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Analysis of Periphyton Features in Post-mining Lakes in Northern Bohemia

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Abstract

One possible way of post-mining landscape restoration is hydric recultivation when the mining lakes emerge. Due to the recent growing numbers of post-mining lakes connected with coal mine suppression, a better understanding of the post-mining lake´s ecosystem is needed. The presented dissertation thesis aimed to study periphyton, which is often a predominant component of the littoral zone of post-mining lakes. The work was conducted in the years 2019–2021 in the three oligo- to mesotrophic post-mining Lakes Medard, Most and Milada in Northern Bohemia. Several periphyton characteristics were assessed: biomass per area, nutrient composition, and autotrophic diversity. Since the periphyton of investigated lakes exists under conditions of severe phosphorus (P) limitation, special focus was paid to the ability of periphyton to acquire P from the water column. Results showed that diatoms, Chlorophyta and Cyanobacteria are the main autotrophic groups of periphyton in all investigated lakes. Their contribution was changing with the increasing trophy and age of the lake from the dominance of diatoms in the highly oligotrophic youngest Lake Medard to the co-dominance of diatoms and Chlorophyta in Lakes Most and Milada. A high fraction of diatom species (25%) in the respective watershed (Ohře river) were found exclusively in the studied post-mining lakes. Contradicting our expectations, specific P uptake affinity decreased in the season and was not correlated with lake water phosphorus concentration. We assume that two main processes concerning the limiting nutrient phosphorus exist: 1) phosphorus acquisition from the lake water and 2) phosphorus internal recycling in the periphyton mats. The obtained knowledge can be used for identifying future changes in the lakes as well as for the prediction of the development of other newly developed lakes established during the restoration of post-mining lands.

Abstrakt

Jednou z možností rekultivace krajiny v minulosti zasažené těžbou je rekultivace hydrická, jež vede ke vzniku unikátních jezer. Ekosystém takto vzniklých jezer nebyl dosud podrobně studován. Cílem předkládané disertační práce je přinést nové poznatky o perifytonu, který je často velmi významným obyvatelem příbřežní zóny jezer vzniklých po těžbě uhlí. Předkládaná práce je zaměřena na unikátní sérii tří různě starých oligo- až mezotrofních jezer Medard, Most a Milada vzniklých po těžbě uhlí na severu České republiky. Experimentální práce byla provedena v rozmezí let 2019 až 2021 a v rámci ní bylo studováno několik parametrů jako například celková biomasa, autotrofní složení a obsah živin v biomase. Jelikož perifyton studovaných jezer přežívá v podmínkách silné limitace fosforem, část studie byla věnována studiu příjmu fosforu perifytonem. Rozsivky, zelené řasy, a sinice byly identifikovány jako hlavní autotrofní organismy perifytonu. Jejich procentuální zastoupení se lišilo s přibývající trofií a stářím jezera od dominance rozsivek v silně oligotrofním nejmladším jezeře Medard do společné dominance rozsivek a zelených řas ve starších jezerech Most a Milada. Ve studovaných jezerech byl nalezen velký počet druhů, které prozatím nebyly v povodí Ohře (kde jsou jezera situována) zaznamenány. V rozporu s naším předpokladem, specifická afinita pro fosfor se snižovala v sezóně a nebyla korelována s koncentrací fosforu ve vodě. Předpokládáme, že vedle příjmu fosforu hraje pro perifyton velkou roli i proces recyklace již získaného fosforu uvnitř nárostu. Získané informace mohou být použity pro modelování budoucího vývoje studovaných jezer i jezer jim podobných vzniklých během rekultivace potěžební krajiny.

Keywords

Post-mining lakes, oligotrophic, periphyton, algae, cyanobacteria, phosphorus cycle, pigment analysis, diversity, autototrophs, benthic

Klíčová slova

Jezera vzniklá po těžbě uhlí, oligotrofní, perifyton, řasy, sinice, koloběh fosforu, analýza pigmentů, diverzita, autotrofové, bentos

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1 Objectives

Gaining knowledge about the newly emerged post-mining lakes is essential since their number will increase in upcoming decades due to the cessation of coal mining. Periphyton, benthic algae and cyanobacterial community, is a prominent component of the post-mining lake´s littoral zones. The overall objective of the present thesis was to explore and evaluate the role of periphyton in the ecosystem of post-mining lakes by describing its temporal changes and spatial distribution.

To fulfil the overall objective of the thesis, several partial goals were delimited:

- To introduce, optimise and apply the following methods on periphyton monitoring:
    - Chemotaxonomical method
    - Phosphorus uptake kinetics
- To describe seasonal changes of the following periphyton features in three post-mining Lakes Milada, Medard and Most in Northern Bohemia:
    - Biomass per area
    - Elemental composition
    - Pigment composition
    - Taxonomic composition of autotrophs
    - Species diversity
    - Growth forms
    - Phosphorus uptake kinetics
- To investigate relationships between the periphyton features and limnological characteristics of the studied lakes.

The thesis consists of three thematically connected studies. **Study I** (**Annex 1**) describes periphyton basic properties such as periphyton biomass per area, nutrient content and autotrophic composition. **Study II** (**Annex 2**) is focused on periphyton´s relation to the limiting nutrient phosphorus. **Study III** (**Annex 3**) deals with periphyton species diversity to underline importance of post-mining lake ecosystems as “hotspots of diversity” in the respective region. In all studies, measured periphyton characteristics are compared to the limnological parameters of the studied lakes.

2 Introduction

2.1 Periphyton

Periphyton is an assembly of water microorganisms living attached to the lakebed. It is composed of multi-layered consortia of photoautotrophs (algae and cyanobacteria) and heterotrophs (bacteria, fungi, and protozoa) with the photoautotrophic microorganisms usually forming the dominant component[1,2]. Periphyton carries out important ecological functions such as nutrient uptake and retention, energy fluxes associated with primary production and respiration and purification of aquatic ecosystems[2–6].

Periphyton has been traditionally investigated in streams[7,8] or wetlands[9,10]. Beside those two ecosystems, periphyton can form prominent biomass also in oligotrophic and mesotrophic lakes[11,12]. Due to its high level of diversity and physiological versatility[13,14] well-developed periphyton can be formed within few weeks[15] and can take part on primary production and nutrient cycles in water bodies, providing the nutrients for other trophic levels in ecosystem[16,17]. In the first stage of post-mining lake formation, submerged macrophytes were identified as pioneer organisms and one of the main sources of organic matter in the littoral zones[18]. We hypothesise that not only submerged macrophytes, but also periphyton has a substantial role in inhabiting the newly developed littoral zone of post-mining lakes.

2.2 Periphyton features as a proxy for the ecosystem of post-mining lakes

Post-mining lakes represent a specific type of man-made ecosystem which increases in numbers due to the popular hydric recultivation and the general trend of coal mining suppression all over the world[19–21]. Several reasons for monitoring of post-mining lakes exists:

- Biodiversity conservation
- Source of water in the landscape
- Recreational opportunities that enhance well-being
- Catchment of water in the landscape
- Prediction of development of:
    - Monitored lake - conservation of water quality
    - Other upcoming post-mining lakes
- Reconstruction of past changes of similar lakes (i.e., moraine-dammed or crater lakes)

Periphyton has already been suggested for biomonitoring of wetlands in Florida[10] or for use as an early warning indicator of nutrient pollution in lakes[22]. Its monitoring is also part of Water Framework Directive[23] and Clean Water Act[24]. As a primary food source for zooplankton, small fish and other small consumers at the base of the food web[25], periphyton acts as a fundament of the ecosystem. Therefore, understanding of the changes in periphyton is critical to determine causes for alterations in communities of higher trophic state (i.e., fish, wading birds) and associated alternation of the entire ecosystem.

2.2.1 Biomass per area

Light, temperature or nutrient (phosphorus or nitrogen) scarcity are considered factors most commonly limiting the primary production and, therefore, also the growth of autotrophs in the freshwater ecosystems[26–28]. The increasing availability of limiting factors, often connected with eutrophication, is therefore followed by an increase in periphyton biomass[29]. Observing changes in the areal periphyton biomass might help to uncover shifts in the limiting factors and therefore serve as an inexpensive and “easy-to-measure” parameter indicating the ecosystem development.

2.2.2 Autotrophic composition on the higher taxonomic level

Once the total biomass is estimated, more detailed analysis might be of additional benefit. Nevertheless, determining the detailed species diversity is difficult to perform and is highly dependent on the skills of taxonomist[30]. On the other hand, sorting the individuals into higher taxonomic units such as Chlorophyta, Cyanobacteria, diatoms and so on, is much easier approach, and it can already bring valuable information about the ecosystem stage. The contribution of autotrophic groups has already been shown to change in response to the aquatic ecosystem changes such as shifts in temperature, concentration of herbicides or other pollutants[31–33]. Periphyton autotrophic composition can be determined directly, by traditional optical microscopy or indirectly, by analysis of group specific pigments.

2.2.3 Nutrient composition

Concerns exist about the benthic algal blooms, which are becoming more common, even in lakes that are considered oligotrophic based on pelagic trophic status indicators[34]. In nutrient enrichment experiments, phytoplankton and periphyton were found to respond differently, suggesting that different nutrients may limit pelagic and benthic primary production, even in the same ecosystem[22]. Therefore, better understanding about the nutrient limitation of periphyton is necessary. By analysis of nutrient composition in periphyton biomass, internal P or N deficiency can be identified[35,36].

In **Study I**, we bring new information about the biomass per area, autotrophic composition using different methods (microscopy, chemotaxonomy) and nutrient content of periphyton in the post-mining lakes in Northern Bohemia. We offer a first insight into the seasonal dynamics of these periphyton features, including data from often neglected winter season.

2.2.4 Contribution of periphyton to the phosphorus cycling

As mentioned previously, the growth of the primary producents in the aquatic ecosystem can be limited by several physical or chemical factors or their combination[26–28]. A very low concentration of phosphorus in the aquatic ecosystem generally suggests P limitation of phytoplankton and periphyton. Different plants and communities, such as periphyton or phytoplankton, differ in their ability to acquire phosphorus from the surrounding[37,38]. Parameter called specific P uptake affinity (*SPUA*) can be used for statistical comparison of those differences as well as for comparison study sites and seasons of one organism[38–40]. Interpretation of *SPUA* is not straightforward due to the unusual units (L $\mathrm{mgOM^{-1}\ h^{-1}}$, OM = organic matter). However, it is analogous to the clearance rate of zooplankton—the volume of water cleared of phosphate per unit of biomass per unit of time[41]. The higher *SPUA* is, the more effective P acquisition is the microorganism/community able. This is beneficial, especially in the low P concentration where the higher *SPUA* compared to the other organisms can give an essential evolution advantage. As a prerequisite to understand the role of periphyton in the P cycling in studied lakes, we monitored changes in the parameters of phosphorus uptake kinetics during the year 2019. Results are summarised in **Study II**.

2.2.5 Periphyton diversity

The littoral zone of lakes is an essential habitat for many species and is denoted as a lake´s compartment with the highest biodiversity[42]. The diversity of periphyton mats can be high, as showed by research in Florida wetlands[14]. Another study recorded that the high species diversity of benthic diatoms reflects a high diversity of other forms of life in the aquatic ecosystem[43]. Functional traits of single species, such as life forms (unicellular, filamentous, colonial) or a form of adherence (mobile, erect, prostrated, stalked), can be observed[44]. The species composition of periphyton and its functional traits are influenced by many abiotic and biotic environmental parameters[45] and can reflect the trophic[46,47] or the successional stage of the aquatic ecosystem. In **Study III,** we offer a detailed analysis of the autotrophic part of periphyton in the post-mining lakes in Northern Bohemia as well as analysis of seasonal shifts in the periphyton species growth forms.

3 Experimental part

All presented work was conducted on periphyton samples from the three post-mining Lakes Medard, Most and Milada. The methods of the presented thesis were addressed in three specific studies that used combinations of various approaches:

Study I: Physical and chemical parameters of the lake environment might change within short periods and, therefore, reflect only current ecosystem condition. In **Study I** we attempt to find “easy-to-measure” periphyton parameters, which have potential to be used as a tool for monitoring the long-term environmental conditions. Key methods used in this study were optical microscopy, high-performance liquid chromatography (HPLC) combined with CHEMTAX and spectroscopy.

Study II: Phosphorus is considered a limiting nutrient for the growth of autotrophs in the freshwater ecosystem. Its increasing concentration is associated with eutrophication and reduction of water quality. Since the post-mining lakes are valuable elements of the landscape and important “biodiversity hotspots”, understanding of the phosphorus traits in this ecosystem is essential. **Study II** describes the ability of periphyton to acquire phosphorus from the water column of post-mining lakes. The key method for this study was use of ^{33}P-labelled orthophosphate in combination with scintillation detection.

Study III: To underline the uniqueness of the post-mining lakes and point out their relevance as an important "biodiversity hotspot" for the respective region, we decided to perform a study aimed at the periphyton biodiversity. Functional traits of single species, such as life (i.e., unicellular, filamentous) or growth form (i.e., prostate, motile), were identified and the seasonal shifts were discussed. The key method for **Study III** was optical microscopy.

Two methods were newly introduced within the experimental part of the proposed dissertation thesis – periphyton pigment detection by high-performance liquid chromatography (**Study I**) and kinetics experiments tracking periphyton P uptake (**Study II**). The implementation of the two above-mentioned methods required a set of preliminary experiments resulting in several optimisation steps. Since the preliminary results of those optimisation steps led to the final methodology, I decided to include these preliminary results within the methodology instead of the results section.

3.1 Study sites and sampling

Three oligo- to mesotrophic post-mining Lakes Medard (493 ha, 59/28 m, flooded in 2016), Most (311 ha, 75/22 m, flooded in 2014), and Milada (area: 252 ha, max/average depth: 25/16 m, flooded in 2010), situated in Northern Bohemia, the Czech Republic, were chosen as study sites (**Fig. 1, Table 1**). Stones covered with periphyton were randomly collected by scuba diving from depths between 0–2 m. Sampling was performed eleven times from February 2019 till October 2021 and consisted of three consecutive weeks in which lakes were visited. Each lake was sampled at two opposite shores, designated as North and South, to cover the heterogeneity of lakes (**Table 1**).

Table 1: GPS location of two sampling sites at each studied lake

GPS	North	South
Lake Medard	50°11'08.8"N 12°36'05.0"E	50°10'38.2"N 12°36'55.4"E
Lake Most	50°32'38.0"N 13°39'36.3"E	50°32'06.5"N 13°38'06.9"E
Lake Milada	50°39'29.9"N 13°56'46.5"E	50°39'08.5"N 13°56'14.0"E

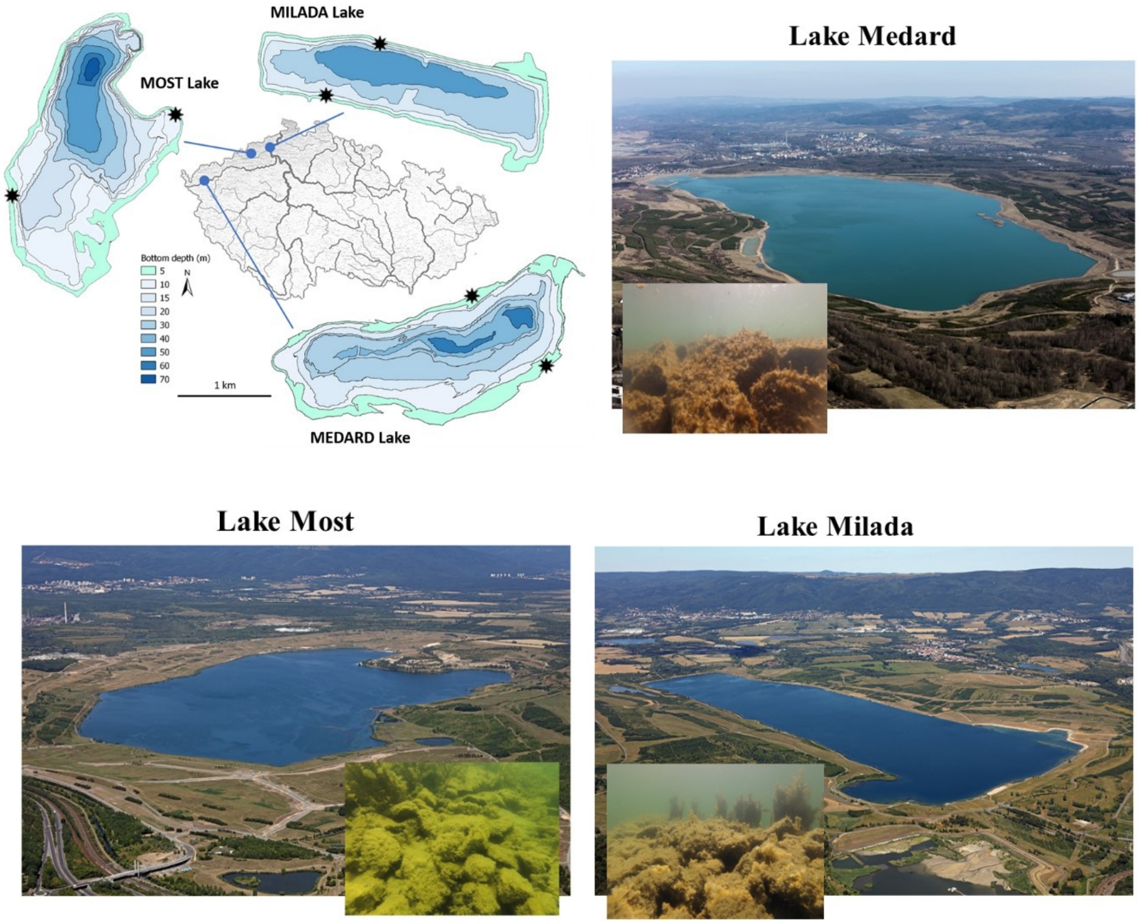

Figure 1: Geographical position of studied Lakes Medard, Most and Milada followed by their aerial view (photos by P. Znachor) with a representative subaqueous picture of periphyton (photos by K. Čapková)

3.2 Periphyton biomass and elemental composition

The quantity of the periphyton biomass per area was determined for all three studies. First, the dry mass (DM) of periphyton homogenate was obtained by drying the sample at 110 °C to the constant weight, followed by burning the sample in the muffle furnace at 500 °C for 2 h to determine the ash mass (AM). Organic matter (OM) was calculated by discriminating AM from DM. Using weight-to-area conversion, stone surface covered by periphyton was estimated by the aluminium foil method[48]. To determine elements composition: total nitrogen, total organic carbon and total phosphorus, samples were stored at –20 °C till the analysis.

3.3 Microscopy

Optical microscopy (Olympus BX 50 light microscope, DIC optics, DP-72 digital camera) was used for the determination of the periphyton species in **Study I** and **III**. Fresh periphytic samples were prepared as well as diatom permanent slides[49,50]. Species composition was assessed using an available determination literature[51]. Nomenclature was updated according to AlgaeBase[52]. The relative abundance of species was estimated using a modified Braun-Blanquet semiquantitative scale of 7–degrees reflecting the individual species biomass to total biomass proportion[53]. The growth forms were distinguished as follows: eukaryotic coccal, eukaryotic filamentous, cyanobacterial coccal, cyanobacterial filamentous, motile, planktic and undifferentiated diatoms. Diatoms were additionally resolved into prostrate, erect, stalked, mobile and planktic.

3.4 High-performance liquid chromatography

3.4.1 Optimisation steps

Samples for **Study I** were collected in the years 2020–2021. A set of samples from the year 2020 was analysed according to the method for marine phytoplankton that was already established in the laboratory in Vienna[54]. However, when the method was applied to periphyton, insufficient resolution of pigment peaks did not allow the resolution of the essential group-specific pigments. Hence, in the spring 2020, I tested a new chromatography method[55]. Newly-introduced HPLC method of Van Heukelem and Thomas (2001) showed better peak resolution **(Table 2)**, therefore, it was favoured above Wright et al. (1991) and introduced in common praxis. As a part of the new method introduction, I prepared a detailed instruction manual which is currently in use in the laboratory in Vienna, where substantial part of the experimental work within **Study I** took place.

Table 2: Comparison of tested methods for determination of periphyton pigments, **YES**, resolved; NO, not resolved, bold indicates resolving new pigments compared to the older method

Pigments	**Wright et al., 1991**	**Van Heukelem and Thomas, 2001**
Chlorophyll c2	NO	NO
Chlorophyll c3	NO	NO
Peridinin	YES	YES
Fucoxanthin	NO	**YES**
Neoxanthin	NO	**YES**
Violaxanthin	NO	NO
19-Hexanoyloxy fucoxanthin	NO	NO
Myxoxanthopyll	YES	YES
Diadinoxanthin	NO	NO
Dinoxanthin	NO	NO
Alloxanthin	YES	YES
Diatoxanthin	YES	YES
Zeaxanthin	NO	**YES**
Lutein	NO	**YES**
Canthaxanthin	YES	YES
Chlorophyll b	YES	YES
Chlorophyll a	YES	YES
α+β carotene	YES	YES

3.4.2 Final methodology

Homogenized periphyton was filtered on glass-fibre filters (GF/C Ederol Company). Pigment extraction was performed in 90% acetone by ultrasonication

(Branson Sonifier 250W) followed by 12 h incubation in the dark at 4 °C. Samples were centrifuged, and the supernatant was taken for spectrophotometric Chl-a analysis at 663 nm[56] and for HPLC. For HPLC, the gradient program according to Van Heukelem and Thomas (2001) with peak detection at 440 nm was applied (Merck-Hitachi LaChrom Elite HPLC System, equipped with L-2455 diode array detector and L-2485 FL-detector). In total, 15 pigment or pigment groups were successfully resolved: the sum of chlorophyll c3 + c2, peridinin, fucoxanthin, neoxanthin, myxoxanthophyll, sum of diadinoxanthin and dinoxanthin, alloxanthin, diatoxanthin, zeaxanthin, lutein, canthaxanthin, chlorophyll b, chlorophyll a and sum $\alpha+\beta$ carotene. Pigment standards (DHI Lab Products, Denmark) were used for the peak identification and calculation of the actual pigment concentrations.

3.5 CHEMTAX analysis

HPLC analysis was followed by data analysis in the matrix factorization program Chemtax 1.95. Chemtax uses factor analysis and the steepest descent algorithm to identify the best fit to the data[57]. Input data for the Chemtax analysis consists of: (a) the initial estimates of the marker pigments to chlorophyll a ratios for each taxonomic group possibly present and (b) the concentration of pigments in the sample. The steepest descent algorithm searches for the best fit to the data, based on the pre-set initial ratios. In other words, program strives to allocate all the present chlorophyll a to the possibly present taxonomic groups with respect to the initial ratios of marker pigments. For my analysis, six major taxonomic groups were delimited based on the microscopical observation and the presence of their marker pigments. Initial ratios for the possibly present taxonomic groups were extracted from the available literature[58,59] and are shown in **Table 3**. For optimisation of the input matrix, series of 60 pigment ratio tables were generated and the final output matrix was calculated based on lower Root Mean Square Error (RMSE) as recommended by Higgins et al (2011). Ratio limits and initial step size and step ratio were set based on recommendations in the Chemtax users´ manual as 10 and 1.3, respectively[60].

Table 3: Initial ratio matrix. Chl-c = chlorophyll c, Peri = peridinin, Fuco = fucoxanthin, Neo = neoxanthin, Myxo = myxoxanthophyll, Diadi = diadinoxanthin, Allo = alloxanthin, Lut = lutein, Cantha = canthaxanthin, Chl-b = chlorophyll b, Chl-a = chlorophyll a

Initial Ratio Matrix											
	Chl-c	Peri	Fuco	Neo	Myxo	Diadi	Allo	Lut	Cantha	Chl-b	Chl-a
Diatoms	0.048	0	0.307	0.001	0	0.086	0	0	0	0	1
Cyanobacteria	0	0	0	0	0.122	0	0	0	0.004	0	1
Chlorophyta	0	0	0	0.037	0	0	0	0.136	0	0.353	1
Dinophyta	0.248	0.43	0	0	0	0.241	0	0	0	0	1
Cryptophyta	0.125	0	0	0	0	0	0.359	0	0	0	1
Xanthophyta	0.123	0	0.392	0	0	0.158	0	0	0.001	0	1

3.6 Phosphorus uptake kinetics

3.6.1 Optimisation steps

Preliminary experiments for **Study II,** where phosphorus uptake kinetics was investigated, were performed in February 2019. The first set of preliminary experiments with the periphyton was conducted to find out the optimal range of the phosphorus concentration for the seasonal study. The goal was to cover a wide range of concentrations and achieve saturation of the phosphorus uptake curve. As a result of preliminary experiments, the range of phosphorus concentrations 1–100 mgP L^{-1} was chosen for the seasonal and inter-lake comparison. However, as I set the range of the tested P concentrations in February, I found that some data lacked saturation in the later seasons. Therefore, both the simplified linear model and the Michaelis–Menten model were fitted across all the data. The more complex model was selected in preference to the simpler one when improving the fit at the probability level of $p < 0.05$ (F test). Michaelis–Menten kinetics was chosen in 23 out of the 35 cases, while the remaining 12 followed the simplified linear model, for illustration of two models, see **Fig. 2**. The periphyton specific P uptake affinity was calculated as ratio of maximum uptake velocity and half-saturation constant (V_{max}/K_s) for Michaelis–Menten model while for the linear model, it was directly equal to P uptake velocity slope (v_{slope}). The implementation of a scintillation methodology for periphyton phosphorus uptake measurement into the laboratory praxis at the Institute of Hydrobiology is one of the crucial results within **Study II**. The introduced method can be used for measuring of phosphorus kinetics of variable types of periphyton (i.e., epilithon, epiphyton, epipelon) from variable habitats.

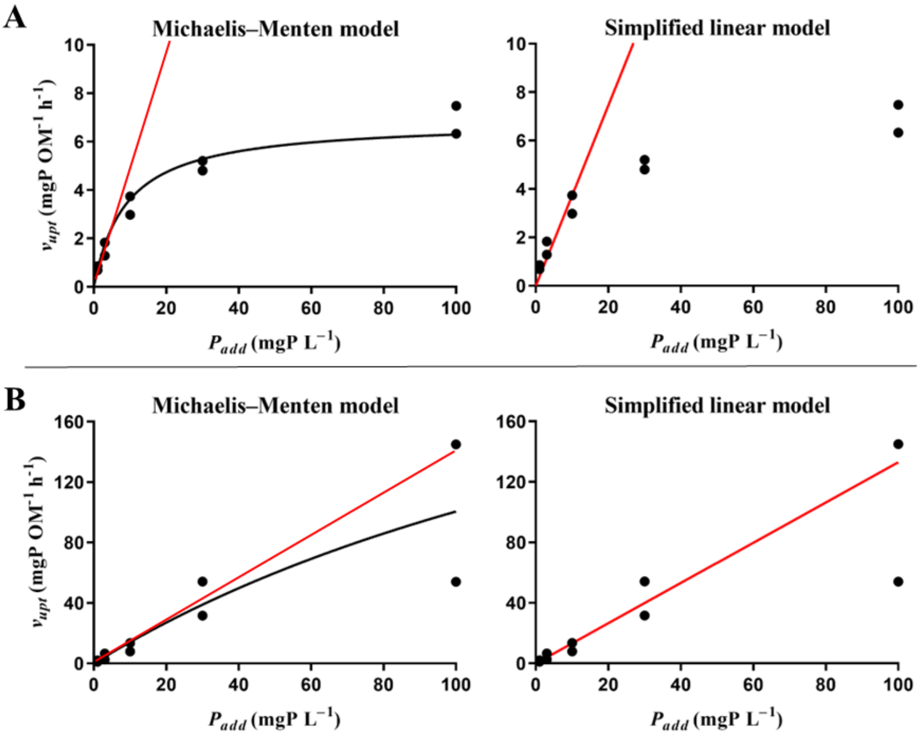

Figure 2: Example of fit by Michaelis–Menten model and linear model. A) Experiment where the Michaelis–Menten was preferred. B) Experiment where the linear model was preferred. Red line indicates the periphyton specific P uptake affinity calculated as V_{max}/K_s for Michaelis–Menten model and v_{slope} for the linear model. v_{upt}, phosphorus uptake velocity, P_{add}, concentration of orthophosphate. Note that the relative weighting by $1/(v_{upt})^2$ was applied, as the error of the v_{upt} was heteroscedastic, accounting for approximately 30 % (average difference between duplicates), irrespectively of P_{add}.

3.6.2 Final methodology

Stones with undisturbed periphyton were placed into plastic containers filled with 100 mL 0.2µm–filtered (cell-free) lake water. The containers were supplemented with five different concentrations of orthophosphate (P_{add}, 1, 3, 10, 30, 100 mgP L^{-1}, added as KH_2PO_4, Sigma-Aldrich Co.) and 20 µl carrier-free ^{33}P–labelled H_3PO_4 in the tracer amount (50–100 kBq; American Radiolabelled Chemicals, Inc., St. Louis, USA). Containers were incubated for 180 min under laboratory illumination (~10 µE m^{-2} s^{-1} PAR) in a gently shaken (120 rev/min) water bath tempered at *in situ* temperature of investigated lakes. At 15–30 min intervals, 0.6 mL aliquots of lake water were transferred into Eppendorf tubes containing 0.6 mL of scintillation cocktail (Ultima GoldTM XR LSC Cocktail, Sigma-Aldrich Co.) and vortexed vigorously. Radioactivity (typically 20–40 thousand counts per minute, c.p.m. per aliquot) was determined with a liquid scintillation counter (Tri-Carb 2900TR, Packard, USA). Phosphorus incorporation into the periphyton biomass manifested as a ^{33}P disappearance from the filtered lake water. The P uptake velocity v_{upt} (mgP gOM^{-1} h^{-1}) of each sample was determined, followed by calculation of specific P uptake affinity *SPUA* (L gOM^{-1} h^{-1}) and other related parameters. For details about P uptake kinetic experiments, see **Study II (Annex II)**.

3.7 Chemical and limnological parameters of studied lakes

Analyses of several limnological parameters were conducted within the presented studies. The measured parameters in the lake water and the methods used are summarised in **Table 4**. Water temperature, pH, O_2, conductivity and euphotic depth were determined *in situ*. The rest of summarised parameters were measured by routine analyses at the chemical department of the Institute of Hydrobiology, as they belong to the basic set of parameters accompanying long-term environmental studies.

Table 4: Methods used for the chemical and limnological analyses

Parameter	Method	Citation
Tropic status index	$\mathrm{TSI\ (Chla)} = 10 \times \left(6 - \frac{2.04 - 0.68 \times \ln \mathrm{Chla}}{\ln 2}\right)$	61
Temperature	Thermometry, YSI EXO 2 multiparametric probe (Xylem Analytics Germany)	
pH	Potentiometry, YSI EXO 2 multiparametric probe (Xylem Analytics Germany)	-
Conductivity	Conductometry, Combo pH/EC HI 98129	-
O_2	Luminescence - optical dissolved oxygen sensor, YSI EXO 2 multiparametric probe (Xylem Analytics Germany)	62
Euphotic depth (Z_{eu})	Photometry, calculated as 1% of surface irradiance, LICOR LI-1400 datalogger (Licor. Lincoln. NE, USA)	63
Acid neutralising capacity (ANC 4.5)	Acid-base titration	64
Dissolved organic carbon	Detection by non-dispersive infrared detector after oxidising to CO_2, Shimadzu TOC/TN analyser (Shimadzu Corp., Japan)	65
Dissolved nitrogen	Chemiluminescence detection after catalytic combustion, Shimadzu TOC/TN analyser (Shimadzu Corp., Japan)	66
Total phosphorus	Flow Injection Analyser with spectrophotometric detection after nitric-perchloric acid digestion	67

Parameter	Method	Citation
Chlorophyll a	Spectrophotometry after acetone extraction	[68]
Dissolved reactive phosphorus	Spectrophotometry - Phosphomolybdate blue method	[69]
Dissolved reactive silica	Spectrophotometry	[70]
N-NO_3	Colorimetry after hydrazine reduction to nitrite	[71]
Cl^{-}	Flow analysis with spectrophotometric detection	[72]
Ca^{2+}	Inductively coupled plasma atomic emission spectrometry	[73]
Mg^{2+}	Inductively coupled plasma atomic emission spectrometry	[73]
Fe	Inductively coupled plasma atomic emission spectrometry	[73]
SO_4^{2-}	Turbidimetric analysis	[74]

3.8 Statistics

For **Study I**, only non-parametric methods were used due to the low number of gathered samples and non-normal distribution of regression residuals of numerous tested linear models. The correlation matrix (Pearson coefficient, $p < 0.001$) for limnological and periphyton parameters and PERMANOVA (Permutational Multivariate Analysis of Variance, $p < 0.05$) analysis for testing the statistical differences between lakes and seasons were performed in R[75] using package vegan[76]. Non-metric multi-dimensional scaling (nMDS) plots based on Gower distances for limnological and periphyton parameters were prepared in Canoco v. 5.1. For **Study II,** all statistic was performed in PRISM v.7 (GraphPad Software), which is commonly used for enzymatic kinetics. Differences between seasons and lakes were evaluated with two-way ANOVA (Analysis of Variance, $p < 0.05$). For **Study III,** all statistic was performed in R[75] using packages vegan[76], EcolUtils[77] and Goeveg[78]. The performed analysis includes PERMANOVA based on Bray-Curtis dissimilarities ($p < 0.05$), ANOVA ($p < 0.05$), correlation matrix (Spearman correlation, $p < 0.05$) and nMDS. Shannon diversity indices[79], diatom trophic[80] and saprobic indices[81] were calculated in Omnidia 6.0.8 software[82]. For details, see the respective study.

4 Results and discussion

4.1 Study I

Lake identity (Medard, Most, Milada) proved to be a significant explanatory variable for all measured periphyton features – biomass per area, nutrient content and taxonomic composition as well as for limnological variables (based on PERMANOVA analysis, for graphical illustration, see **Fig. 3)**. We assume both the trophic status of the lakes and its age play a substantial role in this separation. Periphyton biomass average across all depths ranged between 0.7–7.4 mgOM cm^{-2}, similar to the biomass values recorded for oligotrophic Lake Tahoe[83]. Periphyton growth from spring to autumn and subsequent degradation from autumn to winter was observed for all lakes, probably connected to the changing weather conditions in the temperate climate. Even though the seasonal pattern of lakes was similar, the maximal periphyton biomass recorded in autumn in Lake Medard (2.2 mgOM cm^{-2}) reached less than half of the autumn biomass recorded in Lake Milada (5.2 mgOM

cm^{-2}). In Lake Most, the overall biomass recorded in autumn was 3.9 mgOM cm^{-2}. A relatively high proportion of viable periphyton autotrophs persisted over the winter period in Lakes Medard and Milada (66 and 44 % of maximal values recorded in autumn with autotrophic index 131 and 77, respectively). The changes in the periphytic community longer than one growing season have been studied sporadically. Therefore, the impact of the overwintering periphyton biomass on further periphyton development is unknown. From the extremely high periphyton C:P molar ratios (values ranged from 315 to 1642) persisting P deficiency in all lakes and seasons was deduced[35,36]. Taxonomic analysis revealed that diatoms, Chlorophyta, and Cyanobacteria dominate periphyton. In the youngest Lake Medard, diatoms prevailed, whereas co-dominance of diatoms and Chlorophyta occurred in the older lakes. The contribution of Cyanobacteria did not exceed 25 % and was the highest in autumn. From the limnological parameters, strong effect of Si was identified which suggests silica limitation of benthic diatom growth in the studied lakes even though the open water silica concentrations were relatively high, 0.35–2.85 mg L^{-1},[84]. Other correlations were found with taxonomic composition and Mg^{2+}. Magnesium sulphate ($MgSO_4$) is a common contaminant in mine water[85]. Effect of $MgSO_4$ on the composition of phytoplankton (decrease in taxa richness) has been previously observed in mesocosm experiments and was linked to Mg^{2+} toxicity[86]. We assume that $MgSO_4$, next to the extreme scarcity of nutrients, might have possibly suppressed periphyton growth in Lake Medard where the Mg^{2+} concentration was the highest (~55 mg L^{-1}). For details, see the **Study I** (**Annex I**).

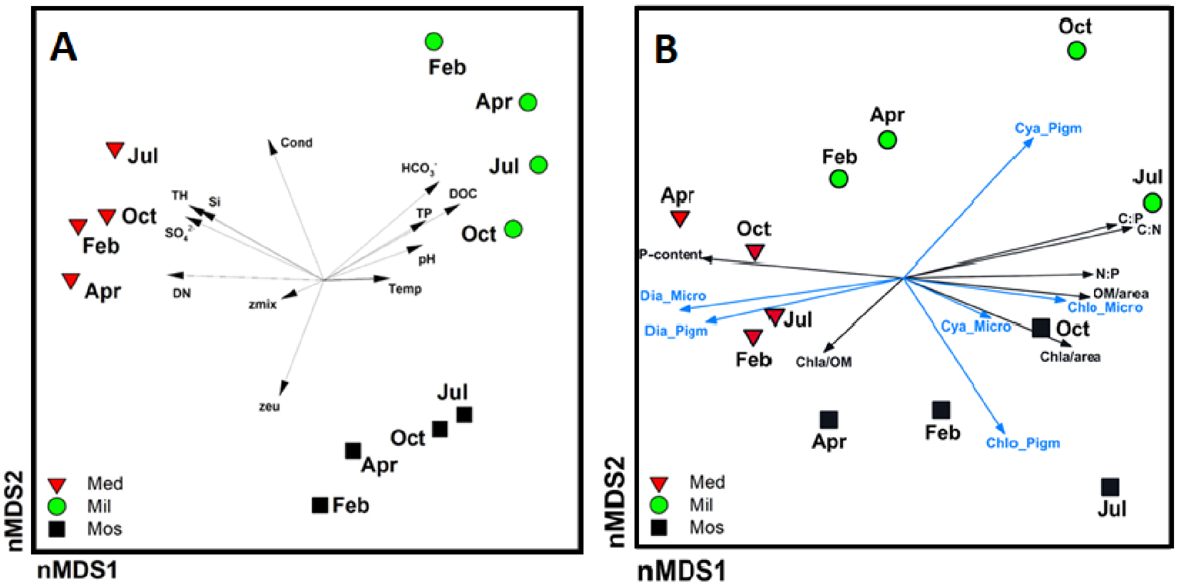

Figure 3: Separation of the lakes based on (A) limnological parameters and (B) periphyton composition. Med = Medard; Mil = Milada; Mos = Most; Temp = surface water temperature; Z_{eu}, = euphotic depth; Z_{mix}, = mixed layer; DN = dissolved nitrogen; TH = Total hardness, Cond = conductivity; DOC = dissolved organic carbon; TP = total phosphorus; Si = dissolved reactive silica, Dia = diatoms; Cya = Cyanobacteria; Chlo = Chlorophyta; Micro = contribution based on microscopy, Pigm = contribution based on pigment analysis; Chla = Chlorophyll a; OM = organic matter (Konopáčová, in revisions)

4.2 Study II

Our study represents the first report on P uptake kinetics by periphyton assemblages naturally growing on stones in temperate oligo- to mesotrophic lakes. Values of specific P uptake affinity (*SPUA*) measured in this study (seasonal range 0.08–3.10 L gOM^{-1} h^{-1}) were roughly one order of magnitude lower than those presented for periphyton in subtropical wetlands[38] and decreased from spring to autumn (p = <0.0001, two-way ANOVA). In **Fig. 4,** winter measurement from the year 2021 was added (which is not included in the publication) to illustrate that the trend of *SPUA* decrease starts already very early in the season. An opposite seasonal trend for *SPUA* was expected in response to strengthened P limitation[87,88] implied by an observed decrease in periphyton P content and increase in C:P molar ratio (**Table 5**). In comparison to periphyton, the P uptake affinity of phytoplankton was increasing. Considering the results, we suggest a possible mechanism underlying a stable coexistence of planktonic and periphytic microorganisms, with plankton prospering mostly in summer and autumn and periphyton in winter and spring. Another possible explanation for the *SPUA* decrease might be linked to the problematic of the diffusion barrier[89] or the higher importance of internal recycling in the periphyton compared to the phytoplankton and, therefore, no need for periphyton to invest into the enzymes for P acquisition from the water column in the later season.

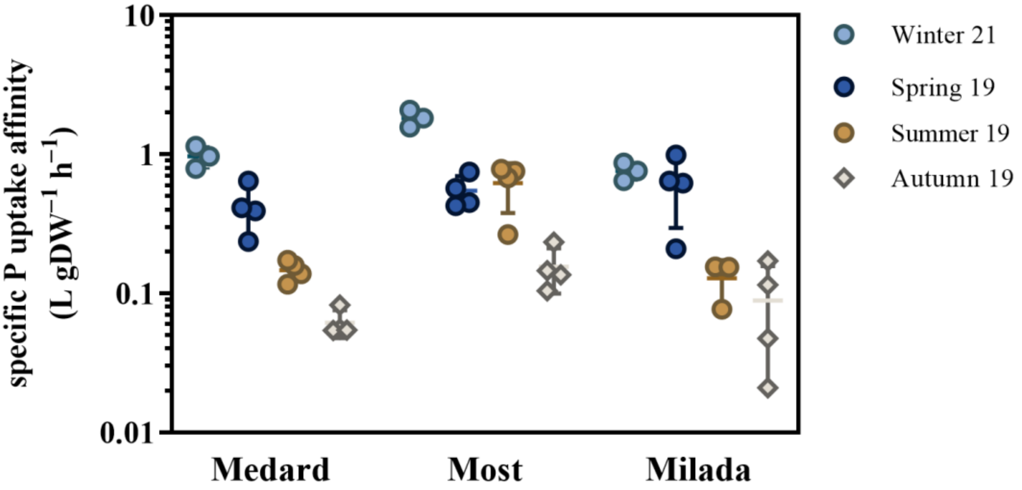

Figure 4: Specific phosphorus uptake affinity of periphyton, 19 – year 2019, 21 – year 2021 (modified from Konopáčová et al., 2021)

Table 5: Seasonal average of C:P molar ratios and P content in periphyton biomass across the studied lakes. All recorded ratios were in the range of P limitation according to Hillebrand et al., 1999. Higher C:P molar ratios indicate higher P deficiency. 19 – year 2019, 21 – year 2021 (modified from Konopáčová et al., 2021)

	Sampling-season averages			
	Winter 21	**Spring 19**	**Summer 19**	**Autumn 19**
C:P molar ratio	433	411	831	802
P content (mgP gOM^{-1})	2.4	3.6	2.2	2.1

4.3 Study III

Study III revealed details on microbial diversity and growth forms of periphytic autotrophs (algae and cyanobacteria) of the studied lakes. Species composition varied significantly among the studied lakes, seasons, and sampling years, whereas sampling sites and depths haven't shown significant impact. This suggests homogeneous composition in the littoral zone of single lakes. High autotrophic diversity was uncovered, as well as unique species which do not occur in other water bodies in the respective watershed of river Ohře. In total, 437 taxa were identified, from which 43 % represented soft algae (Cyanobacteria, Chlorophyta, Rhodophyta, Conjugatophyta, Cryptophyta, Dinophyta, Euglenophyta and Xanthophyta) and 57 % diatoms. Example of species identified during **Study III** are shown in **Fig. 5**. Seasonal changes revealed prevalence of Cyanobacteria and motile forms of diatoms in the late summer in all lakes. This coud lead to the consideration that these life forms are more sucesful in the utilization of nutrients released from the accumulated periphyton biomass. The most important factor infuencing the species composition from tested abiotic variables was Ca^{2+} ($p = 0.015$, PERMANOVA) which offer the intriguing question of the role of this cation in the formation of periphytic mats for future research. The obtained data display that the recultivated water bodies are a "hotspot of microalgal diversity" in Northern Bohemia.

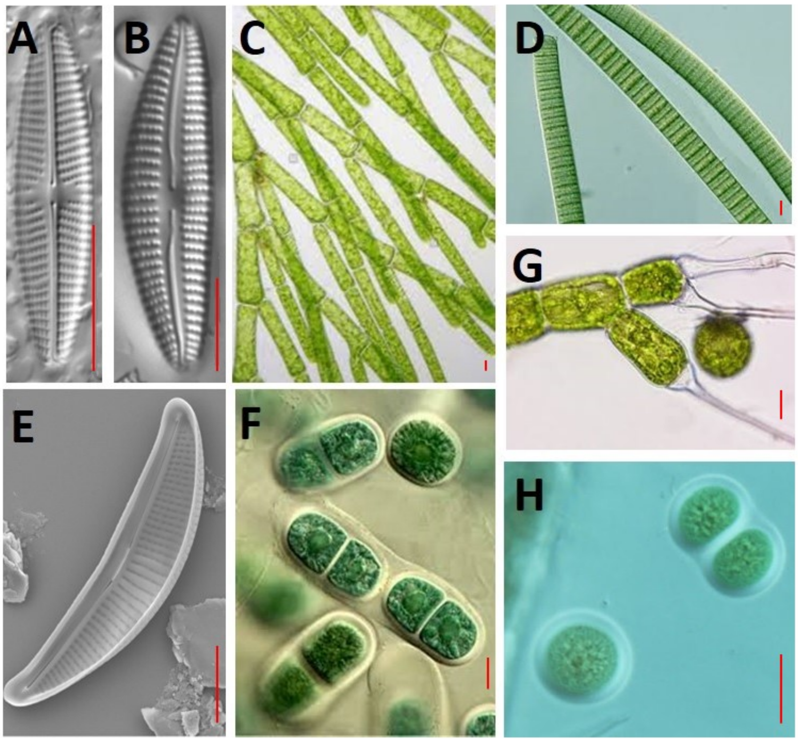

Figure 5: Selected periphyton species: (A) diatom *Navicula libonensis*, (B) diatom *Cymbella subleptoceros*, (C) green algae *Cladophora glomerata*, (D) cyanobacteria *Oscillatoria limosa*, (E) diatom *Halamphora veneta*, (F) red algae *Chroodactylon ornatum*, (G) green algae *Bulbochaeta* sp., (H) cyanobacteria *Chroococcus obliterates*, red line = 10 µm (photos by Tomáš Bešta)

4.4 Synopsis of presented studies

The presented dissertation thesis consists of three studies, whose synopsis is graphically described in **Fig. 6**. **Studies I** and **II** forms together complex information describing periphyton basic features and the relation to the key nutrient in studied lakes, phosphorus. **Study III** brings knowledge on the diversity of so far neglected inhabitants of the littoral zone. Results of this study show that the disturbed littoral zone of post-mining lakes harbours a huge diversity of autotrophic species. The high diversity of diatoms observed in the most oligotrophic Lake Medard indicates that not only quantitative (Medard had the lowest overall biomass) but also qualitative parameters of periphyton must be described. The information gained in **Study III** underlines the importance of conservating post-mining lakes´ ecosystem and, therefore, raises the importance of **Studies I** and **II**.

One major theory emerged by connecting data from all three studies. In contradiction to our expectations, we did not find a correlation between the lake water SRP with C:P molar ratio (**Study I**) or *SPUA* (**Study II**). We assume that additional process, likely phosphorus internal recycling in periphyton mats, plays an essential role next to the phosphorus acquisition from the lake water. In Lakes Most and Milada, we observed seasonal pattern resembling one another. Nutrient uptake from the lake water in winter and spring triggered periphyton growth (*SPUA* was the highest in winter, **Study II**). In summer and even more in autumn, lake water recourses were scarcer, therefore, internal recycling of already obtained phosphorus played a higher role compared to the phosphorus acquisition. Also, as periphyton become thick (up to several mm, our observation), not all cells were probably able to acquire P from the lake water (due to the diffusion barrier, see **Study II**). An increase in mobile forms of diatoms in season (**Study III**) suggests that mobile species might be favoured since they are capable to actively search for nutrients (both internal – within the periphyton mats and external – from the lake water)[90]. We suppose that from summer to autumn, both internal and external phosphorus recourses are exhausted, which manifests in a proportional increase of detritus in the periphyton biomass (increase in autotrophic index, AI, **Study I**).

Seasonal development of periphyton in Lake Medard showed similar trends, however, the overall biomass was substantially lower compared to the Lakes Most and Milada and the seasonal changes were milder. We assume periphyton growth might have been suppressed by Mg^{2+}, which concentration was roughly double compared to the concentrations in the other two lakes and was significantly correlated with taxonomic composition (**Study I**). In **Study III,** single species occurrence was found to be correlated with Ca^{2+}, which might act as factor lowering Mg^{2+} toxicity[85,86]. The influence of different species composition in the lakes was also tested (diatoms in Lake Medard compared to Chlorophyta and diatoms in Lakes Most and Milada), however, no relationship of periphyton features (biomass per area, *SPUA* or C:N:P molar ratio) with taxonomic composition was uncovered.

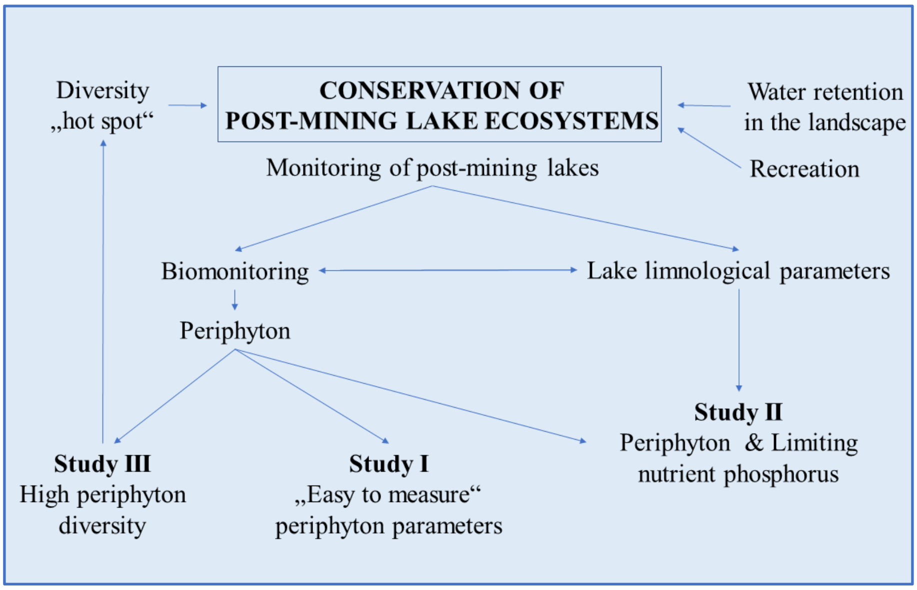

Figure 6: General graphical synopsis of studies presented in the dissertation thesis

5 Conclusion and prospects

Overall, high recorded periphytic biomass covering the littoral belt of investigated lakes (from the shoreline up to 2 m depths) supports our hypothesis that periphyton has a substantial role in colonising a newly developed littoral zone of post-mining lakes. In **Study I**, periphyton parameters with a potential to be used for biomonitoring of post-mining lakes, such as biomass per area or periphyton composition, were described. The relatively high contribution of overwintering periphyton autotrophs in Lakes Milada and Medard turned the attention of our team to the often neglected winter season. **Study II** brings the notion of possible coexistence of phytoplankton prospering in summer and autumn and periphyton prospering in winter and spring. Additionally, we assume that the internal recycling of phosphorus in the periphyton biomass plays an important role. To point out the importance of post-mining lakes as “hotspots of regional biodiversity” periphyton species diversity was described in **Study III**. Altogether, the dissertation thesis forms a consistent package that meets all pre-set goals. The gained information can be applied for predicting successional events in both future and already established post-mining lakes.

The following plans based on the results of the proposed dissertation thesis include increased attention on the winter season. We plan to describe changes in biomass over all depths and periphyton winter activity in processes such as P uptake and primary production. Work already performed, however yet not published, includes measuring primary production as well as employing the introduced methodology on P uptake kinetics for measuring P uptake by occupants of the deeper part of the littoral zone of post-mining lakes (macroalgae *Chara* sp. and *Vaucheria*, sp and submerged macrophyte with the attached epiphyton). Also, the production of the extracellular phosphatases by periphyton in response to the nutrient limitation was measured in the year 2022.

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7 List of student´s published works

7.1 Original publications within the dissertation thesis

Study I

Konopáčová E., Schagerl M., Bešta T., Čapková K., Pouzar M., Štenclová L., Řeháková K. (in revisions) An assessment of periphyton mats using CHEMTAX and traditional methods to evaluate the seasonal dynamic in post-mining lakes. Hydrobiologia,

Q1 - Aquatic Science, IF: 2.82 (2021)

Contribution of E.K. (estimated 70%): contributed to the planning of the study, field sampling, introducing new method for measuring of algal pigments, experimental work, statistical analysis of data, writing of the manuscript and presenting the data on the international conference. Significant part of the study was formed during the internship at Department of Functional and Evolutionary Ecology in University of Vienna.

Study II

Konopáčová E., Nedoma J., Čapková K., Čapek P., Znachor P., Pouzar M., Říha M. and Řeháková K. (2021) Low specific phosphorus uptake affinity of epilithon in three oligo- to mesotrophic post-mining lakes. Front. Microbiol. 12:735498. https://doi.org/10.3389/fmicb.2021.735498

Q1 - Microbiology, IF: 6.06 (2022)

Contribution of E.K. (estimated 70%): contributed to planning the study, field sampling, introducing of the methodology, experimental work, statistical analysis of data, writing of the manuscript and presenting the data on the international conference.

Study III

Bešta T., Mareš J., Čapková K., Janeček K., Štenclová L., Kust A., Říha M., **Konopáčová E.**, Řeháková K. (2023) Littoral periphyton dynamics in newly established post-mining lakes. Aquatic Sciences 85, 21. https://doi.org/10.1007/s00027-022-00914-y

Q1 - Aquatic Science, IF: 2.75 (2022)

Contribution of E.K. (estimated 20%): contributed to the field sampling and writing of the manuscript.

7.2 Another student´s original publications and attended conferences

Journals with impact factor:

Schagerl M., Siedler R., **Konopáčová E.**, Ali S.S. (2022) Estimating biomass and vitality of microalgae for monitoring cultures: a roadmap for reliable measurements. Cells. Aug 8;11(15):2455. https://doi.org/10.3390/cells11152455 Q2 - Cell Biology, Q1 - General Biochemistry, Genetics and Molecular Biology, IF: 7.67 (2022)

Other scientific reviewed journals:

Hrdá K., **Konopáčová E**. and Knotek P. (2021) Effects of polystyrene microparticles, gadolinium salts and their mixtures to soil annelid studied in agar exposure medium. Scientific Papers of the University of Pardubice, Series A; Faculty of Chemical Technology (27):119–136.

Hrdá K., **Konopáčová E.**, Vrzáčková I. and Pouzar M. (2018) Influence of humic acids on zinc oxide nanoparticles and zinc chloride toxicity to Enchytraeus crypticus studied in agar based exposure media. Scientific Papers of the University of Pardubice, Series A, Faculty of Chemical Technology, (24):187-196.

International scientific conferences:

Konopáčová E., Řeháková K., Nedoma J., Pouzar M., Coal Mine Reclamation – The Role of Periphyton Community in Phosphorus Cycling in Oligotrophic Post-Mining Lakes. 17th Annual International Symposium on Environment, 11.-14.7.2022, Athens, Greece

Konopáčová E., Řeháková K., Nedoma J., Pouzar M., Coal mine reclamation – The effect of periphyton community in phosphorus cycling in oligotrophic post-mining lakes, 3rd International Caparica Conference on Pollutant Toxic Ions and Molecules, Lisbon, 4.–7.11.2019, ISBN 978-989-54470-3-9, awarded by Excellent poster presentation prize

National scientific conferences:

Konopáčová E., Řeháková K., Nedoma J., Pouzar M., P uptake traits of epiphyton in oligothropic post-mining lakes Milada, Medard and Most, 62nd Conference of Czech Phycological society, Třeboň, 13.-15.9.2021

Konopáčová E., Periphyton in phosphorus cycling in oligotrophic post-mining lakes, 30. ročník students´ miniconference Chantransia, Horaždovice, 9. - 10. 10. 2019

Konopáčová E., Řeháková K., Nedoma J., Pouzar M., The role of periphyton community in phosphorus cycling in oligotrophic post-mining lakes, 61st Conference of Czech Phycological society, Chlum u Třeboně, 13.-16.9.2020