**University of Pannonia**

Doctoral School of Chemistry and Environmental Sciences

Ph.D. Thesis

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# EFFECTS OF NATURAL ENVIRONMENTAL STRESSORS AND XENOBIOTICS ON AQUATIC MICROORGANISMS

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# 1. Introduction

Freshwaters are among the most threatened ecosystems in the world (Dudgeon et al., 2006); therefore, their significance is unquestionable, and their protection is of high priority and urgency. Climate change is considered a global challenge of the 21st century, affecting not only the atmosphere but extending across all Earth's spheres, including the hydrosphere. Inland surface waters are particularly affected, as their temperature is directly linked to atmospheric temperature (Livingstone & Lotter, 1998). As the consequences of climate change, the global warming, alterations in precipitation patterns and intensity, and increased evaporation result in changes in the hydrological cycle as well as other physical and chemical parameters, including enhanced salinization of aquatic ecosystems (Cañedo-Argüelles, 2020).

A significant portion of our surface waters consists of saline water bodies, among which soda lakes form a distinct group due to their dominance of sodium bicarbonate rather than sodium chloride. Their astatic nature, high hydrological sensitivity, elevated conductivity, large daily temperature fluctuations, high turbidity, as well as high pH and total phosphorus (TP) content make them extreme habitats (Horváth et al., 2013; Stenger-Kovács et al., 2014). Among the effects of climate change on lakes, increasing water temperature and conductivity are particularly pronounced in soda lakes, generating biotic changes such as shifts in seasonal dynamics and floristic composition, and the development of algal blooms (Dokulil, 2014). The dominant organisms in soda lake ecosystems are diatoms (Stenger-Kovács et al., 2014), which provide essential ecosystem services (B-Béres et al., 2023); therefore, understanding their potential responses to climate change is of critical importance.

In addition to climate change, anthropogenic pollution of waters represents a global environmental problem, caused by industrial and commercial waste, agricultural activities, and other human interventions (Owa, 2014). Globally, 3.5–4.6 million tons of pesticides are released into the environment in various forms annually (Zhang et al., 2011). Increasing number of research report their presence in both surface and groundwater, often far from their point of application (Louchart & Voltz, 2007; Gilliom, 2007). These pollutants pose risks not only to target organisms but also to non-target species, potentially impacting entire ecosystems.

In response to the spread of pesticide-induced effects, various water management plans and legal regulations have been established. For example, the Water Framework Directive (WFD) promotes the use of bioindicators for assessing ecological status, among which phytobenthos, including the aforementioned diatoms, is recommended. Due to their sensitivity, diatoms are regularly used not only for ecological assessments but also as test organisms in ecotoxicological studies, for instance, to detect pesticide contamination. Exposure to pollutants can alter numerous physiological properties at the intracellular level (Debenest et al., 2010). In many cases, however, pesticides exert only sublethal effects, which can also be effectively detected using diatoms due to their rigid siliceous frustules, which can deform (e.g., Cattaneo et al., 2004; Cuna et al., 2014). Nevertheless, the processes leading to the formation of teratogenic forms remain unclear, warranting further detailed investigation in this field.

In addition to pesticides, fertilizers also act as pollutants in aquatic environments, enriching water bodies with nutrients (eutrophication) and promoting algal blooms. Harmful algal blooms pose serious problems worldwide (Naselli-Flores & Padisák, 2023). Therefore, the quality and ecological status of natural water bodies, as well as their potential uses, depend heavily on the composition and abundance of algal biomass (O’Neil et al., 2012). Continuous qualitative and quantitative monitoring of algal communities is thus essential for water quality management. While numerous techniques exist for biomass determination, they are often costly, time-consuming, and require significant expertise. Various in vivo methods based on chlorophyll fluorescence detection have also been developed (Kahlert & McKie, 2014). Despite advantages such as speed and non-invasiveness, their effectiveness in capturing algal community diversity remains debatable (Kahlert & McKie, 2014), highlighting the need for further instrument development.

## 2. Objectives

Our knowledge of the global environmental stressors affecting surface waters—such as increasing salinization, rising water temperature, and the growing presence of herbicides—is incomplete or lacks consensus for many organisms. Therefore, the primary aim of my doctoral research is to contribute to resolving these gaps and often conflicting viewpoints through the investigation of aquatic microscopic algae. Within my research, the following specific objectives were defined:

I. **To investigate the potential effects of climate change on benthic diatom species (Chapter 2).**

During the research, we examined the effects of climate change on the photosynthesis of the most characteristic benthic diatom species found in soda lakes of the Carpathian Basin. Based on a climate scenario, we modeled the potential effects of increasing temperature and salinity—globally among the most influential environmental parameters—on three dominant diatom species.

II. **To investigate the potential effects of a plant protection product using a benthic diatom as a model organism (Chapter 3).**

In this study, we examined the sublethal effects of the plant protection product maleic hydrazide through the process of teratogenic form development in a cosmopolitan diatom species, *Gomphonema parvulum*. In addition, we investigated whether the appearance of teratogenic abnormalities was accompanied by changes in other physiological properties.

III. **To develop a measuring instrument for the quantitative estimation of algal biomass (Chapter 4).**

Within the framework of the Aquafluosense project (NVKP_16-1-2016-0049), we developed an instrument based on induced chlorophyll fluorescence for estimating algal density and composition. During the research, we also evaluated the efficiency and sensitivity of the instrument for use in ecotoxicological studies.

# 3. Material and Methods

## 3.1. Prediction of the potential effects of climate change based on the photosynthetic activity of diatom species

Among the dominant and characteristic benthic diatom species of soda lakes (Stenger-Kovács & Lengyel, 2015), three species were isolated for this study: *Nitzschia aurariae*, *N. reskoi*, and *N. supralitorea*. Monocultures of the three *Nitzschia* species were grown in modified DIAT medium under batch culture conditions (Lengyel et al., 2015). The ecophysiological characterization of the species was carried out using a photosynthetron incubation system (Üveges et al., 2011; Lengyel et al., 2015), with three parallel samples placed in Karlsruhe flasks for each cell.

The photosynthetic characteristics of the species were determined along gradients of five environmental parameters (four direct and one indirect):

1. Light (direct): 0 – 8 – 35 – 70 – 110 – 200 – 400 – 800 – 1200 µmol $m^{-2}$ $s^{-1}$
2. Temperature (direct): 5 – 10 – 15 – 20 – 25 – 30 – 35 – 40 °C
3. Chloride ion concentration (direct): 0 – 36 – 437.5 – 875 – 1750 – 3500 – 5250 mg $L^{-1}$
4. Sulfate ion concentration (direct): 0 – 50 – 600 – 1200 – 2400 –3600 –4800 –7200 mg $L^{-1}$
5. Conductivity (indirect): 4000–12000 µS $cm^{-1}$

Each experiment lasted 2 hours for all parameters, during which the dissolved oxygen concentration was measured at the beginning and end using an LDO sensor, and chlorophyll-a content was determined via acetone extraction. The gross photosynthetic activity was then calculated (Wetzel & Likens, 2000). Maximum photosynthetic activity ($P_s$) and the photoadaptation parameter ($I_k$) were determined using the equation described by Platt et al. (1980) in the GraFit software (Leatherbarrow, 2009).To determine optimum values and tolerance ranges, Gaussian curves were fitted to the data, where the peak of the curve indicates the optimum, and the width of the bell curve (σ) represents the tolerance range.

In the ecophysiological experiments, the dependent variable was the average maximum photosynthetic activity (Ps) measured along different ion concentrations, while temperature was used as the explanatory variable in both model selection and modeling.For temperature predictions, the RCP6.0 climate scenario was selected and applied to model a future period (2041–2060) and a historical period (1970–2000) for the Carpathian Basin. Based on the equations and temperature predictions from the RCP6.0 scenario, the average photosynthetic activity of the species was modeled monthly between April and October (the photosynthetically active period, PAP) for the selected past and future periods. QGIS 3.2.3 and GRASS 7.4.1 software were used for analyses and visualization.

### 3.2. Effects of maleic hydrazide on the development of teratogenic forms in a diatom species

For the experiments, cultures of *Gomphonema parvulum* were used, which were propagated and maintained in batch culture using DV medium (Hughes & Lund, 1962). The algal cultures were treated with two concentrations of maleic hydrazide (0.1 and 0.01 mg $L^{-1}$), and a control culture without the plant protection product was also applied. All treatments and measurements were conducted in three replicates. The effects of the plant protection product were examined after the following exposure times: 6, 12, 24, 72, 163, 336, and 672 hours.

At the specified time points, 100 mL subsamples were taken from homogenized cultures, and the following physiological parameters were measured: cell density increase determined by counting in a Bürker chamber; chlorophyll and carotenoid content (Wetzel & Likens, 2000); fucoxanthin content (Wang et al., 2018); photosynthetic activity measured via dissolved oxygen concentration (Wetzel & Likens, 2000); enzyme activity (peroxidase, POD; Imberty et al., 1984); lipid content estimated by microscopic measurement of lipid droplet diameters; and the proportion of teratogenic forms assessed by microscopic analysis of prepared samples following hot hydrogen peroxide digestion of the cells (Ács & Kiss, 2004). In addition, teratogenic valves were classified into types as defined by Lavoie (2017).

### 3.3. Development of a fluorescence-based instrument and its applicability in the ecotoxicological assessment of a herbicide

During instrument development, cultures of the green alga *Raphidocelis subcapitata* and the cyanobacterium *Microcystis aeruginosa* were used. For culture establishment and maintenance, Allen medium (Allen, 1968) and Z8 medium (Andersen, 2005) were applied under batch culture conditions.

Within the framework of the Aquafluosense project, a novel device, the Dichroic Fluorometer System (DFS) was developed. This fluorometer is designed to detect algae in a liquid-phase medium and is equipped with a dedicated sample holder. Samples were excited in a dual-head configuration, using different LEDs in each head (LED1 peak wavelength: 630 nm; LED2 peak wavelength: 470 nm). Emitted fluorescence was measured using silicon photodiodes, with detection wavelengths of 716 nm and 708 nm.

Optimization of the measurements began with the selection of an appropriate microplate color, as this can influence the detected fluorescence signal. To identify the optimal microplate, reflectance was measured in the culture media used for algal cultivation (Allen and Z8) as well as in distilled water using channel 1 of the Dichroic Fluorometer System (DFS) (excitation wavelength: 630 nm; detection wavelength: 716 nm). Fluorescence signals were measured and compared for two different microplate colors (black and white). The necessity of dark acclimation prior to measurement was also evaluated using a monoculture of *R. subcapitata*. Fluorescence intensity was measured after 10 minutes of dark acclimation, and the procedure was repeated without dark acclimation.

For comparison of different quantitative measurement approaches, a threefold dilution series comprising six different concentrations was applied for both *M. aeruginosa* and *R. subcapitata*. At each concentration, optical density, chlorophyll content, cell concentration (determined using a Bürker chamber), and fluorescence signal intensity were measured using prototypes of the FMM and DFS instruments.

Furthermore, the limit of detection (LOD) and the lower limit of quantification (LLOQ) for the fluorometric instruments were defined as the minimum signal corresponding to threefold and fivefold standard deviations of the background, respectively. The upper limit of quantification (ULOQ) for the DFS was defined as the upper measurement limit of the instrument.

Finally, ecotoxicity tests were conducted to assess the harmful effects of the herbicide active ingredient isoxaflutole on algal species. Seventy-two-hour algal growth inhibition tests were performed according to OECD guideline 201 (OECD, 2011) using *R. subcapitata* monocultures. Algal density and chlorophyll content were determined via spectrophotometric optical density measurements. The quantum efficiency of photosystem II (PSII; $F_v/F_p$) and changes in fluorescence intensity (Rfd) of the algal photosynthetic system were measured using the FMM module.

## 4. Key Results in Thesis Points

1. Assessment of the impacts of climate change

1.1. The ecophysiological characterization of the diatom species *Nitzschia supralitorea* was conducted. Its maximum photosynthetic activity was approximately ~4 mg C mg Chl-a$^{-1}$ h$^{-1}$; the thermal optimum was 29.8 °C, while the optimal ion concentrations were 2947 mg L$^{-1}$ for $Cl^{-}$ and 4368 mg L$^{-1}$ for $SO_4^{2-}$ (corresponding to ~8935 µS cm$^{-1}$).

1.2. Based on the average photosynthetic activity of the three most dominant diatom species in soda lakes (*Nitzschia aurariae*, *N. supralitorea*, and *N. reskoi*), it can be concluded that during the 2041–2060 period the photosynthesis of these diatom species will likely be more intensive, irrespective of ion type and concentration, as a result of the temperature increase projected by the RCP6.0 climate scenario. In addition, an extension of their photosynthetically active period (PAP) by approximately two months is expected in the future.

2. Assessment of the effects of the herbicide maleic hydrazide

2.1. The herbicide maleic hydrazide induces the formation of teratogenic morphotypes in the diatom species *Gomphonema parvulum*. When a higher concentration (0.1 mg L$^{-1}$) is applied, these abnormalities appear earlier. The teratogenic alterations are primarily manifested as damage to the diatom valve; in addition, mixed abnormal morphotype categories occur in considerable abundance.

2.2. Maleic hydrazide has no significant effect on cell number increase; the appearance of teratogenic forms does not entail a reduction in reproductive capacity. No relationship was detected between the occurrence of teratogenic forms and pigment content, including chlorophyll, carotenoids, or fucoxanthin. In terms of photosynthetic activity, a hormesis effect was observed, i.e., maleic hydrazide treatment initially exerted a stimulatory effect. The activity of the POD enzyme also increased at the beginning of the treatment. The average diameter of lipid droplets shows a positive correlation with the frequency of occurrence of teratogenic forms.

3. Development of a fluorometer

3.1. During the application of the FluoroMeter, a white microplate proved to be a more suitable sample holder than a black microplate. Furthermore, it was established that dark acclimation is not required prior to the measurement of algal samples.

2. Algal biomass measured by the two prototypes, the FMM (FluoroMeter Module) and the DFS (Dichroic Fluorometer System), shows a strong positive correlation ($r^2 \sim 0.9$) with results obtained using conventional methods (chlorophyll content, optical density, and cell counting with a Bürker chamber). The improved DFS prototype exhibits lower limits of detection (LOD) and lower limits of quantification (LLOQ) than the earlier FMM prototype.

3.3. The developed FluoroMeter proved to be suitable for use in ecotoxicological studies. In the ecotoxicological assessment of isoxaflutole, the fluorescence parameter Rfd (vitality index) showed a stronger dose–response relationship during $EC_{50}$ determination, whereas $F_v/F_p$ proved to be an inappropriate endpoint parameter, given that the active ingredient of isoxaflutole does not exert a toxic effect on this parameter.

# 5. Publication List

## 5.1. Publications related to the PhD thesis

**Diána Lázár**, Eszter Takács, Mária Mörtl, Szandra Klátyik, Attila Barócsi, László Kocsányi, Sándor Lenk, László Domján, Gábor Szarvas, Edina Lengyel, András Székács: Application of a fluorescence-based instrument prototype for chlorophyll measurements and its utility in an herbicide algal ecotoxicity assay. *Water*:15.10 (2023): 1866. https://doi.org/10.3390/w15101866 **IF 3,4: SJR:Q1**

Edina Lengyel, **Diána Lázár**, Attila János Trájer, Csilla Stenger-Kovács: Climate change projections for Carpathian soda pans on the basis of photosynthesis evidence from typical diatom species. *Science of the Total Environment*: 710 (2020): 136241. https//doi.org/10.1016/j.scitotenv.2019.136241 **IF7.963: SJR:D1**

Eszter Takács, **Diána Lázár**, Augustine Siakwa, Szandra Klátyik, Mária Mört, László Kocsányi, Attila Barócsi, Sándor Lenk, Edina Lengyel, András Székács: Ecotoxicological Evaluation of Safener and Antimicrobial Additives in Isoxaflutole-Based Herbicide Formulations. *Toxics*: 12.4 (2024): 238. https://doi.org/10.3390/toxics12040238 **IF4.1: SJR: Q1**

## 5.2. Other publication

Edina Lengyel, Sára Barreto, Judit Padisák, Csilla Stenger-Kovács, **Diána Lázár**, Krisztina Buczkó: Contribution of silica-scaled chrysophytes to ecosystems services: a review. *Hydrobiologia* 850.12 (2023): 2735-2756. https://doi.org/10.1007/s10750-022-05075-5 **IF:2.2 SJR:Q1**

Csilla Stenger-Kovács, Viktória B-Béres, Krisztina Buczkó, Tiba Jassam Kaison Al-Imari, **Diána Lázár**, Judit Padisák, Edina Lengyel: Review of phenotypic response of diatoms to salinization with biotechnological relevance. *Hydrobiologia* 850.20 (2023): 4665-4688. https://doi.org/10.1007/s10750-023-05194-7 **IF: 2.2 SJR:Q1**

### 5.3. Conferences related to the PhD thesis

**Diána Lázár**, Borbála Gémes, Szandra Klátyik, Dániel Csősz, Sándor Lenk, Attila Barócsi, László Kocsányi, Nóra Adányi, Eszter Takács, András Székács. (2019) Algadenzitás in situ meghatározása fluoreszcenciás módszerrel. IX. Ökotoxikológiai Konferencia (Budapest, 2019. november 22.) pp. 14-15

**Diána Lázár**, Borbála Gémes, Szandra Klátyik, Dániel Csősz, Sándor Lenk, Attila Barócsi, László Kocsányi, Nóra Adányi, Eszter Takács, András Székács (2019): Algasűrűség in situ meghatározása, fluoreszcenciás módszerrel, LXI. Hidrobiológus Napok Tihany, 2019. október 2-4

**Diána Lázár**, Borbála Gémes, Szandra Klátyik, Dániel Csősz, Sándor Lenk, Attila Barócsi, László Kocsányi, Nóra Adányi, Eszter Takács, András Székács (2019) Fluorescence instrumentation for rapid, in situ water quality assessment. 11th International Conference on Instrumental Methods of Analysis (IMA-2019) (Athens, Greece, Sep 26-29, 2019) P2-31, p. 211, poster

**Diána Lázár**, Szandra Klátyik, Sándor Lenk, Attila Barócsi, László Kocsányi, Nóra Adányi, Eszter Takács, András Székács (2020): Chlorophyll fluorescence instrumentation for a rapid, in situ measurement of algae density. 26th International Symposium on Analytical and Environmental Problems (online) Szeged, Hungary November 23-24 2020 http://www2.sci.u-szeged.hu/isaep/index_htm_files/PROCEEDINGS_ISAEP_2020.pdf pp. 211-215

**Diána Lázár**, Hubai Katalin, András Székács, Edina Lengyel (2023): Transgenerational physiological effects of an herbicide on the diatom *Gomphonema parvulum*. 11th International Shallow Lakes Conference , Tartu, Észtország, június 10-16.

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