# An assessment of periphyton mats using CHEMTAX and traditional methods to evaluate the seasonal dynamic in post-mining lakes

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**Abstract** This study evaluated periphyton biomass, nutrient content, and taxonomical composition in three nutrient-poor post-mining lakes in the Czech Republic. Two methods, microscopy and chemotaxonomy, were used to determine the taxonomical composition of autotrophs. Both methods identified diatoms, Chlorophyta, and Cyanobacteria as the dominant groups across the lakes. Considerable congruence of the taxonomical methods was found for diatoms and Chlorophyta, however results for Cyanobacteria showed poor correlation. The differences in periphyton features among the lakes were mostly explained by the lake age and trophy. Moreover, high amounts of overwintering biomass show that periphyton development is not established "*de novo*" each year but its current stage is a cumulative result of previous years. Beside the lake age and trophy, limnological characteristics such as Si or $Mg^{2+}$ also affect periphyton taxonomical composition. No correlation of periphytic C:N:P molar ratios with lake water nitrogen and phosphorus, suggests role of additional process to the nutrient uptake, likely internal nutrient recycling in periphyton. These findings are essential in predicting further succession in the examined post-mining lakes and serve as a model for newly formed lakes. As more lakes will be formed within the post-mining recultivation in the short horizon, our study contributes to their successful management.

**Keywords** Algal biomass · Periphyton diversity · Periphytic pigments · Recultivation · Chemotaxonomy · Oligotrophic lake · Seasonal dynamic

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## Introduction

The extensive use of coal started in the middle of the nineteenth century, accompanying the Industrial revolution. Since then, coal has been one of the primary energy supplies for many European industrialised countries (Esposito & Abramson, 2021). Coal mining became a prominent anthropogenic activity that has directly modified the face of many terrestrial ecosystems (Hooke et al., 2012; Larondelle & Haase, 2012). In the north of the Czech Republic, the opencast coal mines are currently transforming the landscape for almost 200 years (Vrablik et al., 2017). Diverse reclamation techniques are used worldwide to relieve the environmental damages caused by coal mining, e.g., reforestation, hydric reclamation or spontaneous succession (Schultze et al., 2010; Søndergaard et al., 2018; Frouz, 2021). As European political statements imply various green stimulus packages containing plans for zero emission economics by mid-century, and reducing of coal mining capacities, the number of post-mining areas is expected to rise in the upcoming decades (Larondelle & Haase, 2012; European Commission, 2018).

Post-mining lakes are being established during the hydric way of reclamation, which represents one of the most popular forms of landscape restoration (Søndergaard et al., 2018). Newly established lakes are artificial surface water bodies often with oligotrophic conditions (Schultze et al., 2010) and different hydrological balance, compared to other man-made water reservoirs and natural lakes. Post-mining lakes commonly do not have any surface in- and outlets. Water supply and exchange mainly depend on precipitation, evaporation and groundwater flow. Therefore, long residence times of some years are characteristic (Axler et al., 1998; Schmidt & Schubert, 2007; Gammons et al., 2013). The long residence time makes the lakes prone to pollution, as pollutants are kept in the water body over long periods. The ecological succession of post-mining lakes depends on many factors, for example, ground and surface runoff water quality, the residence time of water, and the bedrock geology (Gammons et al., 2009). All these factors together influence the final environmental conditions of the fully developed water body.

Monitoring the physical and chemical parameters is a snapshot of the actual environmental conditions, which may change within a short period. Sessile organisms, on the other hand, reflect the long-term conditions of the habitat they colonise (Gaiser, 2009; Marazzi & Gaiser, 2018). For that reason, they are used as biomonitoring tools for decades as an alternative to hydrochemistry. Periphyton is an assembly of benthic photoautotrophs and heterotrophs, which, together with its extracellular polymeric substances, forms a biofilm covering a substrate (Azim et al., 2005). Periphyton monitoring has often been considered to reflect changes in the management of aquatic ecosystems (Hill et al., 2000; Gaiser, 2009) and is part of the Water Framework Directive (European Commission, 2000), where however, only running water periphyton is considered. Next to the long tradition of periphyton research in streams (Stevenson et al., 1996a, b; DeNicola & Kelly, 2014) also studies in wetlands can be found (Gaiser, 2009; Oberholster et al., 2022). Besides those two ecosystem types, periphyton can form prominent biomass also in oligotrophic and mesotrophic lakes (Vadeboncoeur & Steinman, 2002; Liess et al., 2009), especially in systems with extended littoral zones (Azim et al., 2005; Cantonati & Lowe, 2014). By describing the basic periphyton parameters, we can obtain the essential knowledge about such ecosystems. Several crucial parameters such as total biomass, elemental (C:N:P molar ratio) or species composition can be examined and potential changes reported (Gaiser, 2009; Buchaca et al., 2019; Konopáčová et al., 2021). Periphyton biomass was found to be affected by increasing light and nutrient availability in artificial streams (Pacheco et al., 2022). Lambert et al. (2008) recorded a significant increase in periphyton biomass along nutrient enrichment as a result of the recreational development of Laurentian lakes in Canada. Moreover, a decrease in molar C:P ratio was recorded after increasing recreational use. Lambert et al. (2008) hypothesised that periphyton, positioned near the land–water interface, react to the land-derived nutrients. Therefore, measuring periphyton characteristics such as total biomass or C:N:P molar ratios may represent a better tool for early detection of lake perturbation than methods based on pelagic characteristics (Lambert et al., 2008). The autotrophic part of periphyton (algae and cyanobacteria), which usually forms a majority of periphyton biomass (Cantonati & Lowe, 2014; Bešta et al. 2023), is traditionally analysed by light microscopy. However, increasing efforts have been invested to develop more cost- and time-efficient methods. For phytoplankton, a chemotaxonomic method based on the analysis of pigment content using high-performance liquid chromatography (HPLC) combined with the factor analysis program CHEMTAX has been successfully applied for more than 25 years (Mackey et al., 1996; Van Heukelem & Thomas, 2001; Higgins et al., 2011). Several studies attempted to apply this method to freshwater periphyton (Lauridsen et al., 2011; Dalton et al., 2015; Louda et al., 2015; Jerney et al., 2016). However, a confirmation of the accordance of both methods to study periphyton composition is still lacking.

We focused on three recently flooded post-mining Lakes Medard, Most and Milada from the north-western Bohemia, with a well-developed periphytic community. As the predominant substrate is gravel, rock and stone in the littoral zone, macrophytes growth is less favoured and the periphyton community thriving on rocks is the major benthic primary producer. Seasonal changes in periphyton biomass per area and elemental and pigment composition were described. The autotrophic part of periphyton was studied applying two different approaches—traditional taxonomy by microscopic analysis and chemotaxonomy based on pigment determination, including the factorial analysis with CHEMTAX. We hypothesize that the seasonal dynamics of periphyton mats in post-mining lakes are driven by changes in nutrient availability, which affect both the biomass and taxonomical composition of the periphyton communities. CHEMTAX and traditional methods can be used to accurately assess these changes over time. The periphyton characteristics obtained within this study can serve as necessary default information for further monitoring of the ecosystem responses to natural or management changes. Also, the study brings novel and important knowledge complementing the general picture of the large oligotrophic lakes and their development and succession in the initial stages.

## Methods

### Study sites

The study was conducted in the littoral zone of three recently flooded post-mining Lakes Medard, Most and Milada, with moderate to low primary productivity (based on lake water chlorophyll a concentration, see basic lake characteristics in Table 1, Konopáčová et al., 2021). Lakes are located in north-western Bohemia (Czech Republic, Fig. 1). Each lake was sampled at two shores opposite to each other (South, North, see Table 2 for GPS location) to include the heterogeneity of the lakes. All lakes possess a littoral zone with well-developed periphytic community growing on the stone substrate (Fig. 1), reaching approximately 2.5 m depth. Samplings were performed in April, July and October 2020 and February 2021.

### Periphyton sampling

For a vertical profile of biomass distribution in the littoral zone, periphyton was sampled from 0 m up to 2.5 m depth in 0.5 m intervals in April, July and October 2020. In February 2021, the vertical profile sampling was conducted due to severe winter conditions only at one sampling site in depths of 0.5 m and 1.5 m (North for Milada and Most Lakes, South for Medard Lake). Five random stones covered with periphyton were collected at each depth and periphyton was scraped from the collected stones with a scalpel and a toothbrush and gently homogenised. For periphyton biomass per area determination, all harvested biomass was dried at 110°C to the constant weight and the dry mass (DM) was determined, followed by burning samples in the muffle furnace at 500°C for 2 h to determine the ash mass (AM). Organic matter (OM) was calculated by subtracting DM from AM (APHA, 2017). Using weight-to-area conversion, the stone surface covered by periphyton was estimated by the aluminium foil method (Dudley et al., 2001). The total sampled area in each depth was approximately 1,000 $cm^2$ (~ 200 $cm^2$ per stone, 5 replicates). Periphyton biomass in a certain depth was then determined as an average OM per $cm^2$ of the covered part of the stone. The term “periphyton quantity” was used throughout the study to address the average value of all measured depths for the respective lake and season.

For detailed periphyton characterisation (periphyton elemental composition, microscopy and pigment analysis), samples were taken from the 1 m depth, which represented the middle of periphyton littoral zone distribution. Small stones (approximately 4 cm in diameter) covered by periphyton were collected in triplicates and kept separately in the lake water until

**Table 1** Basic characteristics and seasonal averages of selected limnological parameters sampled from the depth of 0.5 m above their deepest points in 2019 (Konopáčová et al., 2021)

| Lake | Flooded (year) | Area (ha) | Max/Avg depth (m) | $Z_{eu}$ (m) | pH | Cond (µS cm$^{-1}$) | TP/SRP (µg l$^{-1}$) | DOC (mg l$^{-1}$) | Chl-a (µg l$^{-1}$) | $NO_3$-N (mg l$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| Medard | 2016 | 493 | 59/28 | 13.8 | 7.8 | 1087 | 4.2/0.3 | 3.1 | 0.9 | 1.2 |
| Most | 2014 | 311 | 75/22 | 15.0 | 8.7 | 532 | 11.9/1.9 | 4.7 | 1.9 | 0.7 |
| Milada | 2010 | 252 | 25/16 | 12.2 | 9.1 | 1034 | 13.7/2.1 | 7.9 | 4.9 | 0.1 |

*Max* maximal; *Avg* average; $Z_{eu}$ euphotic depth; *Cond* conductivity; *TP* total phosphorus; *SRP* soluble reactive phosphorus; *DOC* dissolved organic carbon; *Chl-a* lake water chlorophyll a; $NO_3$-*N* nitrate-nitrogen

analysis. For elemental composition and pigment analysis, organic matter was determined from the defined part of the periphyton and used for normalising the results on OM. All samples were transported in the cooling boxes to the laboratory on the same day.

## Periphyton elemental composition

Samples for the elemental composition (C, N, P) were stored at − 20°C until the analysis, then homogenised using glass beads and analysed. Carbon and nitrogen content was determined using the Shimadzu TOC/TN analyser (Shimadzu Corp., Kyoto, Japan), carbon after oxidising to $CO_2$ by non-dispersive infrared detection and nitrogen after catalytic combustion by chemiluminescence detection. Phosphorus content was measured spectrophotometrically after nitric-perchloric acid digestion (Kopáček & Hejzlar, 1993) using a Flow Injection Analyser (FossTecator, Hoganas, Sweden).

## Periphyton microscopy

Species identification was done by compound microscopy of fresh samples using an Olympus BX 50 light microscope equipped with DIC optics and a DP-72 digital camera. Common identification literature was used (Komárek & Fott, 1983; Krammer & Lange-Bertalot, 1986, 1988, 1991a, b; Ettl & Gaertner, 1988; Hindák, 1996; Komárek & Anagnostidis, 1998, 2005; Krammer, 2000, 2002, 2003; Lange-Bertalot, 2001; Kaštovský et al., 2018a, b). Ten subsamples were randomly taken from each sample of scratched periphyton in amount suitable under the cover glass. The whole area of the cover glass was observed (under magnification ×1,000). The relative abundance of algal species was estimated within 2 days after collection based on the geobotanical Braun-Blanket scale (Braun-Blanquet, 1921), modified for algological purposes (Hindák, 1978). Each found species was assigned an integer value on the 7–degree scale reflecting its relative abundance: 1 (< 0.1%); 2 (0.1–1%); 3 (1–5%); 4 (5–20%); 5 (20–50%); 6 (50–90%); 7 (> 90%). Overall percentage of higher taxonomical groups (diatoms, Chlorophyta, Cyanobacteria etc.) was calculated from the species

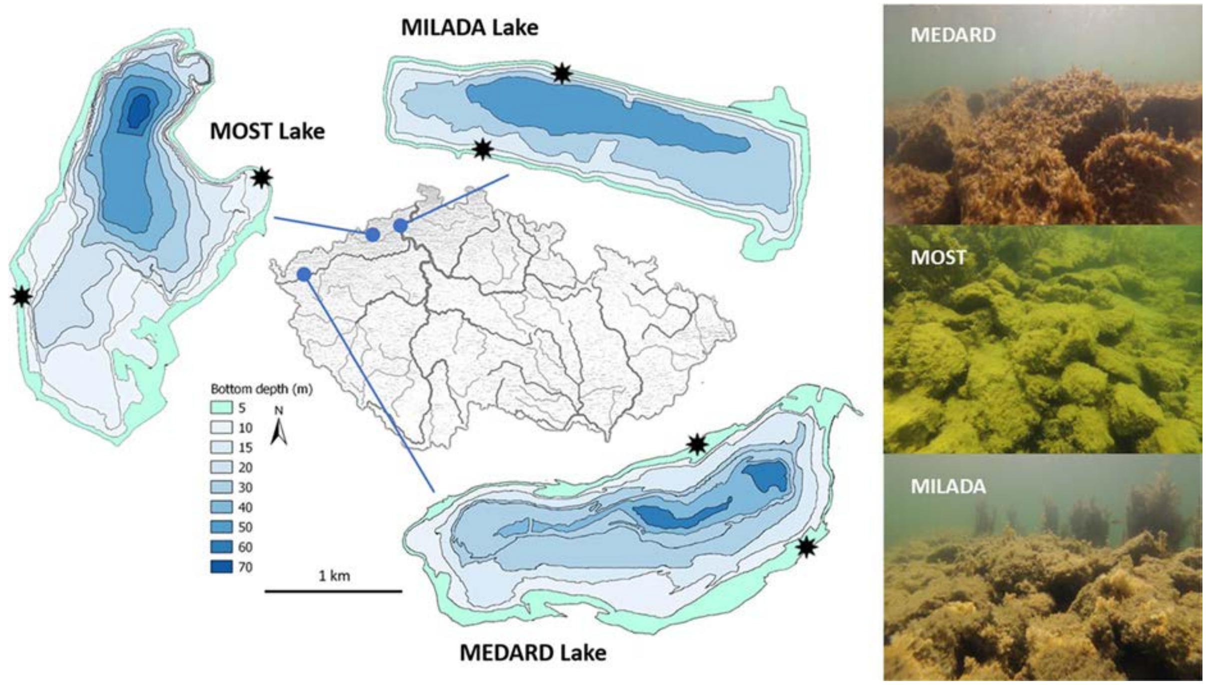

**Fig. 1** Geographical location of the investigated post-mining Lakes Medard, Most and Milada within the Czech Republic, central Europe. Locations of the sampling sites are marked with asterisks. The map is combined with illustrative photos of periphyton from each studied lake

**Table 2** GPS location of two sampling sites at each studied lake

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

frequency using mean of the percentage interval of the corresponding abundance class.

### Periphyton pigment analysis

Periphyton samples were homogenised in 1 l of tap water with a hand blender to break down the periphyton mat structure. The homogenate of a defined volume was then filtered on glass-fibre filters (GF/C Ederol Company) and immediately frozen to assist cell burst and stored in the dark at −20°C until its use for pigment analysis. 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 then centrifuged, and the supernatant was taken for HPLC. For HPLC, the gradient program according to Van Heukelem & 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 (Chl-c), peridinin (Peri), fucoxanthin (Fuco), neoxanthin (Neo), violaxanthin (Vio), myxoxanthophyll (Myxo), diadinoxanthin (Diadi), dinoxanthin (Dino), alloxanthin (Allo), diatoxanthin (Diato), zeaxanthin (Zea), lutein (Lut), canthaxanthin (Cantha), chlorophyll-b (Chl-b), chlorophyll a (Chl-a) and sum $\alpha + \beta$ carotene (Caro). A mixed standard containing 28 pigments (DHI Lab Products, Denmark) was used for peak identification. Furthermore, the actual concentrations of Chl-a, Chl-c, Chl-b and ten carotenoids were calculated based on the external calibration with pure standards (DHI Lab Products, Denmark). The concentrations of the selected pigments (Chl-c, Peri, Fuco, Neo, Myxo, Diadi, Allo, Lut, Cantha, Chl-b and Chl-a) based on Higgins et al. (2011) were used for the following CHEMTAX calculations. Additionally, the concentration of Chl-a in μg per unit OM was normalised per stone area (Chl-a $cm^{-2}$). Also, the periphyton Autotrophic index (AI) was calculated as OM:Chl-a ratio (APHA, 1985). Index values higher than 200 indicate a high proportion of heterotrophs, zoobenthos and/or organic detritus (Weber, 1973; Lowe & Pan, 1996). Chl-a $cm^{-2}$ and AI were grouped with the periphyton elemental composition parameters and further addressed as "qualitative periphyton parameters".

### CHEMTAX calculation

HPLC analysis was followed by data analysis in the matrix factorisation program CHEMTAX 1.95 (Mackey et al., 1996). We delimited six major taxonomical groups according to their specific pigment compositions (diatoms, Cyanobacteria, Chlorophyta, Dinophyta, Cryptophyta, Xanthophyta, Supplementary Table 1). Initial ratios for selected algae groups were based on Lauridsen et al. (2011), which recorded ratios for species of freshwater oligotrophic phytoplankton, except for Xanthophyta ratios, which were taken from Higgins et al. (2011). For optimisation of the input matrix, a series of 60 randomised pigment ratio tables were generated, and the final output matrix (final ratios) was calculated based on lower Root Mean Square Error (RMSE) as recommended by Higgins et al. (2011). Ratio limits, initial step size, and step ratio were set as recommended by Mackey et al. (1997).

### Background limnological parameters

The lake water for chemical analyses was collected at the open water 20 m from the shore at 0.5 m depth using a Friedinger sampler from the locality matching the periphyton samples. Water temperature (Temp), pH and $O_2$ concentration were measured in situ with YSI EXO 2 multiparametric probe (YSI Inc., Yellow Springs, USA). Conductivity (Cond) was measured with Combo pH/EC HI 98129 (Hanna Instruments, Woonsocket, RI, USA). The euphotic depth ($Z_{eu}$) was estimated as the depth of 1% of surface irradiance calculated from vertical light profiles obtained using LICOR LI-1400 datalogger with a spherical quantum underwater sensor LI 193 SA (Licor, Lincoln. NE, USA). The mixing depth ($Z_{mix}$) was defined as the water layer above the persistent thermocline. Lake water Chl-a was determined spectrophotometrically after acetone extraction (CSN EN ISO:10260, 1992). Dissolved reactive silica (Si, Mackereth et al., 1978) and soluble reactive phosphorus (SRP, Murphy & Riley, 1962) were determined spectrophotometrically. Nitrate-nitrogen ($NO_3$-N) was analysed via hydrazine reduction as nitrite (Procházková, 1959). The concentrations of $Ca^{2+}$ and $Mg^{2+}$ were measured with inductively coupled plasma atomic emission spectrometry (ICP AES, CSN EN ISO:11885, 1996). Turbidimetric analysis of $SO_4^{2-}$ followed US EPA Method 375.4. DOC, DN and TP were analysed as described above for the periphyton elemental composition. ANC4.5 (Acid neutralising capacity) determination was based on the norm for surface water of the Czech Republic (CSN EN ISO:9963, 1996). Concentrations of $HCO_3^-$ were assumed to be equal to positive ANC values. The trophic state index (TSI) was determined for each season based on the Secchi depth, the concentration of Chl-a and the concentration of TP (Carlson & Simpson, 1996). Secchi depth was determined as $Z_{eu}/2.8$ (Luhtala & Tolvanen, 2013). The overall seasonal TSI for each lake was defined as an average of TSI based on Chl-a from four seasons measured. TSI based on Chl-a was favoured above others based on Carlson (1977), which suggested using Chl-a based TSI as a primary index since it is a direct estimator of algal biomass in the lake water.

### Statistical analysis

The low number of gathered samples and non-normal distribution of regression residuals of numerous tested linear models even after various types of the data transformation prevent using of parametric correlation statistics in the data processing. As an initial Permanova (Permutational Multivariate Analysis of Variance, $P < 0.05$) based on periphyton parameters proved the difference of lake sites to be insignificant ($R^2 = 0.0208$, $F = 1.2911$, $P = 0.267$), we treated them as replicates in all following statistical analysis. The tests of significant differences between seasons and lakes were done with Permanova ($P < 0.05$). The correlation between selected limnological parameters (ANC4.5, $Ca^{2+}$, Conductivity, DN, DOC, Chl-a, $Mg^{2+}$, $NO_3$-N, $O_2$, pH, Si, $SO_4^{2-}$, SRP, TP) and periphyton parameters (biomass, C:N:P molar ratios, Chl-a $cm^{-2}$, AI and taxonomical composition) was calculated by Spearman's rank correlation. $Z_{eu}$, $Z_{mix}$ and temperature were excluded from the analysis since we assume no effect of $Z_{eu}$ and $Z_{mix}$ on samples coming from depth 1 m. Temperature copy effect of season. Due to the high number of tests, results with $P < 0.001$ were considered as significant.

Correlation between the results gained by traditional microscopy and chemotaxonomy was tested with another non-parametric correlation matrix for the three main autotrophic groups present (diatoms, Chlorophyta and Cyanobacteria). As Streptophyta cannot be resolved by pigment analysis from Chlorophyta (due to the high similarity of pigment content), we grouped them into Chlorophyta also in the microscopy. Both correlation matrices and Permanova were performed in R (Oksanen et al., 2019; R Core Team, 2019).

To uncover seasonal differences for single periphyton parameters, we tested each parameter separately with Kruskall–Wallis test (Dunn's multiple comparison post-test, $P < 0.001$, Supplementary Table 2). Kruskall–Wallis test and figures showing seasonal changes in selected periphyton parameters (Figs. 2, 3, 4) were prepared in PRISM v.7 (GraphPad Software).

## Results

### Periphyton quantity: vertical biomass profile

The area exclusively covered by periphyton (> 90%) reached 1.5 m depth for Lakes Medard and Milada and 2 m depth for Lake Most. In depths > 2.5 m, periphyton biomass was generally lacking. A sharp decline in periphyton biomass was observed at depths > 1.5 m in Lakes Medard and Milada and 2 m in Lake Most (Supplementary Fig. 1). Periphyton biomass in all lakes significantly increased from spring to autumn and decreased from autumn to winter ($P = 0.0007$, Table 3, Fig. 3). The amount of maximum biomass reached in autumn, differed significantly between the lakes. The periphyton biomass

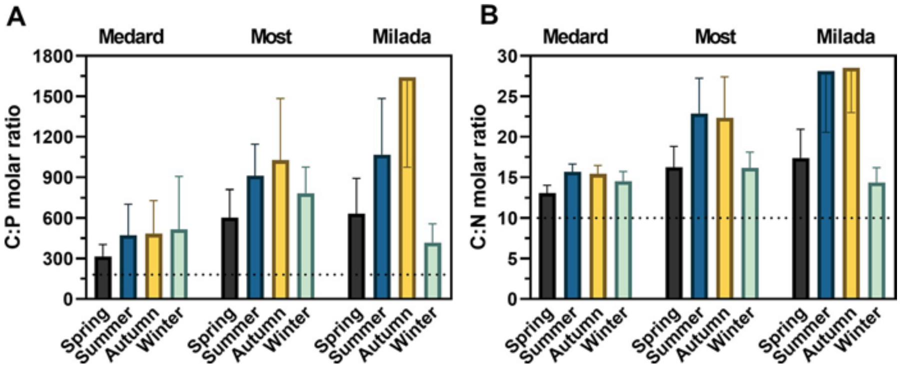

**Fig. 2** Seasonal changes in periphyton C:P and C:N molar ratios. Dashed line indicates border of **A** phosphorus and **B** nitrogen depletion in the periphyton biomass (given by Hillebrand & Sommer, 1999)

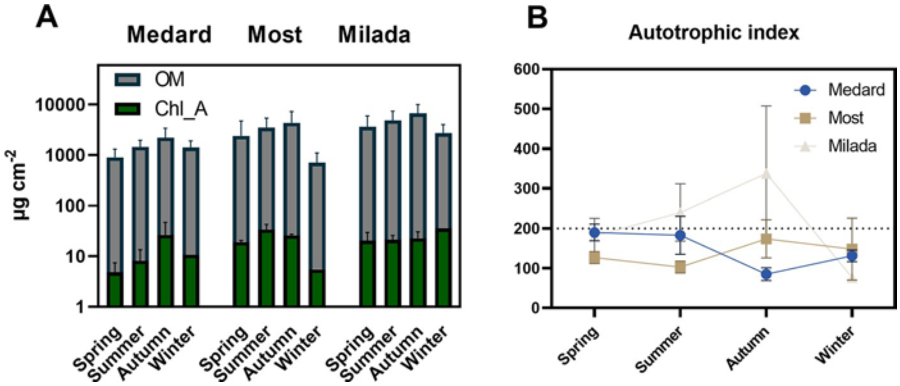

**Fig. 3** Relation of organic matter and Chl-a content. **A** Periphyton organic matter (OM) and Chlorophyll a (Chl-a) per area in the depth of 1 m. Columns show averages of three values from two sampling sites and standard deviation. **B** Periphyton Autotrophic Index. Each dot represents the average of three values from two sampling sites with its standard deviation

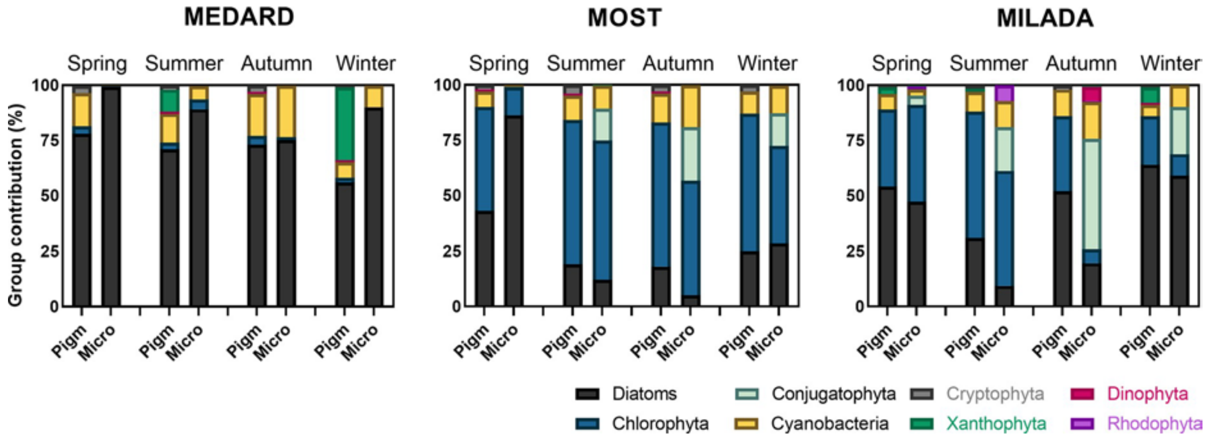

**Fig. 4** Seasonal changes in periphyton taxonomic contribution. Expressed as the average value of three replicates from two sampling sites. Pigm—algal group contribution according to the Chemtax. Micro—algal group taxonomic contribution according to the microscopy. Note that in taxonomical composition based on pigments, the group Chlorophyta and Conjugatophyta cannot be resolved due to the very similar pigment composition

**Table 3** Seasonal changes of periphyton biomass in three studied lakes

| Periphyton biomass (OM mg $cm^{-2}$) | | | | | | Autumn | |
|---|---|---|---|---|---|---|---|
| | Spring | Summer | Autumn | Winter | *P* | Min biomass/depth | Max biomass/depth |
| Medard | $0.9^{a}$ | $1.4^{b}$ | $2.2^{b}$ | $1.4^{ab}$ | **0.0007** | 1.8 (shoreline + 0.5 m) | 3.2 (1 m) |
| Most | $2.3^{a}$ | $3.1^{b}$ | $3.9^{b}$ | $0.7^{a}$ | **0.0003** | 2.3 (2 m) | 7.4 (shoreline) |
| Milada | $3.6^{a}$ | $4.3^{ab}$ | $5.2^{b}$ | $2.5^{a}$ | **0.0003** | 3.0 (1 m) | 7.0 (0.5 m) |

Values of maximal and minimal biomass within the vertical profile are shown for the season with the significantly highest recorded biomass (autumn). The difference in periphyton biomass between seasons was tested with Kruskall–Wallis test, $P < 0.001$, Dunn's multiple comparisons post-test. *P*-values in bold indicate statistically significant results

recorded in autumn in Lake Medard (2.2 mg OM $cm^{-2}$) reached less than half of the autumn biomass recorded in Lake Milada (5.2 mg OM $cm^{-2}$, Table 3). In Lake Most, the overall biomass recorded in autumn was 3.9 mg OM $cm^{-2}$, Table 3). The differences among the minimum and maximum biomass amounts recorded along the vertical profiles indicate an uneven distribution of biomass in the littoral zone of all three lakes (Table 3, Supplementary Fig. 1).

### Periphyton quality: elemental composition

Extremely high periphyton C:P molar ratios ranging from 315 to 1,642 and N:P molar ratios from 13 to 29 (Supplementary Table 2, Fig. 2) indicated P + N limitation in all lakes and seasons. P limitation is indicated when C:P > 180 and > 369 whereas N limitation when C:N > 10 and > 11 according to the optimal C:N:P ratios by Kahlert (1998) and Hillebrand & Sommer (1999), respectively. The higher C:P and C:N molar ratios are, the deeper the P and N deficiency in the periphyton is indicated. The average C:P molar ratio in Lake Medard was ~ 420 and did not significantly change throughout the study period. Molar C:N ratios in Lake Medard significantly increased from spring to summer ($P \le 0.0001$, Supplementary Table 2). In Lake Most, both C:P and C:N showed two significantly varying periods indicating different degree of nutrient limitation, spring + winter (C:P ~ 694 and C:N ~ 16) and summer + autumn (C:P ~ 969 and C:N ~ 23). A comparable pattern was also observed in Lake Milada with average values C:P ~ 525 and C:N ~ 16 in spring + winter and C:P ~ 1355 and C:N ~ 29 in summer + autumn season (Supplementary Table 2, Fig. 2).

### Periphyton quality: pigment analysis

The HPLC analyses resolved 18 pigments, from which 12 were calibrated via external standards to determine the absolute concentration of pigments in the periphyton (Supplementary Table 3). The highest pigment concentrations were recorded for the ubiquitous pigments Chl-a, Chl-b and fucoxanthin (Supplementary Table 3). Ten pigments were found in all studied lakes (Chl-c, Peri, Fuco, Neo, Diadi, Allo, Lut, Cantha, Chl-a, Caro). Chlorophyll b was characteristic only for Lakes Milada and Most and has not been detected in Lake Medard. Myxoxanthophyll was detected only at Milada Lake in autumn however, this pigment was present in all investigated replicates. Peridinin, which is associated with some Dinophyta, appeared as well in all investigated lakes, although Dinophyta were recorded by microscopy only in Milada Lake. Canthaxanthin was not present during the winter season at any investigated lake. Pigments peridinin, neoxanthin, myxoxanthophyll, alloxanthin and canthaxanthin were present in concentrations $< 0.1$ µg mg $OM^{-1}$ (Supplementary Table 3). Besides using the pigments for chemotaxonomy, we focused on describing seasonal changes in Chl-a concentration. Two different approaches were chosen: Chl-a per $cm^{-2}$ values, which accompany the changes of the total biomass per defined area of the lakebed, and Autotrophic index (AI), which describes the contribution of autotrophs in periphyton. In Lake Medard, the highest Chl-a content per area (26.5 µg $cm^{-2}$) and simultaneously the lowest AI (85) was recorded in autumn (Fig. 3, Supplementary Table 2). In Lake Most, Chl-a content per area significantly increased in the summer (34.1 µg $cm^{-2}$, $P = 0.0007$), however, changes in AI remained insignificant (Fig. 3, Supplementary Table 2). Lake Milada's highest Chl-a content and lowest AI were recorded in winter, however seasonal changes for both values remained insignificant (Supplementary Table 2).

### Taxonomical composition of autotrophs

The most prominent algal groups presented in all three lakes, confirmed by both microscopy and pigment analysis, were diatoms, Chlorophyta and Cyanobacteria (Fig. 4). Correlation of percentual proportions of the main autotrophic groups given by pigment analysis and microscopy were significant for Chlorophyta ($R^2 = 0.846$ and $P = 0.001$) and diatoms ($R^2 = 0.672$ and $P \leq 0.001$, Table 4, Fig. 5). Results for Cyanobacteria given by pigment analysis and microscopy significantly differed ($P = 0.245$) and showed very low correlation ($R^2 = 0.130$, Table 4, Fig. 5). In general, supported by both taxonomical methods, in Lake Medard, periphyton was dominated by diatoms during the whole year with contribution > 56%. Lakes Most showed codominance of diatoms and Chlorophyta, with unclear seasonal patterns. Lake Milada showed codominance of diatoms and Chlorophyta with the highest contribution of Chlorophyta in summer (significant by pigment analysis, $P = 0.0008$, Supplementary Table 2). A relatively high contribution of the representatives of Conjugatophyta was recorded by microscopy in Lakes Most and Milada (up to 24 and 50%, respectively). No Conjugatophyta were recorded in Lake Medard. Due to the inability of pigment analysis to resolve Chlorophyta and Conjugatophyta, the percentual contribution of Conjugatophyta was included in the Chlorophyta group (Fig. 4). Groups Rhodophyta (~ 3%) and Dinophyta (~ 6%) were microscopically observed exclusively in Lake Milada. Pigment analysis showed different results for these two groups. Rhodophyta were not defined as a single CHEMTAX group because of the very low abundance. Peridinin, reflecting the group Dinophyta, was present in all lakes, however, yielding only very low Dinophyta contribution 1%. Additionally, in Lake Medard, a relatively high contribution of Xanthophyta (33%) was identified by pigment analysis, however not observed by microscopy.

**Table 4** Correlation between taxonomical determination by microscopy and HPLC/CHEMTAX calculated by non-parametric correlation matrix, values in bold indicate statistically significant results ($P < 0.05$, $n = 12$)

| Group | Microscopy vs HPLC/CHEMTAX | | |
|---|---|---|---|
| | $R$ | $R^2$ | $P$ |
| Chlorophyta | 0.92 | 0.846 | **< 0.001** |
| Diatoms | 0.82 | 0.672 | **0.001** |
| Cyanobacteria | 0.36 | 0.130 | 0.245 |

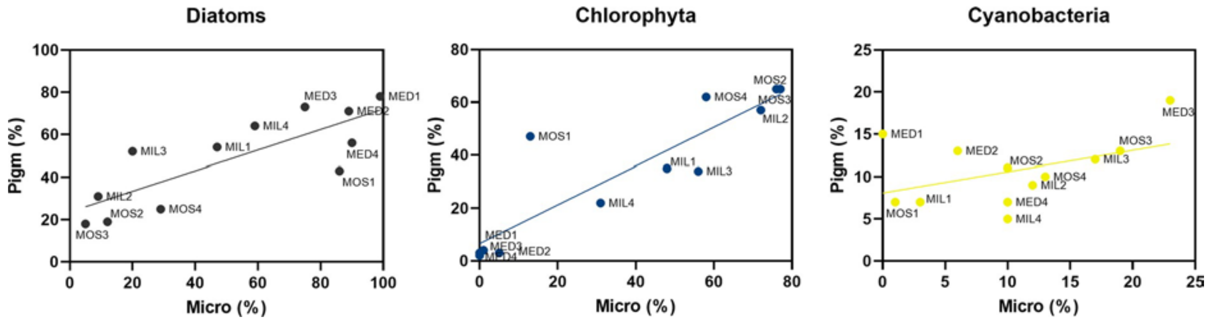

**Fig. 5** Correlation of chemotaxonomy and microscopy. Note that for method comparison, groups Chlorophyta and Cojugatophyta cannot be resolved by chemotaxonomy, therefore, they were also merged in microscopy. Diatoms and Chlorophyta shows relatively good congruence of methods ($R^2$ given by non-parametric correlation matrix were 0.672 and 0.846 for diatoms and Chlorophyta, respectively), results of Cyanobacteria were significantly different and showed poor correlation ($R^2$ = 0.130), *MED* Medard, *MOS* Most, *MIL* Milada, 1, spring, 2, summer, 3, autumn, 4, winter

### Relation between limnological and periphyton parameters

All three lakes differed significantly concerning the limnological parameters ($R^2$ = 0.875, $P$ = 0.001, Table 5). Differences among the seasons were also significant, nevertheless, seasons explained only a small part of variability ($R^2$ = 0.106, $P$ = 0.001, Table 5). The overall TSI calculated as the average of TSI based on Chl-a from four seasons measured was 28, 36 and 44 for Medard, Most and Milada, respectively (Supplementary Table 5, Supplementary Fig. 4). The average values of limnological parameters are summarised in Supplementary Table 4, the relation of the individual limnological parameters to the seasons and lakes is shown in Supplementary Fig. 2. Similar pattern concerning the lakes and seasons was found based on periphyton parameters (quantity, quality and taxonomical composition). Most of the variability was again explained by the lake ($R^2$ = 0.383, $P$ = 0.020 for periphyton quality and quantity and $R^2$ = 0.607, $P$ = 0.001 and $R^2$ = 0.778, $P$ = 0.001 for taxonomical composition determined by microscopy and chemotaxonomy, respectively). Less variability was explained by season, and seasonal differences were mostly not significant (Table 5, Supplementary Fig. 3).

Several correlations were found between individual limnological and periphyton parameters (Table 6, Supplementary Table 6). Overall periphyton biomass was positively correlated with pH and DOC and negatively with DN (Table 6). Considering the correlation of limnological parameters with taxonomical composition of periphyton, differences in the results of the two taxonomical methods (microscopy and chemotaxonomy), make evaluation of results difficult. Both taxonomical methods, however, show significant positive correlation of diatoms and negative correlation

**Table 5** Statistical differences between lakes and seasons based on Permanova analysis of limnological and periphyton parameters

| | Limnological parameters | | | Periphyton quality + quantity | | | Taxonomical composition | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | MICRO | | | PIGM | | |
| | $R^2$ | $F$ | $P$ | $R^2$ | $F$ | $P$ | $R^2$ | $F$ | $P$ | $R^2$ | $F$ | $P$ |
| Lake | 0.875 | 143.11 | **0.001** | 0.383 | 4.63 | **0.020** | 0.607 | 41.19 | **0.001** | 0.778 | 18.49 | **0.001** |
| Season | 0.106 | 11.61 | **0.001** | 0.369 | 2.97 | 0.077 | 0.349 | 15.79 | **0.001** | 0.096 | 1.52 | 0.276 |
| Residual | 0.018 | | | 0.248 | | | 0.044 | | | 0.126 | | |

Values in bold indicate statistically significant results ($P < 0.05$)

*Micro* taxonomical composition determined by microscopy, *Pigm* taxonomical composition determined by pigment analysis

**Table 6** Significant results of non-parametric correlation matrix between limnological variables and measured periphyton parameters

| Quantity and quality | Si (mg $l^{-1}$) | $Mg^{2+}$ (mg $l^{-1}$) | DN (mg $l^{-1}$) | $SO_4^{2-}$ (mg $l^{-1}$) | DOC (mg $l^{-1}$) | $Ca^{2+}$ (mg $l^{-1}$) | Cond (µS $cm^{-1}$) | pH |
|---|---|---|---|---|---|---|---|---|
| Biomass (mg OM $cm^{-2}$) | − 0.63 | − 0.45 | **− 0.87** | − 0.22 | **0.86** | − 0.64 | − 0.36 | **0.86** |
| Taxonomical composition | | | | | | | | |
| Pigm_Diatoms | **0.87** | **0.89** | 0.59 | **0.83** | − 0.49 | 0.46 | 0.78 | − 0.44 |
| Pigm_Chlorophyta | **− 0.91** | **− 0.95** | − 0.64 | **− 0.83** | 0.55 | − 0.45 | **− 0.86** | 0.31 |
| Micro_Diatoms | **0.92** | 0.75 | **0.84** | 0.56 | − 0.76 | 0.63 | 0.62 | − 0.62 |
| Micro_Chlorophyta | **− 0.91** | **− 0.85** | − 0.71 | − 0.68 | 0.68 | − 0.54 | − 0.69 | 0.45 |
| Micro_Conjugatophyta | − 0.63 | − 0.63 | **− 0.84** | − 0.54 | **0.84** | **− 0.83** | − 0.48 | 0.51 |

*R* values are displayed and only parameters with at least one significant correlation are shown ($P < 0.001$, in bold). For complete data, see Supplementary Table 6. Pigm—algal group contribution according to the Chemtax. Micro—algal group taxonomic contribution according to the microscopy

*DN* dissolved nitrogen; *DOC* dissolved organic carbon; *Cond* conductivity

of Chlorophyta with Si ($P = 0.0003$ for diatoms and $P \leq 0.0005$ for Chlorophyta, Table 6). Chlorophyta were additionally negatively correlated with $Mg^{2+}$ ($P = 0.0005$, Table 6). Several other correlations were found for one or another taxonomical method and are summarised in Table 6. No correlation of periphyton quantitative and qualitative parameters (overall biomass, elemental composition) with its taxonomical composition (which would be supported by both taxonomical methods) was found (Supplementary Table 7).

## Discussion

### Periphyton quantity: vertical biomass profile

Periphyton biomass varied from 0.7 to 7.4 mg OM $cm^{-2}$ and reached up to 2 m depth. Unequal distribution of periphyton biomass was recorded within the vertical profile from shoreline up to 2 m depths, however, no light limitation in the depths 0–2.5 m is expected in any studied lake and season since the water transparency remained high throughout the year ($Z_{eu}$ ~ 13 m, Supplementary Table 4). The decrease of periphyton biomass in the depths below 2 m was rather associated with increasing presence of submerged macrophyte *Myriophyllum* sp., macroalgae *Vaucheria* sp. and stonewort *Chara* spp. (our observation, Vejříková et al., 2022). The lowest overall biomass (biomass across all depths) was recorded for the Lake Medard, which was identified as the most oligotrophic (TSI = 28), and it's amount increased with increasing lake trophy, reaching its highest values in Lake Milada, identified as mesotrophic (TSI = 44). Similar biomass values to the ones from our study were recorded for oligotrophic Lake Tahoe (~ 2 mg OM $cm^{-2}$, Atkins et al., 2021).

Considering the seasonal changes, biomass values significantly increased from spring to autumn and decreased from autumn to winter in all lakes. Those changes were likely linked to the seasonal weather dynamics in the temperate zone. Interesting results was that substantial amount of biomass was able to overwinter in an active state (based on autotrophic index, AI, Weber 1973; APHA, 1985; Lowe & Pan, 1996). Especially significant overwintering biomass was measured in Lake Medard and Milada, where it formed on average 66% (Lake Medard) and 47% (Lake Milada) of maximal biomass recorded in autumn. In Lake Most, overwintering biomass formed only 17% of its autumn maximum. Therefore, the colonization process does not need to start "*de novo*" every year. Instead, the initial conditions differ from year to year, considering especially the instant stage of the total lake's succession and particular seasonal characteristics. This probably explains the differences in total biomass of three studied lakes, with the lowest values in the youngest Lake Medard and highest in the oldest Lake Milada. However trophic state index shares the same pattern as lake age (Lake Medard > Lake Most > Lake Milada), and therefore we cannot identify which of those factors is the determining one.

Three significant correlations were identified between the single limnological parameters and periphyton biomass: positive correlation with DOC and pH and negative with DN. DOC (2.97–8.62 mg $l^{-1}$) is likely reflecting the trophic state of lakes. Correlation between pH and periphyton biomass could suggest that changes in pH are affecting the availability of nutrients, similarly, as described in soil (Ferrarezi et al., 2022) and ultimately impacting periphyton growth. For example, DN, which was negatively correlated with periphyton biomass, might have been less available in lower pH occurring in Lake Medard compared to the other two lakes. Xiang et al. (2009) found reduction in soil N availability as a result of a increase in soil pH. To our knowledge no study describes changes in nutrient availability connected with pH for periphyton. pH is also known to have a major influence on bacterial community composition in freshwater systems (Lindstrom et al., 2005; Llirós et al., 2014). As periphyton is a consortium of autotrophs and heterotrophs, pH effect might be directing the bacterial part of periphyton. Nonetheless, as we have no data on heterotrophic part of periphyton, further research would be needed to support this hypothesis. However, as the reported pH values (8–9) were common for lakes worldwide (Michelutti et al., 2002; Liess et al., 2009; Keshri et al., 2017), the effect of pH might be also indirect.

## Periphyton quality

Concerning the nutrient limitations, two optimal molar C:N:P ratios for periphyton, from which nutrient deficiency in the periphyton can be deducted, can be found in the available literature (119:17:1 according to Hillebrand & Sommer, 1999 and 158:18:1 according to Kahlert, 1998). Applying both optimal ratios, persisting P + N deficiency was indicated (Supplementary Table 2, Supplementary Fig. 1) in all lakes and seasons. However, studied lakes differed in the degree of limitation. The lowest deficiency was recorded for Lake Medard, even though the lake was identified as the most oligotrophic. Also, Liess et al. (2009) investigating periphyton of oligotrophic lakes in Sweden found that the lakes with lower SRP showed as the ones with lower C:P molar ratios and so the lowest P deficiency in the biomass, compared to the lakes with higher SRP. Both C:P and C:N molar ratios in Lake Medard were lowest in the spring, suggesting that Medard's periphyton might use the released nutrients from the degraded macrophytes (e.g., *Myriophyllum* sp. biomass was completely decomposed during autumn and winter season, our observation) and the upwelling nutrients during winter mixing. The assumption was supported by the increased lake water SRP and DN recorded from autumn to winter (from < 0.5 to 3 µg $l^{-1}$ and 1.2 to 1.3 mg $l^{-1}$, respectively). Macrophytes and their seasonal biomass degradation has been previously identified as important sources of phosphorus and nitrogen contributing to the nutrient cycling in the littoral zone of lakes (Pieczyńska, 1993; Asaeda et al., 2000).

In Lake Most, both 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 (Supplementary Table 2, Supplementary Fig. 1). Similar trend of the two periods was also observed in Lake Milada. The extreme summer depletion of available N + P in both Milada and Most Lakes was likely linked to the periphyton growth from spring to summer and also by consequent formation of the diffusion barrier (i.e., limited surface for nutrients entering the biofilm, Riber & Wetzel, 1987), which make nutrient acquisition from the lake water less effective. As no correlation between molar C:N:P ratios and lake water SRP and DN found by our study, we assume that two main processes play a role when considering the nutrient depletion/availability for periphyton growth: (a) internal recycling and (b) nutrient uptake from the water column (Fong et al., 1994; Sickman et al., 2009; Konopáčová et al., 2021). We further assume that the proportional contribution of these two processes change within the season. The nutrient acquisition is prominent in the late winter and spring and is exchanged by an internal recycling in the later season, when external recourses of nutrients are exhausted, and the diffusion barrier effect is higher. This hypothesis is supported by findings in Konopáčová et al. (2021) where decreasing specific P uptake affinity of periphyton in a season was found. In Lakes Most and Milada, nutrient acquisition was the most effective in spring yielding periphyton growth from spring to autumn. Nutrient resources (both external and internal) were however not able to cover requirements of newly developed biomass. This brought periphyton in the high nutrient deficiency manifesting as high C:N and C:P molar ratios followed by an increase in autotrophic index, AI in autumn likely caused by an accumulation of detritus in the biomass (Weber, 1973; Lowe & Pan, 1996). We assume same principles play a role in Lake Medard, however the extreme P scarcity (SRP near to zero) connected with lake trophy or/and younger lake age prevent substantial periphyton growth in Lake Medard. Consequently, also the diffusion barrier effect is less prominent. Therefore, rather mild periphyton seasonal dynamics compared to Lakes Most and Milada can be observed in Lake Medard.

To consider a possible effect of the different taxonomical composition of periphyton, we tested the correlation of taxonomical composition and C:N:P molar ratios. No significant effect of taxonomical composition on C:N:P molar ratios was revealed (Supplementary Table 7). Also, no correlation was identified for lake water Chl-a, commonly used as a proxy for phytoplankton biomass and periphyton parameters (Supplementary Table 6), therefore we cannot support idea of direct competition of periphyton with phytoplankton about the light and nutrients (Jäger & Diehl, 2014; Rodríguez & Pizarro, 2015).

### Periphyton taxonomical composition

Supported by both taxonomical methods (microscopy and chemotaxonomy), diatoms, Chlorophyta and Cyanobacteria were identified as major contributors to the autotrophic part of the periphyton (Fig. 4). Whereas results for diatoms and Chlorophyta were in agreement, results for other taxonomical groups (i.e., Cyanobacteria, Dinophyta, Xanthophyta) showed significant differences. A similar discrepancy between periphyton pigment and microscopical taxonomical determination as in our study was described by Lauridsen et al. (2011) and even stronger divergence between the methods was stated by Havens et al. (1999). Recorded differences might have been caused by the low accuracy of one of the tested methods, which is according to our hypothesis caused by following conditions or their combination: (a) Pico-sized algae are not adequately recognised by traditional microscopy but are included by pigment analysis (Mackey et al., 1996). Also, fragile algae taxa are mechanically destroyed and no longer observable. (b) High heterogeneity of samples (e.g., smaller coccal algae are outgrown by large filaments) and detritus further complicate its precise microscopic evaluation. (c) Incorrect results of chemotaxonomy might be obtained by setting inaccurate initial pigment ratios to Chl-a (Higgins et al., 2011; Simmons et al., 2016). Ratios for freshwater periphyton are lacking, for that reason, phytoplankton ratios are being applied, with the majority ratios obtained from cultures (Lauridsen et al., 2011; Dalton et al., 2015). Since phytoplankton ratios might differ from the ratios of periphytic algae, inaccurate results might be obtained. Setting initial pigment ratios for benthic algae is particularly needed before we can apply the method for routine evaluation of periphyton. To our knowledge, no ratios for chemotaxonomy exist for freshwater periphyton taxonomical units so far.

When we focus on the lakes comparison, in the youngest Lake Medard, the autotrophic part of the periphyton consisted mainly of diatoms over the whole sampling season. In Lake Most, taxonomical composition differed according to the season. In spring diatoms were identified as the dominant autotrophic group, replaced by Chlorophyta in the rest of the sampling season. In the oldest Lake Milada, the dominant autotrophic groups of periphyton were diatoms and Chlorophyta. Contribution of Cyanobacteria didn't exceed 25% in any of lakes studied. The situation that the strongly oligotrophic lake harbours diatoms and with an increasing trophy of lake diatoms are replacement by Chlorophyta is in agreement with experiments by Iannino et al. (2020), which found the replacement of diatoms by Chlorophyta and Cyanobacteria when P concentration increased. The contribution of Cyanobacteria was similar for all studied lakes regardless of their trophic state. Current models of lake eutrophication based on pelagic indicators do not take into account littoral greening and benthic mats formation (Vadeboncoeur et al., 2021). The benthic algal blooms (formed often by filamentous Chlorophyta and Cyanobacteria) might also occur in lakes identified as oligotrophic (Vadeboncoeur et al., 2021). Therefore, novel monitoring approaches are required to describe the prominent benthic mats formation.

Several correlations of periphyton taxonomical composition with limnological parameters were observed (Table 6). However, differences in the significant correlations gained for the two tested taxonomical methods (microscopy and chemotaxonomy) make evaluating results difficult. Three clear correlations were significant by both methods: positive effect of Si on the contribution of diatoms (silica cell walls) and negative on contribution of Chlorophyta and the negative effect of $Mg^{2+}$ on the contribution of Chlorophyta. The strong effect of Si suggests silica limitation of diatom growth in the studied lakes even though the open water silica concentrations were relatively high (0.35–2.85 mg $l^{-1}$, Martin-Jezequel et al., 2000). Considering $Mg^{2+}$, which concentrations in studied lakes varied from 26.0 to 55.7 mg $l^{-1}$, magnesium sulphate ($MgSO_4$) is a common contaminant in mine water (van Dam et al., 2010). Its effect 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 $Mg^{2+}$ is an additional factor along the lake trophic state and age, affecting the developed of periphyton in the studied lakes. No direct correlation with periphyton biomass may be caused by the complexity of periphyton assemblages. Whereas some periphytic algae might be sensitive to $Mg^{2+}$ some others might be unaffected. To support the theory of $Mg^{2+}$ inhibitory effect on the periphyton growth, laboratory experiments with $Mg^{2+}$ addition/removal would be source of valuable information. In a recent study Bešta et al. (2023) we have identified $Ca^{2+}$ as an important driver of periphyton species diversity. Since in literature, $Ca^{2+}$ is listed as a factor possibly reducing $Mg^{2+}$ toxicity (van Dem et al., 2010; Mooney et al., 2020), novel and interesting questions about the role of $Ca^{2+}$ and $Mg^{2+}$ on periphyton community has been stated.

## Conclusion

Microscopical identification of individual species is commonly used for bioindication purposes (Kitner & Poulíčková, 2003; Schaumburg et al., 2004; Peng et al., 2020). Nevertheless, this method is cost-demanding, time-consuming and often influenced by the workers experience. Therefore, efforts are invested in development of suitable alternatives. Estimation of periphyton biomass, C:N:P content, and composition of taxonomic groups of autotrophs are commonly considered. Yet, responses of these parameters to the shifts in the ecosystem need to be investigated first. The current study brought first knowledge about ecological demands and seasonal dynamics of the periphyton assemblage dominating the littoral zone of newly established post-mining lakes.

In contradiction to our original hypothesis, that the periphyton seasonal dynamics is driven by nutrient availability, we conclude that seasonal changes in periphyton biomass were related to the changes in the seasonal weather dynamics in the temperate zone. As periphyton in studied lakes exist in persistent P + N limitation, we insist that the role of lake water nutrients availability is important. We assume, that importance of nutrient availability might have been masked by the effect of nutrient internal recycling within the periphyton biomass. Only very limited number of literature dealing with internal recycling in periphyton mats can be found, therefore, we encourage further research on this topic.

As we found significant proportion of overwintering viable biomass in the studied lakes, we state that the current periphyton condition is a cumulative result of the periphyton development in the past years. We bring two most significant variables explaining the variability of periphyton parameters between the lakes: trophic state and age of the lake. Further Si and $Mg^{2+}$ concentrations were significantly affecting periphytic taxonomical composition. The combination of mentioned factors might be an important explanatory to the periphyton biomass development, and their specific roles should be further investigated.

The traditional microscopical approach for taxonomical identification was compared with chemotaxonomy and both techniques came to quite similar conclusion concerning the percentual contribution of the most abundant taxonomical groups Chlorophyta and diatoms. However, the results for other taxonomical groups such as Cyanobacteria or Dinophyta, differed significantly. Therefore, we encourage caution when comparing the results gained by these two methods. The results of the study can serve for future monitoring of described post-mining lakes as it offers detailed information about the periphyton community in the current conditions of studied lakes. They further add knowledge to the development of similar lakes in their initial stage.

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**Declarations**

**Conflict of interest** The authors declare that they have no conflict of interest.

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