, quasi steady-state F_s and F'_m were determined to calculate the electron transport rate (ETR) of *S. polyrhiza* plants. The RLC plotted using ETR values corresponding to 15 incident PPFD levels was used to determine two further parameters ETR_{max} and α.

3.1.2. Customized chlorophyll fluorescence measuring routine in L. gibba

In this customized protocol, we illuminated the already light adapted *L. gibba* plants for 60 s under blue actinic irradiation and measured the light-adapted F_s and F'_m. The plants were then put into 20 minutes of dark adaptation to fully oxidize their PSII. Subsequently, we measured the dark-adapted ChlF levels (i.e., F_o and F_m) following the same protocol given in section 3.1.1. Two ChlF-based endpoints i.e., F_v/F_o and Y(II) were calculated for *L. gibba* plants.

3.2. Measurement of growth inhibition

We used ChlF images of *L. gibba* cultures taken on the 0^{th} and 3^{rd} days representing the dark-adapted F_m to determine their relative growth rate (RGRs) in terms of the total surface area and frond numbers according to the following formula (OECD, 2006):

$$RGR_X = [\ln(X_f) - \ln(X_i)]/3$$

where X denotes the growth parameter (area or number of fronds), X_i is the initial value of the respective growth parameter on day 0 and X_f is the final value of the respective growth parameter on day 3.

3.3. Data processing and statistical analyses

We fitted non-linear regression models by means of the “drc”-package (Ritz et al., 2015), in R statistical environment (R Core Team, 2015) and in RStudio (RStudio Team, 2023) to estimate the effective concentrations (EC) resulting in 20% (EC_{20}) and 50% (EC_{50}) inhibition of the respective endpoint. We evaluated the sensitivity of ChlF-based endpoints based on their calculated mean ranks for EC_{20} and EC_{50} values in *S.*

polyrhiza. Similarly, the sensitivity of measured growth- and ChlF- based endpoints in *L. gibba* was compared by their median EC_{20} and EC_{50} values using paired sample Wilcoxon test in OriginPro 2016. A Spearman's correlation of the calculated EC_{50} values for *L. gibba* was also measured using the 'corrplot' package (Wei & Simko, 2021) in RStudio.

4. RESULTS AND DISCUSSION

4.1. Comparative responsiveness of ChlF-based endpoints

As a result of the treatments, ChlF parameters of *S. polyrhiza* were affected in the applied concentration range in terms of all the three reference toxicants. The calculated EC_{20} and EC_{50} values for different ChlF-based endpoints showed considerable differences ranging between 1.0–3.7 mg L^{-1} (EC_{20}) and 1.8–20.6 mg L^{-1} (EC_{50}), respectively. According to our results, ETR_{max} and Y(II) were the most responsive endpoints while F_v/F_m and Rfd were the least responsive in Ni treated *S. polyrhiza* plants. A hormetic effect was observed for most of the ChlF parameters when the Ni concentration was <1.25 mg L^{-1}. This stimulatory effect ranged up to 10% as compared to control in the case of calculated ETR_{max}.

The derived EC_{20} values for Cr(VI) were found to be ranging between 0.7–2.5 mg L^{-1} and EC_{50} values to be between 1.8–9.0 mg L^{-1}. Cr(VI) resembled the effects of Ni in that ETR_{max} was the most sensitive endpoint while Rfd and F_v/F_m were the least sensitive ones. Unlike Ni, Cr(VI) did not cause "true" (i.e., >105%) hormetic response in most of the endpoints except Rfd where the stimulation was more than 102% of the control.

Similarly, the calculated EC_{20} and EC_{50} values for NaCl treatments ranged between 7.4–13.2 g L^{-1} and 9.7–15.5 g L^{-1}, respectively. In line with our heavy metal treatments, the highest sensitivity was recorded for ETR_{max}, whereas Rfd and F_v/F_m were the least sensitive endpoints. Similar to Ni treatments, hormetic responses were observed for several ChlF-based endpoints including F_v/F_o, Y(II), F'_v/F'_o, and ETR_{max} at lower NaCl concentrations.

We compared the overall sensitivity of tested ChlF endpoints in *S. polyrhiza* using the mean ranks of the calculated EC_{20} and EC_{50} values. In terms of both EC_{20} and EC_{50}, the order of sensitivity indicated ETR_{max}, Y(II) and F'_v/F'_o to be the most responsive endpoints followed by F_v/F_o. On the other hand, F_v/F_m was the least responsive endpoint along with other light-adapted endpoints including F'_v/F'_m, Rfd and α.

In general, F_v/F_m and Rfd are amongst the frequently used parameters in plant eco physiology research. In fact, F_v/F_m is the most frequently applied parameter in the literature related to ChlF analyses (Guidi et al., 2019; Vidaković-Cifrek & Tkalec, 2023). However, in our study, both F_v/F_m and Rfd were among the least sensitive endpoints. On the other hand, F_v/F_o proved to be more sensitive than F_v/F_m even though both ratios were calculated using the same basic ChlF levels, i.e., F_o and F_m, but F_v/F_o performs over a larger dynamic range than F_v/F_m (Lichtenthaler et al., 2005). Therefore, careful consideration must be taken into account when relying exclusively on F_v/F_m in a toxicological study as this endpoint proved to be quite robust when it comes to plant responses to toxic effects.

4.2. Applicability of the customized ChlF measurement protocol

Similar to *S. polyrhiza*, photosynthetic inhibition was also recorded in terms of both ChlF-based endpoints F_v/F_o and Y(II) in *L. gibba* after exposure to 12 toxic elements. Based on the calculated EC_{50} values, the following order of phytotoxicity was established for the tested metals and metalloids:

F_v/F_o: Cr(VI) > Cu > Ag > As(III) > Cd > Hg > Ni > Se(IV) > Se(VI) > Cr(III) > Zn > As(V)
Y(II): Ag > Cr(VI) > Cu > As(III) > Cd > Hg > Ni > Se(VI) > Se(IV) > Cr(III) > As(V) > Zn

The computed EC_{20} for the two ChlF-based endpoints did not differ substantially according to the paired sample Wilcoxon signed ranks test ($p = 0.176$, $Z = 1.412$). The calculated medians were 2.665 mg L^{-1} (interquartile range: 0.47–7.13 mg L^{-1}) for F_v/F_o and 3.210 mg L^{-1} (interquartile range: 0.81–11.06 mg L^{-1}) for Y(II). However, in terms of EC_{50}, a significant difference was observed ($p = 0.021$, $Z = 2.275$) in their sensitivity. Overall, F_v/F_o proved to have a higher sensitivity with a calculated median EC_{50} of 5.340 mg L^{-1} (interquartile range: 1.41–11.86 mg L^{-1}) as compared to 5.755 mg L^{-1} for Y(II) (interquartile range: 2.09–17.37 mg L^{-1}), respectively. The maximal hormesis stayed below 105% of the respective control and, thus, the EC values were estimated using non-hormetic 3-parameter log-logistic models.

The calculated EC values for the dark- and light-adapted ChlF-based endpoints (i.e., F_v/F_o and Y(II), respectively) derived from the customized 2-step measurement protocol showed an overall strong correlation ($\rho = 0.97$) indicating their strong interdependence despite being measured separately. However, the order of sensitivity of the dark- and light-adapted ChlF-based endpoints in *L. gibba* was the opposite for most of the tested elements contrasting to our results with *S. polyrhiza*. Since we measured the dark-adapted F_v/F_o following the same darkening period (20 minutes), this measurement was practically not influenced by adopting the customized protocol. Therefore, it should have been the applied light-adapted endpoint i.e., Y(II) that showed decreased responsivity.

A possible reason for the altered sensitivity of Y(II) is that it can be significantly affected by even slight differences in the measuring conditions (Murchie & Lawson, 2013). Although we allowed the plants to adapt to the blue LED source for 60 s before applying the saturation pulse, still this shorter period could lead to a different state of PSII photochemistry as compared to the standard protocol. Deriving the ChlF parameters from the customized protocol sometimes resulted in negative values, or summing up the 3 quenching parameters contradicted to the theoretical expectation of Y(II)+Y(NPQ)+Y(NO) = 1 (Irfan et al., unpublished results). These observations pointed to the unsuitability of the customized protocol in such calculations. Based on the results of this study, measuring the dark-adapted ChlF-based endpoints, especially F_v/F_o, would be preferable in phytotoxicity studies when a quick comparison is required in terms of toxicity potential of different environmental stressors.

4.3. Comparative sensitivity of growth- and ChlF- based endpoints

We also recorded clearly developed growth inhibitory effects in our *L. gibba* test plants exposed to the applied toxicants for 3 days. Overall, the following order of phytotoxic potential of the applied toxicants was observed based on the calculated EC_{50} values:

RGR_{frond}: Ag> Cu > Hg > Cd > As(III) > Ni > Cr(VI) > Se(IV) > Se(VI) > Cr(III) > Zn >As(V)
RGR_{area}: Ag > Hg > Cu > Cr(VI) > Cd > Se(VI) > As(III) > Ni > Zn > Se(IV) > Cr(III) > As(V)

The paired sample Wilcoxon signed ranks tests showed highly significant differences for both EC_{20} and EC_{50} ($p < 0.001$, $Z = 3.02$). The resulting median values for EC_{20} were 2.64 mg L^{-1} (interquartile range: 0.81–5.32 mg L^{-1}) for RGR_{frond} and 0.365 mg L^{-1} (interquartile range: 0.13–3.45 mg L^{-1}) for RGR_{area}, respectively. Similarly, in terms of EC_{50}, the corresponding medians were 4.065 mg L^{-1} (interquartile range: 1.74–7.96 mg L^{-1}) for RGR_{frond} and 1.75 mg L^{-1} (interquartile range: 0.38–4.28 mg L^{-1}) for RGR_{area}. Hormesis was observed in terms of growth-based parameters including As(III), As(V), Cu, Ni, Se(IV) and Se(VI), however, to keep data processing uniform within the dataset, we modelled the responses using 3-parameter log-logistic model without a hormetic component.

The results for growth inhibition-based endpoints were consonant with metal phytotoxicity literature where RGR_{area} was comparatively more sensitive than RGR_{frond} in duckweeds (Oláh et al., 2018; Markovic et al., 2021). Frond area is a continuous variable as an opposite to the quantile nature of frond number. This difference contributes significantly to the higher sensitivity of the RGR_{area}. A typical duckweed response is that daughter fronds may reach smaller size under stress since less resources can be devoted to frond expansion, or due to stress-induced morphogenic responses (Potters et al., 2007). This way, the newly-formed but smaller fronds have a relatively less contribution to the increment in total frond area resulting in stronger growth inhibition, while frond number growth may still be less inhibited.

In terms of the tested metals and metalloids, we obtained a similar order of sensitivity for the more sensitive growth- and ChlF-based parameters i.e., RGR_{area} and F_v/F_o, respectively. The median EC_{20} values for RGR_{area} and F_v/F_o were 0.365 mg L^{-1} (interquartile range: 0.13–3.45 mg L^{-1}) and 2.665 mg L^{-1} (interquartile range: 0.47–7.13 mg L^{-1}), respectively, showing a seven-fold difference (paired sample Wilcoxon signed ranks tests $p = 0.042$, $Z = 2.045$). Similarly, median values in case of EC_{50} for RGR_{area} and F_v/F_o, showed a three-fold difference with the respective values of 1.75 mg L^{-1} (interquartile range: 0.38–4.28 mg L^{-1}) and 5.340 mg L^{-1} (interquartile range: 1.41–11.86 mg L^{-1}) according to the paired sample Wilcoxon signed ranks tests ($p = 0.042$, $Z = 2.045$). In addition to the generally higher sensitivity of RGR_{area}, some toxicant-specific patterns were also observed. For instance, F_v/F_o proved to be more sensitive than RGR_{area} when we compared EC_{50} in the case of Cr(VI). Additionally, in the case of As(V), both the EC_{20} and EC_{50} for F_v/F_o were lower than those for RGR_{area}. Similarly, As(III) treatments resulted in comparable sensitivities of RGR_{area} and F_v/F_o in terms of the calculated EC_{20} and EC_{50} values.

Despite the lower sensitivity of ChlF-based endpoints, the calculated EC_{50} values for F_v/F_o and Y(II) in *L. gibba* were strongly correlated to the ones for the growth-based endpoints (i.e., RGR_{frond} and RGR_{area}). Interpreting inhibition of ChlF-based endpoints is not as simple as growth-based endpoints as the former group indicates operability of certain physiological processes of the plants. Thus, if those physiological processes are not affected directly by the applied toxicant, a weaker or delayed sensitivity may occur in the ChlF-based endpoints (Brain & Cedergreen, 2009; Alkimin et al., 2019).

Additionally, the sensitivity of ChlF-based endpoints can also be influenced by irradiation conditions as the test plants exposed to toxicants under sub-saturating irradiance promote up-regulation of PSII repair and ROS scavenging systems compared to higher irradiation levels (Christensen et al., 2003; Wilkinson et al., 2015). As a result of these comparisons, it could be concluded that ChlF- and growth rate-based endpoints might not have the same sensitivity pattern to a specific concentration range. Based on our results, therefore, it is recommended to be very thoughtful before using ChlF-based endpoints as sole method to measure phytotoxic effects.

4.4. Multi-well plate-based vs ISO/OECD standard duckweed tests

The sensitivity of our multi-well plate-based test setup was compared to the reported results by a previous study with a standardized growth inhibition test protocol (Naumann et al., 2007) that used *L. minor*. We found that our setup was less sensitive than the standardized one conducted in accordance with the ISO protocol (ISO, 2005). Their toxicity experiments lasted for 7 days and had 9 metals common with our *L. gibba*-based study including Ag, As(III), As(V), Cd, Cr(VI), Cu, Hg, Ni and Zn. The median EC_{20} and EC_{50} values of the 9 tested metals were significantly lower (EC_{20} = 0.086 mg L^{-1}, EC_{50} = 0.683 mg L^{-1}) in the study by Naumann et al., (2007) compared to ours (EC_{20} = 2.27 mg L^{-1}, EC_{50} = 3.21 mg L^{-1}). The calculated

EC_{20} values in the two studies showed only weak correlation (Spearman's ρ = 0.55, p = 0.125), while this correlation was comparatively stronger between the EC_{50} values (Spearman's ρ = 0.78, p = 0.012).

The RGR_{frond} and RGR_{area} EC_{50} values in the present study, when compared to the values previously obtained in our lab following the OECD (2006) protocol and using the same *S. polyrhiza* clone, were considerably higher (Oláh et al., 2015, 2016; Hepp et al., 2016). In aquatic plants, ChlF endpoints are typically thought to be comparably sensitive to growth-based endpoints (Ralph et al., 2007; Brain & Cedergreen, 2009). However, our results with *S. polyrhiza* contradicted these observations. Regardless of being the most responsive endpoints, the calculated EC_{50} values for ETR_{max} and Y(II) were considerably higher in the present study as compared to growth-based endpoints using the same *S. polyrhiza* clone. The calculated EC_{50} values for the most responsive ETR_{max} in the case of Ni and Cr(VI) were 10-fold higher than EC_{50}s of growth-based endpoints in OECD-conform tests, that ranged between 0.18–0.2 mg L^{-1} (Oláh et al., 2015). Similarly, in NaCl treatments, the present EC_{50} values for ETR_{max} were 2-3 times higher than those for the growth-based endpoints corresponding to 3.45 and 4.51 g L^{-1} for RGR_{area} and RGR_{frond}, respectively (Hepp et al., 2018).

In our plate-based setup, the tests were performed using 4 mL of medium volume for a total exposure time of 3 days as compared to 100 mL vessels used for treatments lasting for 7 days in standard OECD and ISO protocols. Firstly, the plate-based test may result in lower dose (that is toxicant to biomass ratio) at the same nominal toxicant concentrations. Secondly, a shorter exposure time may also cause lower sensitivity of phytotoxicity assays. Regarding different exposures, three-day-long Cd treatments in *S. polyrhiza* resulted in three times higher calculated EC_{50} values for the growth-based endpoints than the seven-days-long treatments under the same experimental conditions (Oláh et al., 2014).

We performed morphometric measurements using ChlF-based images taken by the PAM fluorometer as an easy and time efficient method, however, chlorotic areas of fronds could significantly bias the calculated frond area in this case. Camera photos capture both the healthy and the chlorotic parts of the fronds. Due to no or low ChlF signal from chlorotic regions, on the other hand, ChlF-based images do not indicate plant surface in these parts. Because of these undetected regions, the total surface area could appear to be considerably lower while still maintaining the frond count. This can result in a widening gap between the sensitivities of the two growth-based endpoints.

Despite the comparatively lower sensitivity and methodological constraints, ChlF-based phenotyping techniques can increase the throughput of toxicity assays by providing a non-invasive tool for physiological monitoring. Furthermore, the utilization of imaging techniques in ChlF allowed for the simultaneous measurement of duckweed growth and photosynthetic responses in phytotoxicity studies, resulting in a considerable reduction in testing duration. Finally, using the small-scale multi-well-plate-based testing system in combination with ChlF imaging technique duckweed phytotoxicity assays, can facilitate simultaneous screening of large sample series or multiple duckweed species/clones within a short duration.

5. NEW SCIENTIFIC RESULTS

1. This study compared the most widely used ChlF-based endpoints to evaluate their suitability to replace classic growth-based endpoints. The results didn't support the replacement of growth-based endpoints by the ChlF-based ones, but rather favored joint measurement of both endpoint groups to maximize the information obtained from toxicological tests.
2. The results of this study proved that F_v/F_o is a more sensitive endpoint than the frequently used F_v/F_m and, hence, reporting F_v/F_o should also be promoted instead or in addition to the latter parameter.
3. Measuring ChlF using our customized protocol significantly affected the sensitivity order of the endpoints and resulted in an overall lesser sensitivity of the light-adapted Y(II) as compared to the dark-adapted F_v/F_o. This fact, together with the more standardized way of measuring dark-adapted ChlF parameters, favors using those parameters as endpoints in duckweed-based phytotoxicity tests.
4. Hormetic patterns were found to be prevalent in our results with duckweeds in case of both growth- and ChlF-based endpoints, though even the maximal stimulation was relatively low with the metallic elements. These results suggest that hormesis, especially in case of the more responsive parameters, may play a considerable role in shaping the concentration-response patterns and thus should be considered when analyzing duckweed responses to environmental stimuli.
5. The short-term and less cost-intensive tissue-culture plate-based toxicity test system can be applied in duckweed toxicity assay in case a large number of

species/ecotypes/chemical agents is to be tested within a limited timescale and limited space availability, however, the sensitivity of the tests is lower as compared to standard OECD or ISO test systems.

6. As a baseline for further research of this kind, this study produced a detailed phytotoxicity dataset for *L. gibba* obtained in a multi-well-plate-based configuration for 12 environmentally significant metals and metalloids.

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MTMT ID: 10095099

List of publications related to the dissertation

Foreign language scientific articles in international journals (2)

1. **Irfan, M.**, Mészáros, I., Szabó, S., Oláh, V.: Comparative Phytotoxicity of Metallic Elements on Duckweed Lemna gibba L. Using Growth- and Chlorophyll Fluorescence Induction-Based Endpoints.
   Plants-Basel. 13 (2), 1-16, 2024. ISSN: 2223-7747.
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2. Oláh, V., Hepp, A., **Irfan, M.**, Mészáros, I.: Chlorophyll Fluorescence Imaging-Based Duckweed Phenotyping to Assess Acute Phytotoxic Effects.
   Plants-Basel. 10 (12), 1-19, 2021. ISSN: 2223-7747.
   DOI: http://dx.doi.org/10.3390/plants10122763
   IF: 4.658

List of other publications

Foreign language international book chapters (1)

3. **Irfan, M.**, Qadir, A., Ali, H., Jamil, N., Ahmad, S. R.: Vulnerability of Environmental Resources in Indus Basin After the Development of Irrigation System.
In: Irrigation - Water Productivity and Operation, Sustainability and Climate Change. Ed.: Ricart Sandra, Rico Antonio M., Olcina Jorge, IntechOpen, London, 81-99, 2019. ISBN: 9781789846775
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Foreign language scientific articles in international journals (7)

4. Oláh, V., Kosztankó, K., **Irfan, M.**, Barnáné Szabó, Z., Jansen, M. A. K., Szabó, S., Mészáros, I.: Frond-level analyses reveal functional heterogeneity within heavy metal-treated duckweed colonies.
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5. Bilal, M., Qadir, A., Yaqub, A., Hassan, H. U., **Irfan, M.**, Aslam, M.: Microplastics in water, sediments, and fish at Alpine River, originating from the Hindu Kush Mountain, Pakistan: implications for conservation.
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6. Oláh, V., **Irfan, M.**, Barnáné Szabó, Z., Sajtos, Z., Ragyák, Á., Döncző, B., Jansen, M. A. K., Szabó, S., Mészáros, I.: Species- and Metal-Specific Responses of the Ionome of Three Duckweed Species under Chromate and Nickel Treatments.
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7. Tariq, Z., **Irfan, M.**, Qadir, A.: Barrages influencing microplastics distribution and ingestion; A case study.
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9. Arif, M. M., Qadir, A., Ahmad, S. R., Baqir, M., **Irfan, M.**: Occupational Stress among Medical and Paramedical Staff in Tertiary Care Hospitals Based on Observational Study.
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Foreign language abstracts (1)

11. Barnáné Szabó, Z., **Irfan, M.**, Hepp, A., Mészáros, I., Oláh, V.: Comparison of different non-destructive phenotyping approaches to assess acute phytotoxic effects of arsenic treatments in duckweed tests.
    In: XVI. Kárpát-medencei Környezettudományi Konferencia = 16th Carpathian Basin Conference for Environmental Sciences: absztrakt kötet = abstract book. Szerk.: Cseresznyés Dóra, Király Csilla, Eötvös Loránd Tudományegyetem Természettudományi Kar, Budapest, 240-245, 2021. ISBN: 9789638221827

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