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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, se v průběhu sezóny specifická afinita pro fosfor snižovala 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, autotrophs, benthic

Klíčová slova

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

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List of abbreviations

Allo	Alloxanthin
ANOVA	Analysis of variance
ANC 4.5	Acid neutralising capacity
AM	Ash mass
Cantha	Canthaxanthin
Chl-a	Chlorophyll a
Chl-b	Chlorophyll b
Chl-c	Chlorophyll c
Chlo	Chlorophyta
Cond	Conductivity
Cya	Cyanobacteria
Dia	Diatoms
Diadi	Diadinoxanthin
DM	Dry mass
DN	Dissolved nitrogen
DOC	Dissolved organic carbon
Fuco	Fucoxanthin
HPLC	High-performance liquid chromatography
K_m	Half-saturation constant
Lut	Lutein
Med	Lake Medard
Micro	Contribution based on microscopy
Mil	Lake Milada
Mos	Lake Most
Myxo	Myxoxanthophyll
Neo	Neoxanthin
OM	Organic matter

P_{add}	Added concentration of phosphorus
Peri	Peridinin
PERMANOVA	Permutational multivariate analysis of variance
Pigm	Contribution based on pigment analysis
SPUA	Specific phosphorus uptake affinity
SRP	Soluble reactive phosphorus
RMSE	Root Mean Square Error
Temp	Temperature
TH	Total hardness
TN	Total nitrogen
TOC	Total organic carbon
TP	Total phosphorus
V_{max}	Maximum phosphorus uptake velocity
v_{upt}	Phosphorus uptake velocity
Z_{eu}	Euphotic depth
Z_{mix}	Mixed layer

1 Objectives

Gaining knowledge about the newly emerged post-mining lakes is essential since their number will increase in the 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´s 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 the 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

In this thesis, I have studied an ecosystem of post-mining lakes situated in Northern Bohemia. Compared to the other post-mining lakes (for example, in Germany or Poland), which suffer from extreme acidification, lakes in Northern Bohemia are not acidic and offer a habitat for the broad scale of aquatic organisms. Since it is not feasible to measure the limnological parameters with sufficient frequency (e.g., daily, weekly), limnological analyses cannot capture short-term changes in the critical factors. However, long-living aquatic organisms or assemblages can integrate the information from longer periods. In this dissertation thesis, I summarise my attempts to study periphyton, a prominent community in the littoral zone of post-mining lakes. For the potential use of periphyton for biomonitoring, first, the starting conditions must be delineated. I describe seasonal changes in periphyton biomass per area, elemental and pigment composition and the relation of periphyton to the limiting nutrient, phosphorus. Periphyton diversity is also pointed out to underline the uniqueness of the post-mining lakes ecosystem for the respective region.

2.1 History and current stage of coal mining in Europe

While it has been mined in a small amount since ancient times, coal was only an occasional heat source until the 18th century. The extensive use of coal took its turn in the mid of 19th century, and coal has been for many years one of the primary energy supplies for many industrialised European countries (Esposito and Abramson, 2021). Coal mining has become a prominent anthropogenic activity directly modifying the face of terrestrial ecosystems (Hooke et al., 2012; Larondelle and Haase, 2012). For example, in Northern Bohemia the opencast coal mines have been influencing and transforming the landscape for more than 150 years (Hendrychová and Kabrna, 2016; Vrablik et al., 2017). The extensive rise of coal production in the Czech Republic is illustrated in **Fig. 1**, reaching its peak in 1984 (Czech Statistical Office, 2012). Across all years, brown coal mining situated in Northern Bohemia formed around 78 % of the total brown coal production in the Czech Republic (Czech Statistical Office, 2012). The inhabitants of mining regions not only exploit the land, but a long tradition of post-mining reclamation in European countries exists. The obligation to return mined landscapes to their original use by reclamation was enshrined in mining law #146 from 1854 by the Austrian Hungarian Emperor Franz Joseph I (Macdonald et al., 2015). Reclamation plans that aim to return the land and watercourses to an acceptable environmental state and productive use are an integral part of any mining project in the European Union (European Commission et al., 2018). Hydric recultivation represents, besides foresting and agriculture recultivation, one of the popular forms of landscape recovery (Nixdorf et al., 2005; Søndergaard et al., 2018). The number of post-mining areas in Europe is expected to rise throughout the upcoming decades due to the coal mining suppression associated with the European Union's Green Deal (European Commission, 2018; Larondelle and Haase, 2012).

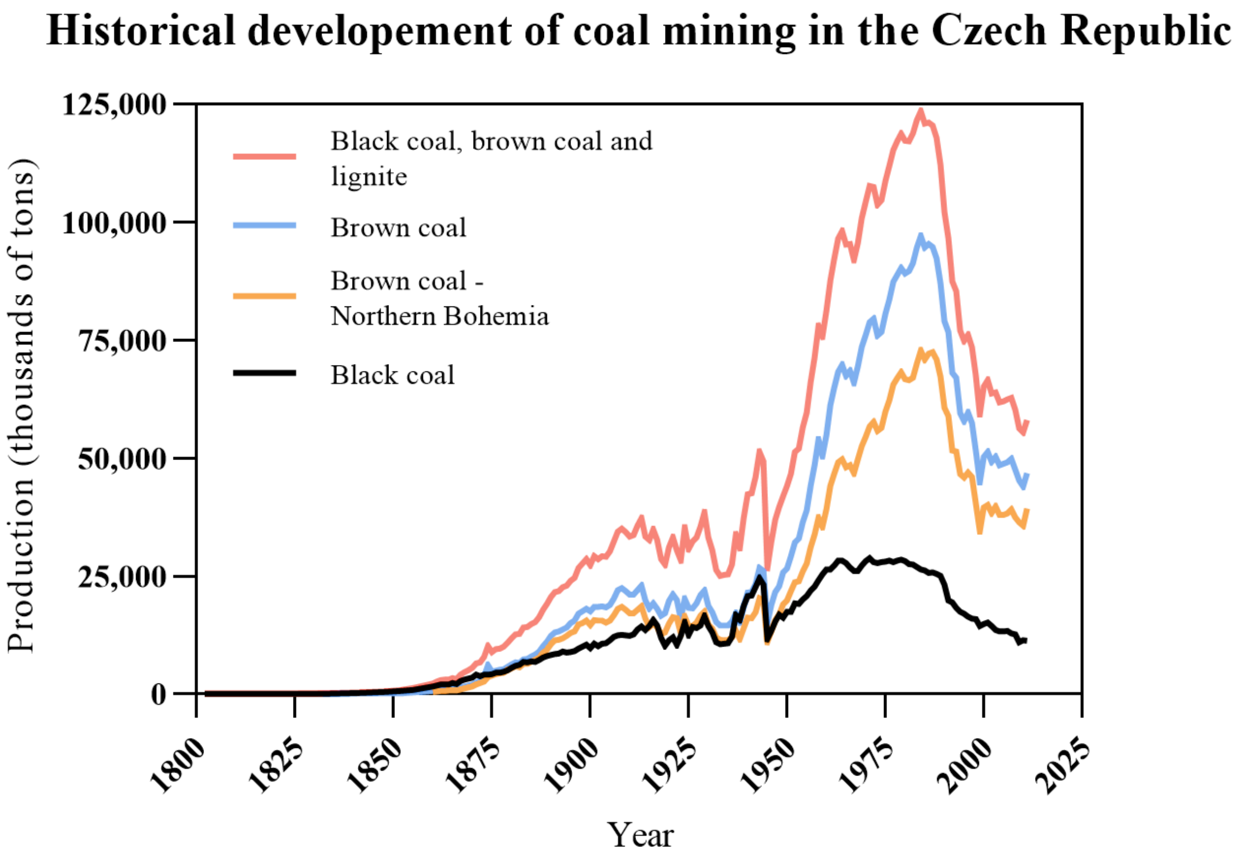

Figure 1: Coal mining in the Czech Republic from 1800 to 2021 (Czech Statistical Office, 2012).

2.2 Ecosystems of post-mining lakes

Thousands of lakes created from the flooding of abandoned mines occur across every inhabited continent (Blanchette and Lund, 2016). These artificial surface water bodies resemble crater lakes of volcanic or meteor-impact origin rather than co-occurring lakes, which tend to be shallower and more nutrient-rich (Blanchette and Lund, 2016). Compared to the valley-dammed reservoirs, where the water level rapidly fluctuates, fully flooded post-mining lakes possess a more stable water level, which allows for the development of littoral zone vegetation. The post-mining lakes ecosystem conditions result from several factors such as water quality, catchment interaction, size, location or morphology. Many post-mining lakes suffer from high acidity as a consequence of pyrite decay followed by the leaching of toxic compounds from the substratum. Such conditions hamper the development of living organisms (Oszkinis-Golon et al., 2021). However, lakes with neutral or even alkaline water exist as well (Sienkiewicz and Gąsiorowski, 2019). Regarding nutrient concentrations, newly raised post-mining lakes are often oligotrophic (i.e., nutrient-poor) due to their young age and high volume-to-surface area ratio (Gammons et al., 2009). However, it has been observed that post-mining lakes established decades ago tend to eutrophicate (Sienkiewicz and Gąsiorowski, 2016).

Many further post-mining lakes will be created by hydric recultivation due to the suppression trend concerning non-renewable resources (European Commission, 2018). For example, the private mining company Sokolovská uhelná a.s. active in the Sokolov region of Northern Bohemia has already recultivated several areas and given rise to water bodies of variable sizes. Flooding of pits Jiří and Družba is planned in the region for the year 2036, which should give rise to an extraordinarily big lake with a maximal depth of 93 m and a water volume of 515 mils. m^3. Several similar extensive projects exist worldwide (Kabir et al., 2015; Sloss, 2013). Therefore, monitoring of already established post-mining ecosystems is essential to perform. It has been found that the post-mining lakes of different geographical locations are similar in many biotic and abiotic aspects depending on the mining type and substrate (Gammons et al., 2009). Thus, a description of a particular lake succession stage can reasonably predict the general successional development in a particular type of post-mining lake. Furthermore, the opportunity to study the primary succession of ecosystems of such size is at least uncommon, and data might be used for the reconstruction of past changes in the older lakes.

The physical, chemical and limnological characteristics of post-mining lakes are often studied from the beginning of their establishment (Schroeter and Gläβer, 2011), but their biological assemblages are studied less. Post-mining lakes, which do not suffer from extreme acidity, form a completely new, underexplored habitat for a wide range of organisms. Post-mining lakes in Northern Bohemia harbour several species rare for the Czech Republic, both in the lake and the closest surrounding (Frouz et al., 2007). A high level of submerged macrophytes diversity was observed in the post-mining lakes in Poland (Oszkinis-Golon et al., 2021). In Germany, lakes were identified as important migratory points for various protected migratory birds (Deshaies, 2018). Newly emerged post-mining lakes are valuable elements of the landscape not only for the ability to increase regional biodiversity. In general, lakes act as well as agents for water retention in the region with a noticeable impact on the local precipitation and temperature (Scott and Huff, 1996). Considering the socioeconomic importance, the potential recreation use for the region´s inhabitants is undeniable. Former coal mining regions across Europe are found to be substantially poorer than comparable regions in the same country (Esposito and Abramson, 2021). Therefore, developing a potentially attractive place for tourism could benefit the respective region. For all the above mentioned, a monitoring process must take place to uncover ecosystem changes (i.e., eutrophication) and consequently help conserve the so-called “good quality of water”.

2.3 Periphyton

Two major groups of microorganisms exist in aquatic ecosystems – freely floating phytoplankton and attached periphyton. The relative importance of each group depends mainly on environmental conditions (nutrients, light, temperature) in the exact aquatic ecosystem (Brothers et al., 2016; Wetzel, 2001). Even though in some systems, periphyton can dominate over the phytoplankton, less attention has been paid to its examination. 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 (Azim et al., 2005; Cantonati and Lowe, 2014). 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 (Azim et al., 2005; Battin et al., 2016; Brothers et al., 2016; Dodds, 2003a; Wyatt et al., 2019). The submerged periphyton community offers a hiding place and a food source for fish and zooplankton (Vander Zanden et al., 2006). Several subgroups of periphyton exist, called according to the substrate to which they are attached, i.e., epilithon = covering rocks, epiphyton = covering submerged macrophytes, epipelon = live on or in association with fine-grained substrata or sand (Azim et al., 2005).

Periphyton has been traditionally investigated in streams (DeNicola and Kelly, 2014; Stevenson et al., 1996) or wetlands (Gaiser, 2009; Oberholster et al., 2022). Besides those two ecosystems, periphyton can form prominent biomass also in oligotrophic and mesotrophic lakes (Liess et al., 2009; Vadeboncoeur and Steinman, 2002). Due to its high level of diversity and physiological versatility (Fierer et al., 2010; Gaiser et al., 2011), well-developed periphyton can be formed within a few weeks (Johnson et al., 1997) and can take part in primary production and nutrient cycles in water bodies, providing the nutrients for other trophic levels in the ecosystem (Hart and Robinson, 1990; Lamberti, 1996). 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 (Oszkinis-Golon et al., 2021). 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.

2.4 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 number due to the popular hydric recultivation and the general trend of coal mining suppression all over the world (Larondelle and Haase, 2012; Říhová Ambrozová and Ivanova, 2013; Søndergaard et al., 2018).

Several reasons for post-mining lakes monitoring exist:

- Biodiversity conservation
- Source of water in the landscape
- Recreational opportunities that enhance well-being
- Catchment of water in the landscape
- Prediction of the 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 the biomonitoring of wetlands in Florida (Gaiser, 2009) or for use as an early warning indicator of nutrient pollution in lakes (Ozersky and Camilleri, 2021). Its monitoring is also part of the Water Framework Directive (European Commission, 2000) and Clean Water Act (Federal Water Pollution Control Act, 1948). As a primary food source for zooplankton, small fish and other small consumers at the base of the food web (Vander Zanden et al., 2006), periphyton acts as a fundament of the ecosystem. Therefore, understanding 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.

2.4.1 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 freshwater ecosystems (Dodds, 2006; Francoeur et al., 1999; Wilcock et al., 2002). The increasing availability of limiting factors, often connected with eutrophication, is therefore followed by an increase in periphyton biomass (Pacheco et al., 2022). The relationship is, however, not linear and once the eutrophication causes a notable increase in phytoplankton biomass, periphyton biomass decreases due to the lower availability of light (Cantonati and Lowe, 2014). On the other hand, when the nutrient load decreases, a reverse process to eutrophication, called oligotrophication or structural shift, can be observed, accompanied by a periphyton biomass increase (Brothers et al., 2016). Taken from the other end, following changes in the areal periphyton biomass might help uncover shifts in the limiting factors and therefore serve as an inexpensive and "easy-to-measure" parameter indicating the ecosystem development. Sampling techniques vary depending on the type of periphyton (epilithon, epiphyton, epipelon). The epilithic periphyton is usually sampled by removing core sediment of smaller diameters, from which the attached biomass is removed by knife or toothbrush for analyses or experiments (Douglas, 1958). Periphyton areal biomass is then easy to estimate by determining the amount of dry mass (DM) or organic matter (OM) per sampled area (Lakatos et al., 1998; Romanów and Witek, 2011).

2.4.2 Autotrophic composition on the higher taxonomic level

Once the total biomass is estimated, a 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 the taxonomist (Culverhouse et al., 2003). Records exist that benthic diatoms diversity indices, which are traditionally used for monitoring rivers (Charles et al., 2021), tend to be higher at intermediate levels of pollution rather than at low levels of pollution (Blanco et al., 2012; Ector and Rimet, 2005; Rensburg et al., 2008). On the other hand, sorting the individuals into higher taxonomic units, such as Chlorophyta, Cyanobacteria, diatoms and so on, is a 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 aquatic ecosystem changes such as shifts in temperature, concentration of herbicides or other pollutants (Bondar-Kunze et al., 2021; Carles et al., 2021; Konschak et al., 2021). For example, in response to the pollutant wastewater, the relative abundance of diatoms has decreased, whereas Cyanobacteria increased (Carles et al., 2021). Periphyton autotrophic composition can be determined directly, by traditional optical microscopy or indirectly, by analysis of group-specific pigments. For pigment analysis, spectroscopy or high-performance liquid chromatography (HPLC) can be used, from which HPLC is favoured due to its ability to distinguish a higher spectrum of pigments. HPLC pigment analysis is based on the simultaneous detection of extracted pigments, usually at wavelength ~ 440 nm. Different elution programs exist, originally developed for phytoplankton (Mendes et al., 2007; Sanz et al., 2015; Van Heukelem and Thomas, 2001; Wright et al., 1991), varying in their mobile phases, columns or gradient program and the use for periphyton samples has to be tested.

2.4.3 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 (Vadeboncoeur et al., 2021). 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 (Ozersky and Camilleri, 2021). The difference in the limiting factor for the growth of these two groups was also found in several upland lakes in Great Britain (Maberly et al., 2002). Therefore, a better understanding of the nutrient limitation of periphyton is necessary to uncover details about the benthic algal bloom formation. The nutrient composition of periphyton biomass can be easily detected with commonly used methods for the determination of nitrogen, carbon and phosphorus (Bisutti et al., 2004; Kopáček and Hejzlar, 1993; Muñoz-Huerta et al., 2013). Applying optimal C:N:P molar ratios from the literature, P or N deficiency in the periphyton can be identified (Hillebrand and Sommer, 1999; Kahlert, 1998). On the ecosystem level, seasonal changes in the periphyton ratios can identify changing availability or scarcity of P or N in the water column.

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 the often neglected winter season.

2.4.4 Contribution of periphyton to the phosphorus cycling

As mentioned previously, the growth of the primary producers in the aquatic ecosystem can be limited by several physical or chemical factors or their combination (Dodds, 2006; Francoeur et al., 1999; Wilcock et al., 2002). The gradual change in the availability of limiting factors might be followed by changes in periphyton composition. Both rapid decrease and rapid growth of biomass (benthic algal blooms) can be observed. As the latter can lead to a consequent decrease of water quality, an understanding of the reaction kinetics to the changes in the limiting factors is needed.

A very low total phosphorus concentration (TP) in the aquatic ecosystem suggests P limitation of phytoplankton and periphyton. Moreover, plants and microorganisms can acquire P only in the form of soluble inorganic orthophosphate (Wetzel, 2001), which usually forms around 10–15 % of TP. The concentration of soluble inorganic orthophosphate in the nutrient-poor water is difficult to estimate. The most common method to estimate soluble inorganic orthophosphate is the determination of soluble reactive phosphorus (SRP). In low concentrations (~SRP <3 μg L^{-1}), this method, however, overestimates the results (Dodds, 2003b; Tanaka et al., 2006). Due to the uncertainty in soluble inorganic orthophosphate concentration, neither *in situ* P uptake velocity can be directly measured.

Instead, the kinetics parameters of P uptake are measured in the experimental setup, and the P uptake velocity in the ecosystem can be calculated back. A method commonly used for investigation of P uptake kinetics of phytoplankton or periphyton is based on kinetics experiments with the scintillation detection of PO_4^{3-} (Hwang et al., 1998; Scinto and Reddy, 2003; Wolfe and Lind, 2010). At the initial time, variable concentrations of unlabelled PO_4^{3-} are added into the filtered lake water surrounding the periphyton sample together with a tracer amount of ^{33}P–labelled PO_4^{3-}. Aliquots of the lake water are then taken at regular intervals. The signal of ^{33}P in aliquots becomes detectable by mixing with a scintillation cocktail, which consists of a mixture of organic aromatic compounds. Scintillation cocktail transforms adsorbed energy of β–emission (resulting from the decay of ^{33}P) into detectable photons in the range of wavelengths 375–430 nm. The emission of photons per minute is proportional to the number of decays of ^{33}P per minute, which is, in turn, proportional to ^{33}P–PO_4^{3-} concentration. The resulting scintillation signal is in the units "counts per minute", c.p.m (L'Annunziata et al., 2020). The P uptake velocity (calculated from changes of c.p.m signal in time) is dependent on the present PO_4^{3-} concentration – the higher the PO_4^{3-} concentration is, the higher is the P uptake velocity. The dependency is not linear, and it can be approximated by the saturation curve of Michaelis-Menten kinetics. From the saturation curve, maximal P uptake velocity, V_{max} (mgP gOM^{-1} h^{-1}), half-saturation (Michaelis) constant, K_m (mgP L^{-1}) and their ratio V_{max}/K_m called specific P uptake affinity, *SPUA* (L gOM^{-1} h^{-1}) can be calculated (Vahtera et al., 2010).

Different plants and communities, such as periphyton or phytoplankton, differ in their ability to acquire phosphorus from the surrounding (Christiansen et al., 2016; Scinto and Reddy, 2003). As it is independent of the PO_4^{3-} concentration, specific P uptake affinity can be used for statistical comparison of differences between, for example, lakes and seasons (Hwang et al., 1998; Scinto and Reddy, 2003; Wolfe and Lind, 2010). *SPUA* describes the ability of an investigated microorganism/community to acquire P into its biomass. Its interpretation is not straightforward due to the unusual units (L $mgOM^{-1}$ h^{-1}). 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 (Thingstad and Rassoulzadegan, 1999). 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**.

2.4.5 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 (Vadeboncoeur et al., 2011). Most of the post-mining lakes colonisation and succession studies have focused on phytoplankton, zooplankton, invertebrates, and fish (Bylak et al., 2019; Nixdorf et al., 1998; Steinberg et al., 1998). Although the periphyton often forms dominant biocenosis in the littoral zone of oligotrophic post-mining lakes, the investigation, according to my knowledge, is missing. The diversity of periphyton mats can be high, as shown by research in Florida wetlands (Gaiser et al., 2011). Another study recorded that the high species diversity of benthic diatoms reflects a high diversity of other forms of life in the aquatic ecosystem (John, 2003). Detailed analysis and identification of individual periphytic species is challenging, however, valuable information about the shifts of periphyton functioning in the ecosystem can be obtained. Functional traits of single species, such as life forms (unicellular, filamentous, colonial) or a form of adherence (mobile, erect, prostrated, stalked), can be observed (Dunck et al., 2022). The species composition of periphyton and its functional traits are influenced by many abiotic and biotic environmental parameters (Li et al., 2020) and can reflect the trophic (Cattaneo, 2011; Stancheva and Sheath, 2016) or the successional stage of the aquatic ecosystem. As already highlighted in several studies, eutrophication was found to lead to lower taxonomic diversity (Hillebrand and Sommer, 2000; Worm et al., 2002). Considering the composition, periphyton of eutrophic localities was found to be inhabited by non-diatom filaments accompanied by stalked and mobile diatoms (DeNicola and Kelly, 2014; DeNicola et al., 2006; Fisher et al., 2006). 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 an 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 the current ecosystem condition. In **Study I**, we attempt to find "easy-to-measure" periphyton parameters, which have a potential to be used as a tool for monitoring long-term environmental conditions. Key methods used in this study were optical microscopy, high-performance liquid chromatography 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 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

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 (**Table 2**), were chosen as study sites (**Fig. 2, Fig. 3**). Littoral zone of studied post-mining lakes is covered with geotextiles which isolate the water column from the sediment. An artificially delivered stone substrate is covering the geotextile up to 3 m depth and serves as an erosion barrier and, at the same time, as a suppressing factor for the development of macrophytes. Thanks to these circumstances, huge mats of periphyton community (also known as epilithon) covers have been developed (**Fig. 3**).

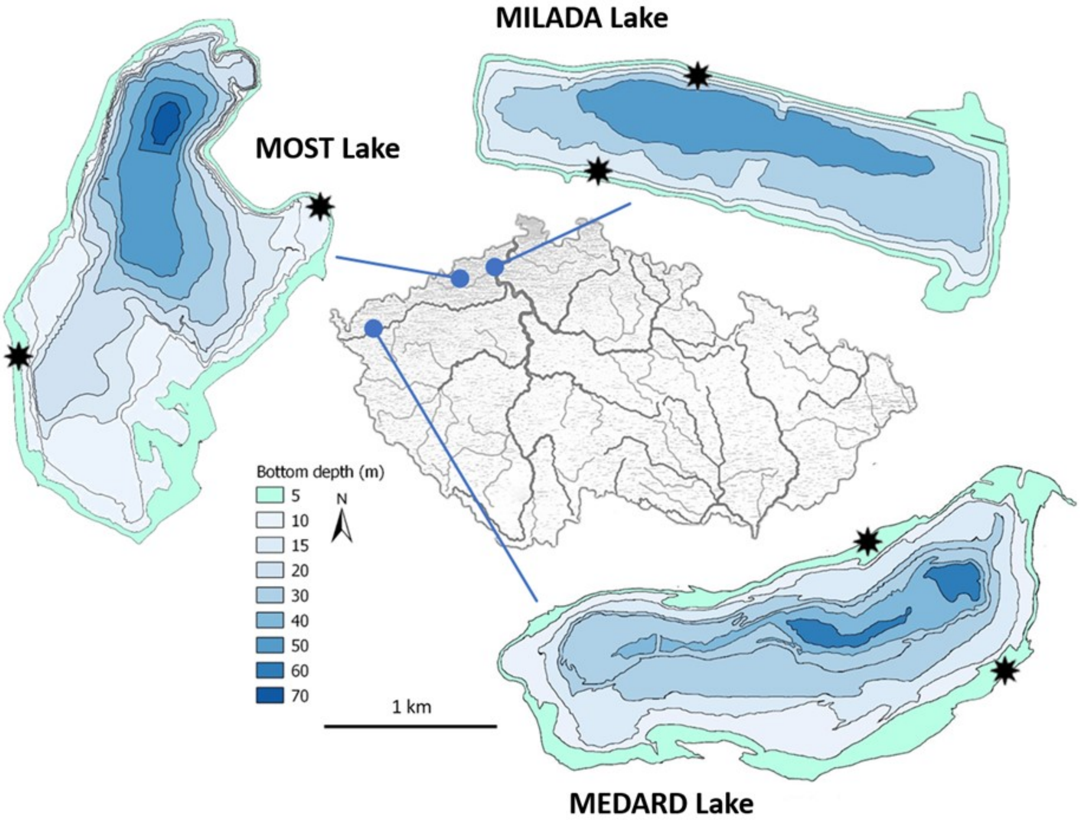

Figure 2: Geographical position of the post-mining Lakes Medard, Most and Milada

Lake Medard

Lake Most

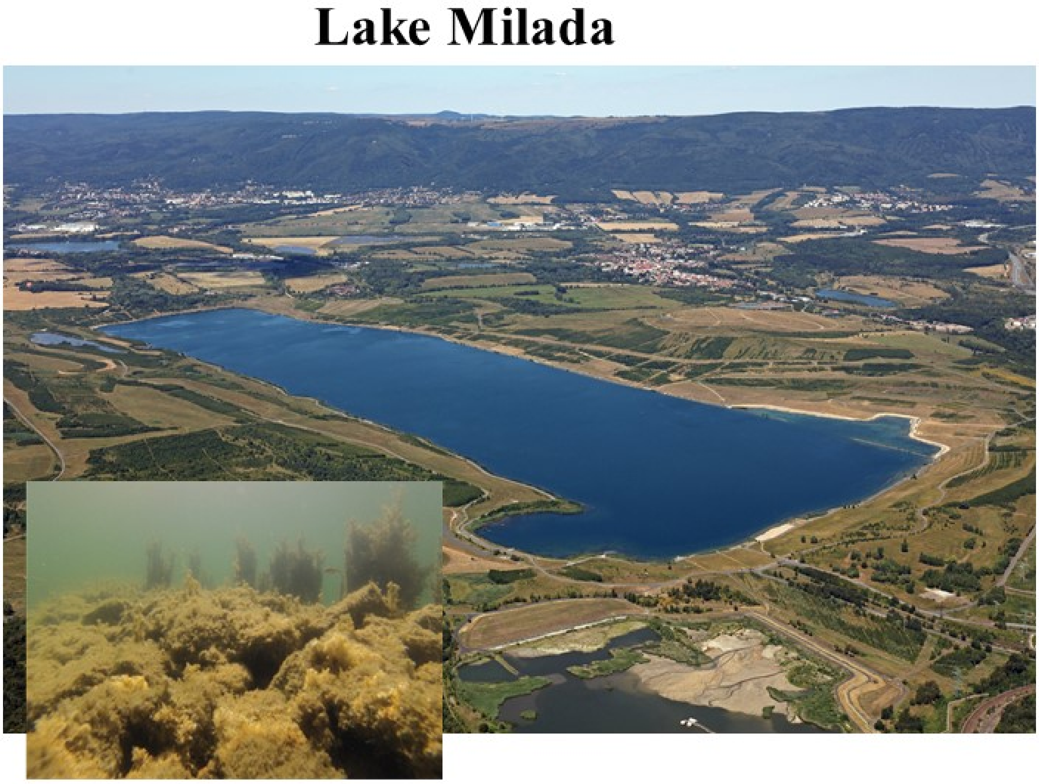

Figure 3: Aerial view of studied Lakes Medard, Most and Milada (photos by P. Znachor) combined with a representative subaqueous picture of periphyton (photos by K. Čapková)

3.2 Sampling

Stones covered with periphyton were randomly collected by scuba diving from depths between 0–2 m. For the microscopy, pigment analysis and biomass and elemental composition, periphyton samples were scraped off a defined area of stone with a scalpel and a toothbrush and gently homogenised (Douglas, 1958). For the P uptake experiment, whole stones with periphyton cover were collected to avoid biofilm disruption (the course of a biological process might have been affected by the biofilm disruption). Sampling was performed eleven times from February 2019 till October 2021. Specific sampling periods varied between the studies (**Table 1)**. Each sampling month 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 2**).

Table 1: Summary of sampling campaigns performed at the Lakes Medard, Milada and Most

Sampling Year	Sampling Month	Study
2019	February	Study II + III
2019	April	Study II + III
2019	July	Study II + III
2019	October	Study II + III
2020	April	Study I+II
2020	July	Study I+II
2020	October	Study I+II
2021	February	Study I+II

Sampling Year	Sampling Month	Study
2021	April	Study I
2021	July	Study I
2021	October	Study I

Table 2: 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

3.3 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, the stone surface covered by periphyton was estimated by the aluminium foil method (Dudley et al., 2001). To determine elements composition: total nitrogen (TN), total organic carbon (TOC) and total phosphorus (TP), samples were stored at –20 °C till the analysis and then homogenised using glass beads. TOC and TN were analysed with TOC/TN Shimadzu analyser (Shimadzu Corp., Japan), TOC after oxidising to CO_2 by non-dispersive infrared detection and TN after catalytic combustion by chemiluminescence detection. TP was determined spectrophotometrically after nitric-perchloric acid digestion (Kopáček and Hejzlar, 1993).

3.4 Microscopy

Optical microscopy (Olympus BX 50 light microscope, DIC optics, DP-72 digital camera) was used for the determination of the periphyton higher autotrophic groups (diatoms, Chlorophyta, Cyanobacteria etc.) in **Study I** and for the detailed taxonomical classification in **Study III**. For both studies, fresh periphytic samples were prepared, as well as permanent diatom slides (Fott, 1954; Houk, 2003). Species composition was assessed using available determination literature (Ettl and Gärtner, 2013; Hindák F., 1996; Kaštovský et al., 2018; Komárek, 2013; Komárek and Anagnostidis, 2005, 1998; Komárek and Fott, 1983; Krammer, 2002, 2003; Krammer and Lange-Bertalot, 1991b, 1988, 1986, 1991a; Lange-Bertalot, 2001). Nomenclature was updated according to AlgaeBase (Guiry and Guiry, 2021). The relative abundance of species was estimated using a modified Braun-Blanquet semi-quantitative scale of 7–degrees (**Table 3**), reflecting the individual species biomass to total biomass proportion (Hindák, 1978). The growth forms were distinguished as follows: eukaryotic coccal (e.g., *Chlamydocapsa* spp.), eukaryotic filamentous (e.g., *Spirogyra* spp.), cyanobacterial coccal (e.g., *Chroococcus* spp.), cyanobacterial filamentous (e.g., *Phormidium* spp.), motile (e.g., *Trachelomonas* spp.), planktic (e.g., *Scenedesmus* spp.) and undifferentiated diatoms. Diatoms were additionally resolved into prostrate (e.g., *Cocconeis* spp.), erect (e.g., *Fragilaria* spp.), stalked (e.g., *Gomphonema* spp.), mobile (e.g., *Navicula* spp.) and planktic (e.g., *Cyclotella* spp.).

Table 3: Braun-Blanquet semi-quantitative scale

Degree	Percentage of biomass (%)
1	<0.1
2	0.1–1
3	1–5
4	5–20
5	20–50
6	50–90
7	>90

3.5 High-performance liquid chromatography

3.5.1 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 (Wright et al., 1991). 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 (Van Heukelem and Thomas, 2001). A comparison of methods is shown in **Table 4**. Newly-introduced HPLC method showed better peak resolution and resolved two groups of previously coeluting pigments – fucoxanthin + neoxanthin and zeaxanthin + lutein. Therefore, this method was favoured above Wright et al. (1991) and introduced in common praxis in the laboratory in Vienna, where a substantial part of the experimental work within **Study I** took place. As a part of the new method introduction, I prepared a detailed instruction manual (mobile phase preparation, starting the machine, loading the elution program etc.) which is currently in use in the laboratory in Vienna.

Three sets of peaks were coeluting even with the newly introduced method: chlorophyll c2+c3; violaxanthin and 19-hexanoyloxy fucoxanthin; diadinoxanthin and dinoxanthin (**Table 4**). For subsequent chemotaxonomical analysis, we dealt with co-eluting pigments as follows: chlorophyll c2+c3 were treated as a sum of chlorophyll c, diadinoxanthin and dinoxanthin were also treated as a sum since they are linked to the same algal group. Pigments violaxanthin and 19-hexanoyloxy fucoxanthin were excluded from the further calculations. The coelution of the above-mentioned pigments didn't restrict us from applying pigment analysis on periphyton samples.

Table 4: 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

Pigments	Wright et al., 1991	Van Heukelem and Thomas, 2001
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

3.5.2 Final methodology

Homogenised 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 (Lorenzen, 1967) 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, the sum of diadinoxanthin and dinoxanthin, alloxanthin, diatoxanthin, zeaxanthin, lutein, canthaxanthin, chlorophyll b, chlorophyll a and the sum α+β carotene. Pigment standards (DHI Lab Products, Denmark) were used for the peak identification and calculation of the actual pigment concentrations.

3.6 CHEMTAX analysis

HPLC analysis was followed by data analysis in the matrix factorisation program Chemtax 1.95. Chemtax uses factor analysis and the steepest descent algorithm to identify the best fit to the data (Mackey et al., 1996). 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, the 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 (Higgins et al., 2011; Lauridsen et al., 2011) and are shown in **Table 5**. For optimisation of the input matrix, a series of 60 pigment ratio tables were generated, and the final output matrix was calculated based on a 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 (Mackey et al., 1997).

Table 5: 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

3.7 Phosphorus uptake kinetics

3.7.1 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. This concentration range was found to cover the whole saturation curve of P uptake, which is favourable when measuring P uptake kinetics.

An essential step for the study was to plot P uptake velocity against P_{add}, from which specific P uptake affinity can be later calculated. 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 an illustration of two models, see **Fig. 4**. The periphyton specific P uptake affinity was calculated as maximum uptake velocity divided by 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}).

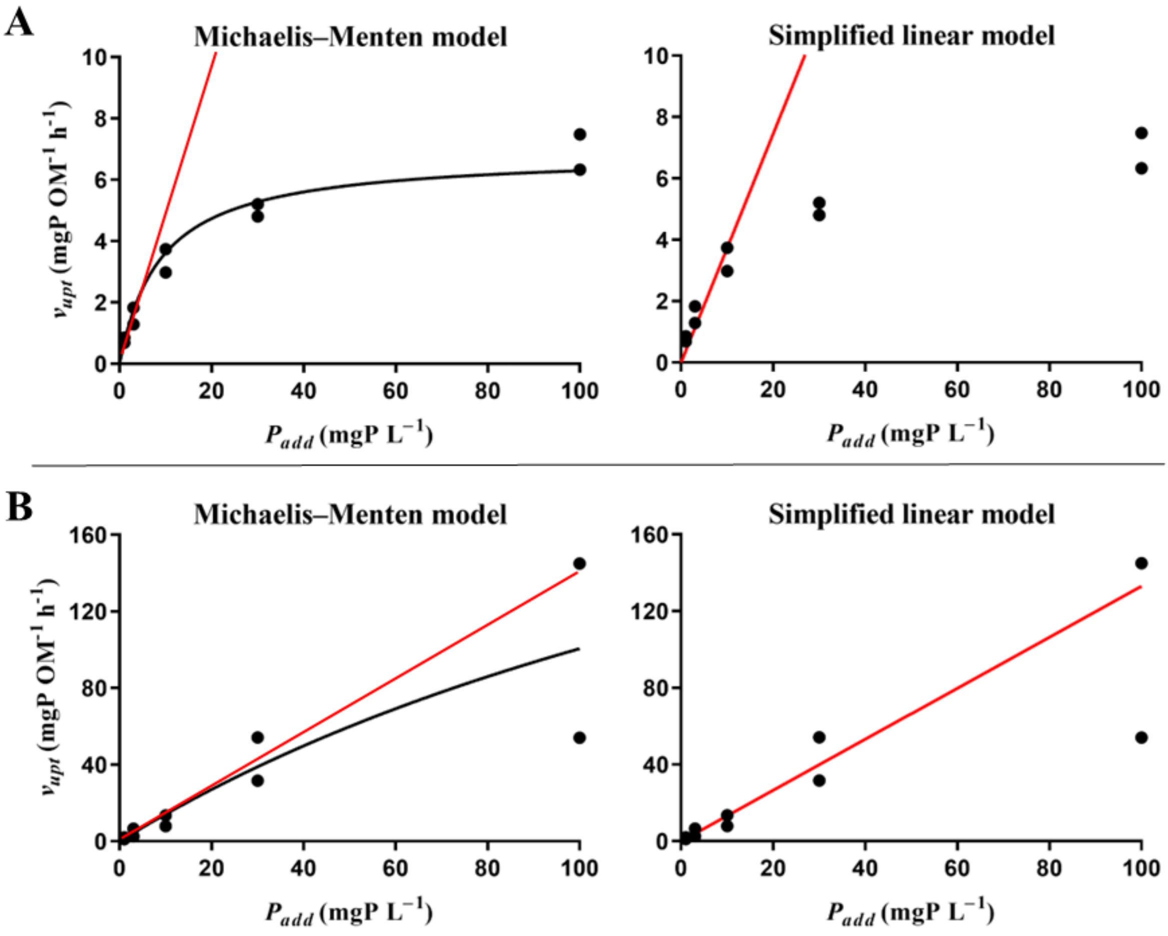

Figure 4: Example of fit by Michaelis–Menten model and linear model. A) Experiment where the Michaelis–Menten was preferred. B) Experiment where the linear model was preferred. The red line indicates the periphyton specific P uptake affinity calculated as V_{max}/K_s for Michaelis–Menten model and v_{slope} 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}.

As a control, I conducted an experiment without added periphyton (empty testing vessels/plain rock in the vessel) to test that the disappeared ^{33}P–labelled H_3PO_4 from the lake water was not adsorbed to the surface of the plastic testing vessel or the rocks instead of taken up by biological periphyton uptake process. Neglectable adsorption on the surface of the testing vessel or the surface of the stone substrate was recorded.

Due to the greater demands on the sample volume for the spectroscopic method (Kopáček and Hejzlar, 1993; Murphy and Riley, 1962) and low accuracy in low P concentration (Dodds, 2003b; Tanaka et al., 2006), it would not be possible to process the same amount of sample as when using the scintillation method (~144 kinetics experiment per season). The implementation of a scintillation methodology for periphyton phosphorus uptake measurement into the laboratory praxis at the Institute of Hydrobiology is, therefore, 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 different habitats – not only from the post-mining lakes.

3.7.2 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 an *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 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 the calculation of specific P uptake affinity *SPUA* (L gOM^{-1} h^{-1}) and other related parameters. For comparison of periphyton and seston contribution on the P traits in studied lakes, the same experiment was performed with seston. For details about P uptake kinetic experiments, see **Study II (Annex II)**.

SAFETY CONCERNS – Isotope ^{33}P is a β–emitter with low energy (order of magnitude lower than ^{32}P) and half-time approximately 25 days. The maximal reach of irradiation, when stored in glass, is 0.23 mm, therefore, the danger of irradiation is considered very low. It is necessary, however, to wear laboratory gloves to avoid direct contact with the skin (Redman, 2020). Laboratory waste contaminated with ^{33}P was stored for six months and then thrown into communal waste.

3.8 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 6**. Water temperature, pH, O_2, conductivity and euphotic depth were determined *in situ*. The rest of the 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 6: Methods used for the chemical and limnological analyses

Parameter	Method	Citation
Tropic status index	$\text{TSI (Chla)} = 10 \times (6 - \frac{2.04 - 0.68 \times \ln \text{Chla}}{\ln 2})$	(Carlson, 1977)
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)	(DIN ISO 17289, 2014)
Euphotic depth (Z_{eu})	Photometry, calculated as 1% of surface irradiance, LICOR LI-1400 datalogger (Licor. Lincoln. NE, USA)	(Kirk, 1994)

Parameter	Method	Citation
Acid neutralising capacity (ANC 4.5)	Acid-base titration	(CSN EN ISO 9963, 1996)
Dissolved organic carbon	Detection by a non-dispersive infrared detector after oxidising to CO_2, Shimadzu TOC/TN analyser (Shimadzu Corp., Japan)	(CSN EN ISO 7827, 2013)
Dissolved nitrogen	Chemiluminescence detection after catalytic combustion, Shimadzu TOC/TN analyser (Shimadzu Corp., Japan)	(Nydahl, 1978)
Total phosphorus	Flow Injection Analyser with spectrophotometric detection after nitric-perchloric acid digestion	(Kopáček and Hejzlar, 1993)
Chlorophyll a	Spectrophotometry after acetone extraction	(CSN EN ISO 10260, 1992)
Dissolved reactive phosphorus	Spectrophotometry - Phosphomolybdate blue method	(Murphy and Riley, 1962)
Dissolved reactive silica	Spectrophotometry	(Mackereth et al., 1978)
$N\text{-}NO_3$	Colorimetry after hydrazine reduction to nitrite	(Procházková, 1959)
Cl^-	Flow analysis with spectrophotometric detection	(CSN EN ISO 15682, 2002)
Ca^{2+}	Inductively coupled plasma atomic emission spectrometry	(CSN EN ISO 11885, 1996)
Mg^{2+}	Inductively coupled plasma atomic emission spectrometry	(CSN EN ISO 11885, 1996)
Fe	Inductively coupled plasma atomic emission spectrometry	(CSN EN ISO 11885, 1996)
SO_4^{2-}	Turbidimetric analysis	(US EPA Method 375.4, 1999)

3.9 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, $p < 0.05$) for testing the statistical differences between lakes and seasons were performed in R (R Core Team, 2019) using package vegan (Oksanen et al., 2019). 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 statistics were 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 statistics were performed in R (R Core Team, 2019) using packages vegan (Oksanen et al., 2019), EcolUtils (Salazar, 2020) and Goeveg (Goral and Schellenberg, 2017). 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 (Shannon and Weaver, 1964), diatom trophic (Rott et al., 1999) and saprobic indices (Rott et al., 1997) were calculated in Omnidia 6.0.8 software (Lecointe et al., 1993). 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. 5)**. 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 (Atkins et al., 2021). 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}. Periphyton autotrophic index suggests an auto-heterotrophic to autotrophic composition of periphyton (Crossey and La Point, 1988; Lakatos et al., 1998) with an accumulation of death biomass and detritus in Lakes Most and Milada in autumn (Lowe and Pan, 1996; Weber, 1973). 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 (Hillebrand and Sommer, 1999; Kahlert, 1998). Periphyton C:P and C:N molar ratio in Lake Medard did not change significantly, whereas in Most and Milada Lakes, C:P and C:N molar ratios clearly showed two separate periods – spring and winter with higher N+P availability and summer and autumn with extreme N+P deficiency.

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, a strong effect of Si was identified ($p < 0.0003$, non-parametric correlation matrix), 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} (Martin-Jézéquel et al., 2000). Other correlations were found with taxonomic composition and Mg^{2+}. Magnesium sulphate ($MgSO_4$) is a common contaminant in mine water (van Dam et al., 2010). The 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 (Mooney et al., 2020). 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}).

As well as above-described results with high environmental relevance, a comparison of two methods for taxonomic determination of periphyton autotrophic groups was an objective of the study**.** Overall, both tested methods for periphyton taxonomic composition, traditional microscopy and chemotaxonomy, showed similar outcomes concerning groups Chlorophyta and diatoms (non-parametric correlation matrix, R^2 = 0.846 and 0.672 for Chlorophyta and diatoms, respectively). A very low correlation was observed for Cyanobacteria, moreover, results for Cyanobacteria significantly differed. For details, see **Study I** (**Annex I**).

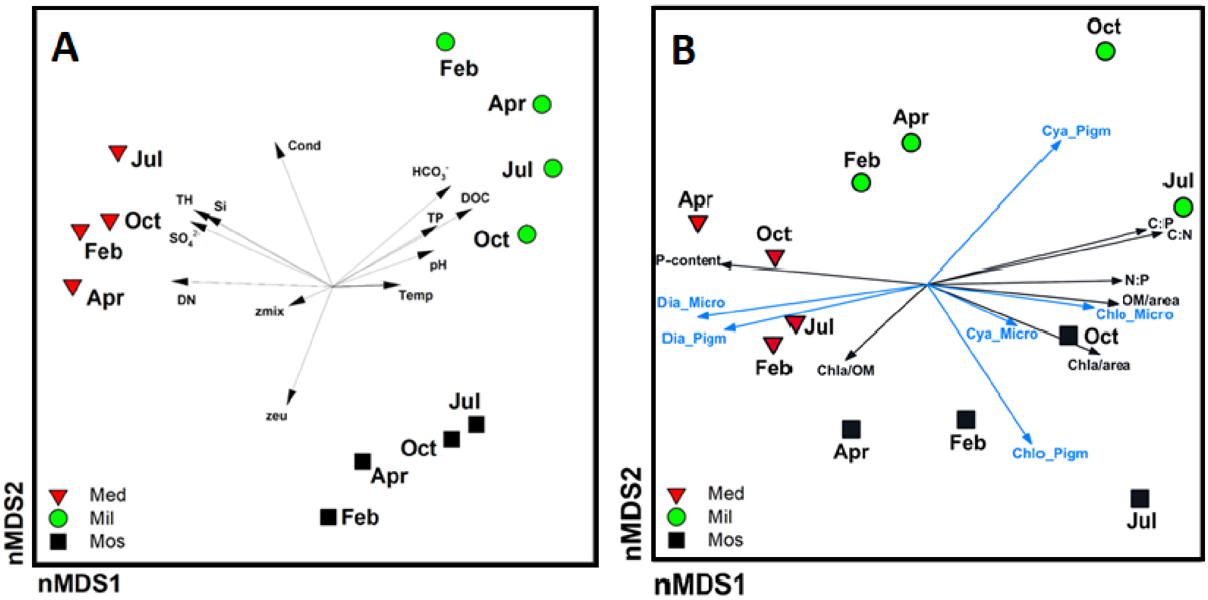

Figure 5: Separation of the lakes based on (A) limnological parameters and (B) periphyton composition. Med = Medard; Mil = Milada; Mos = Most; Temp = surface water temperature; Z_{eu}, = euphotic depth; Z_{mix}, = mixed layer; DN = dissolved nitrogen; TH = Total hardness, sum of Ca^{2+} and Mg^{2+}; 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

The first application of the introduced methodology for P uptake kinetics was the monitoring of seasonal changes in phosphorus uptake dynamic of periphyton from the post-mining lakes in Northern Bohemia. Our study represents the first report on P uptake kinetics by periphyton assemblages naturally growing on stones in temperate oligo- to mesotrophic lakes. Regarding the seasonal changes of parameters of phosphorus uptake in the studied post-mining lakes, maximum uptake velocity (V_{max}, seasonal range 1.9–129.0 mgP gOM^{-1} h^{-1}) decreased by an order of magnitude from April to October, while half-saturation constant (K_S, seasonal range 3.9–135.0 mgP L^{-1}) did not show any consistent temporal trend. 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 (Scinto and Reddy, 2003) and decreased from spring to autumn (p = <0.0001, two-way ANOVA). The among-system differences in kinetic parameters might be attributed to the differences in abiotic and biotic drivers affecting P uptake in compared ecosystems, in the species composition of periphyton, environmental conditions, and morphological and limnological variances between studied lakes. In **Fig. 6,** 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 (Cáceres et al., 2019; Krumhardt et al., 2013), implied by an observed decrease in periphyton P content and increase in C:P molar ratio (**Table 7**). 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 mainly 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 (Riber and Wetzel, 1987) 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.

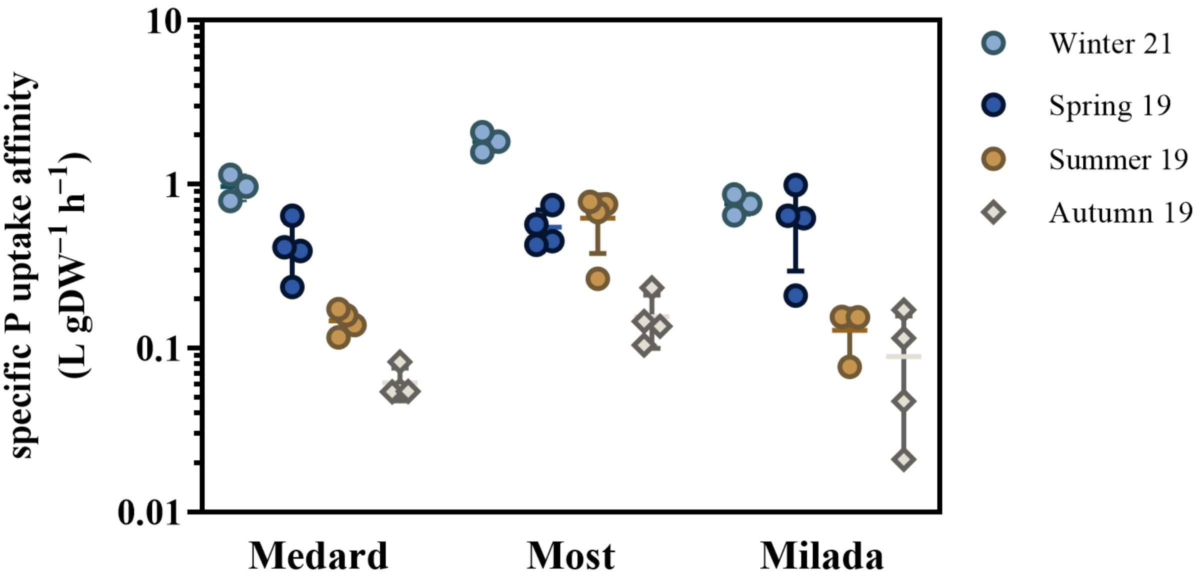

Figure 6: Specific phosphorus uptake affinity of periphyton, 19 – year 2019, 21 – year 2021 (modified from Konopáčová et al., 2021)

Table 7: 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 (mg P g OM^{-1})	2.4	3.6	2.2	2.1

4.3 Study III

Several studies exist describing the abundance and diversity of macrophytes, fish, or zooplankton in post-mining lakes (Bylak et al., 2019; Deshaies, 2018; Frouz et al., 2007; Nixdorf et al., 2005; Oszkinis-Golon et al., 2021). Prominent biomass of periphyton was so far neglected in published diversity studies on post-mining lakes ecosystem. Therefore, **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 a 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 % were represented by soft algae (Cyanobacteria, Chlorophyta, Rhodophyta, Conjugatophyta, Cryptophyta, Dinophyta, Euglenophyta and Xanthophyta) and 57 % by diatoms. Examples of species identified during **Study III** are shown in **Fig. 6**. The Shannon index values were 3.1–4.1 and 0.7–3.3 for diatoms and soft algae, respectively. Considering the soft algae, higher diversity was observed in the older Lakes Most and Milada, whereas higher diversity of diatoms, as well as their proportion in periphyton biomass, was recorded in Lake Medard. Seasonal changes revealed the prevalence of Cyanobacteria and motile forms of diatoms in the late summer in all lakes. This could lead to the consideration that these life forms are more successful in the utilisation of nutrients released from the accumulated periphyton biomass.

The most important factor influencing 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. The data could serve for the monitoring and sustainability of reclamation mining pits and can be used for the monitoring of water quality according to the recommendation of the Water Framework Directive (European Commission, 2000).

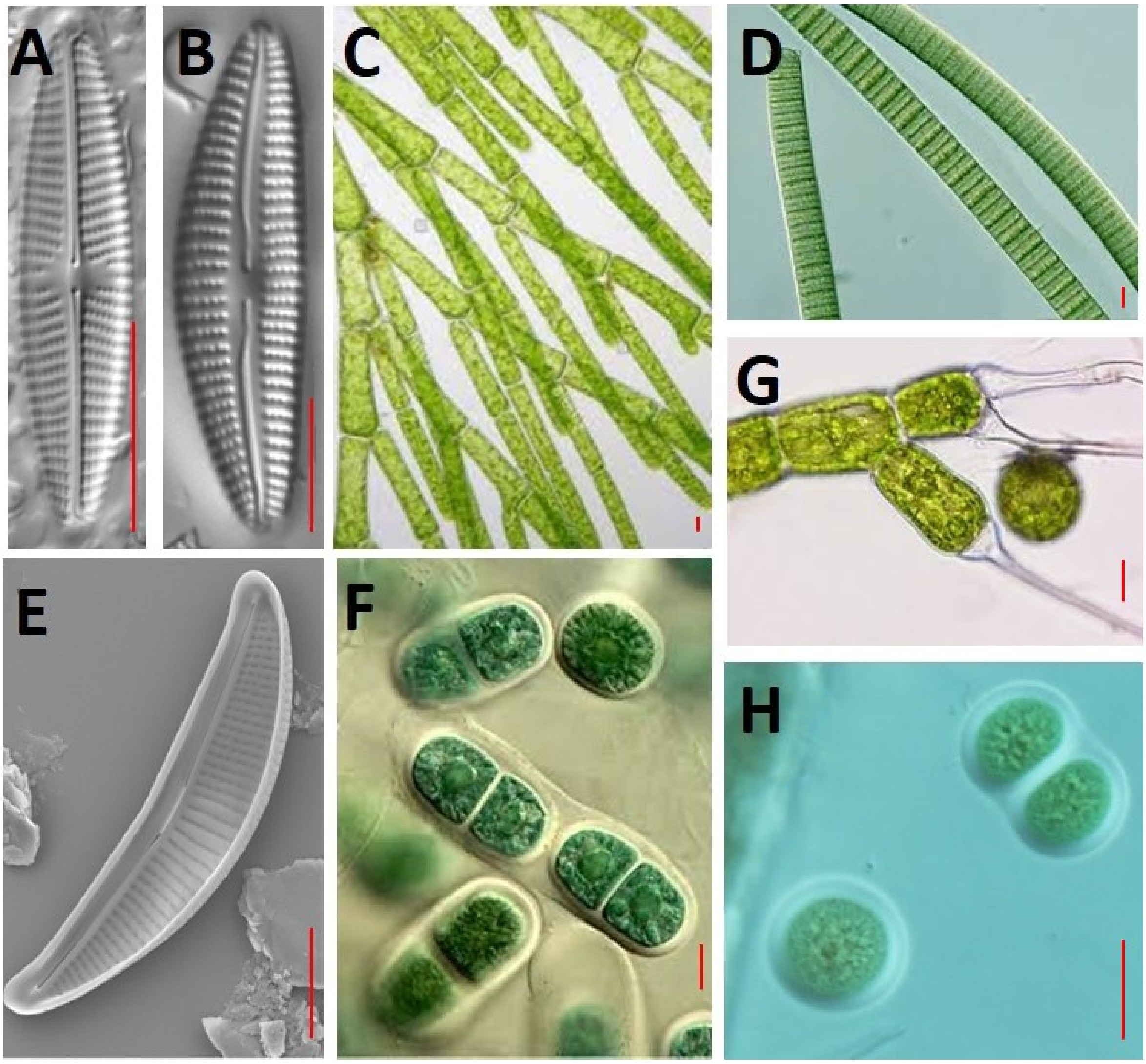

Figure 7: Selected autotrophic species found in periphyton: (A) diatom *Navicula libonensis*, (B) diatom *Cymbella subleptoceros*, (C) green algae *Cladophora glomerata*, (D) cyanobacteria *Oscillatoria limosa*, (E) diatom *Halamphora veneta*, (F) red algae Chroodactylon ornatum, (G) green algae *Bulbochaeta* 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. 8**. All three studies offer comprehensive information and a holistic approach to the periphyton community at the early stages of post-mining lakes succession. Post-mining lakes were proven to be important elements of the stricken landscape, and several reasons for their conservation exist, such as recreation, water retention or increase of biodiversity in the landscape. To protect these specific ecosystems, we need to monitor them to understand their dynamics. Periphyton, one of the prominent components in the nutrient-poor systems of post-mining lakes, was studied in the presented dissertation thesis. **Studies I** and **II** form 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 conserving post-mining lakes´ ecosystems 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 an 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 patterns 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 resources 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) (Pringle, 1987). We suppose that from summer to autumn, both internal and external phosphorus resources 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 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 a factor lowering Mg^{2+} toxicity (Mooney et al., 2020; van Dam et al., 2010). 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.

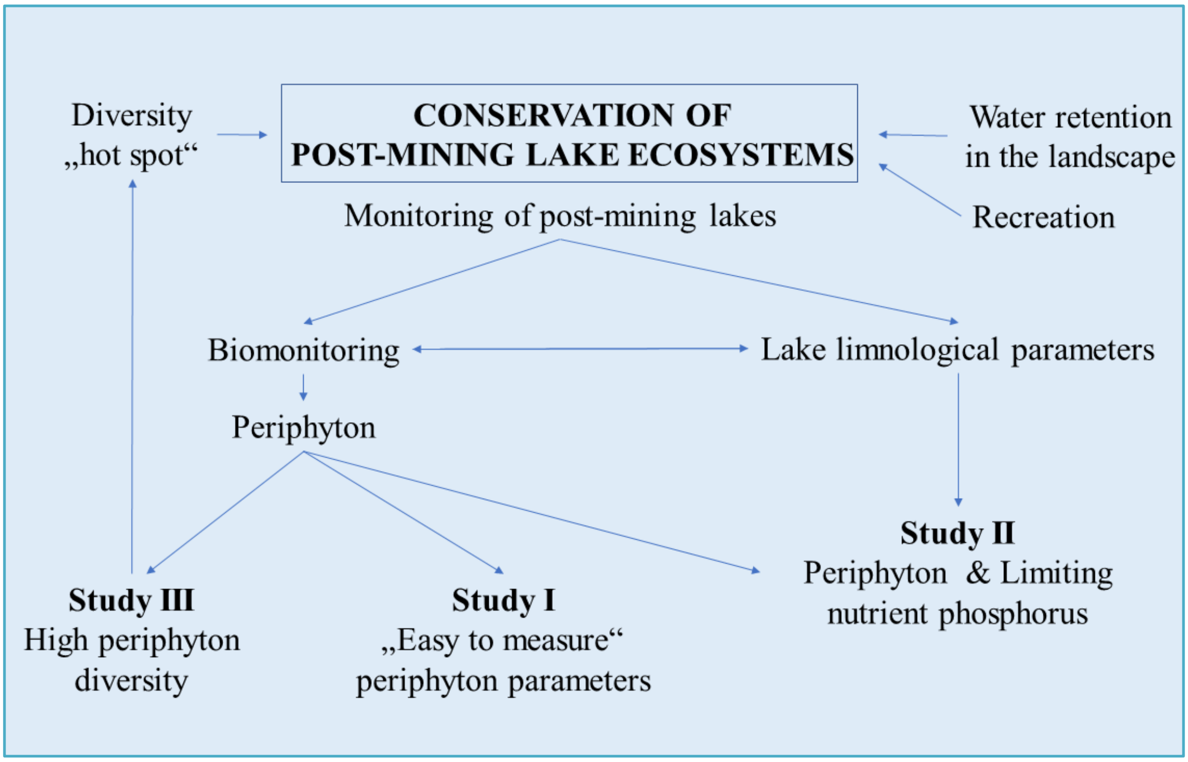

Figure 8: General graphical synopsis of studies presented in the dissertation thesis

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. Differences in periphyton features between the post-mining lakes of different age of flooding and seasonal changes were uncovered and discussed. In **Study I**, periphyton parameters with the potential to be used for biomonitoring 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**, which deals with the relationship between periphyton and the limiting nutrient phosphorus, 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 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 to the winter season, with planned sampling in the year 2023. 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

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