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Periphyton Developed on Artificial Substrates: Effect of Substrate Type and Incubation Depth[1]

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Abstract—The aim of this study was to assess the effect of substrate type and incubation depth on periphyton that had developed on artificial substrates. Uniform rectangular tiles made out of artificial substrates: glass, ceramic, willow tree and yew tree, were fixed on a floating buoy and deployed at three different depths in a photic zone of the Sava Lake (Belgrade, Serbia). Non-taxonomic attributes in the developed biofilm were estimated week-by-week from the start of the experiment in July, until its end in September 2014. Through assessment of substrate type and depth of incubation effect we concluded that these parameters for the fact influence periphyton development and composition. Glass was preferred by autotrophic component over ceramic and wooden substrates. In general, substrate type effect was diminished by increasing incubation depth. When non-taxonomic parameters are to be used in biomonitoring studies, our results suggest that glass substrate and shallow layer of water column (up to 50 cm) for incubation should be preferred.

Keywords: periphyton, artificial substrates, glass, wood, ceramic, depth, chlorophyll *a*

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INTRODUCTION

Periphyton is a biofilm that forms on both natural and artificial submerged substrates, and it is composed mostly of algae and Cyanobacteria, as well as bacteria, fungi and microinvertebrates (grazers). Its complex structure includes also mucilage and organic detritus, along with an inorganic component originating from different types of particles. Periphyton's sensitivity to environmental conditions makes this complex community suitable for the assessment of the current ecological conditions of the ecosystem [1].

Artificial substrates has been described as a promising tool for more practical and reliable approach in biomonitoring studies based on periphyton [2, 3], but still it's unclear which substrate and what incubation depth should be preferably used. Influence of substrate type and light conditions (dependable on incubation depth) on periphyton characteristics is for a long time evident [4–6]. And although Wetzel [7] strongly suggested that generalizations in periphyton studies are premature and misleading since reciprocal metabolic interactions between attached algae and substrata are neglected, even in contemporary studies different kinds of substrates are employed in monitoring studies, leading to general knowledge. Glass is for the fact the most often used artificial substrate for developing and studying periphyton, but other substrates such as ceramic and plastic are also popular, while wood as a substrate is usually neglected [4, 8]. Still, very few studies comparatively describe substrate influence on periphyton development [8–11], thus this question is still to be debated. Potapova and Charles [12] assessed applicability of different substrates in algae based water quality monitoring, and found that when autecological features of algae are monitored it is appropriate to use various substrates, but when non-autecological attributes are used, periphyton should be collected from single substrate. Although [13] suggested that Non-Taxonomic Periphyton Index (NTPI) can be effectively used as a supplementary tool in water quality assessment (especially because of its advantages in terms of cost efficiency and practical relevance), as far as our knowledge reaching, no studies were preformed to assess applicability of different substrates when non-taxonomic

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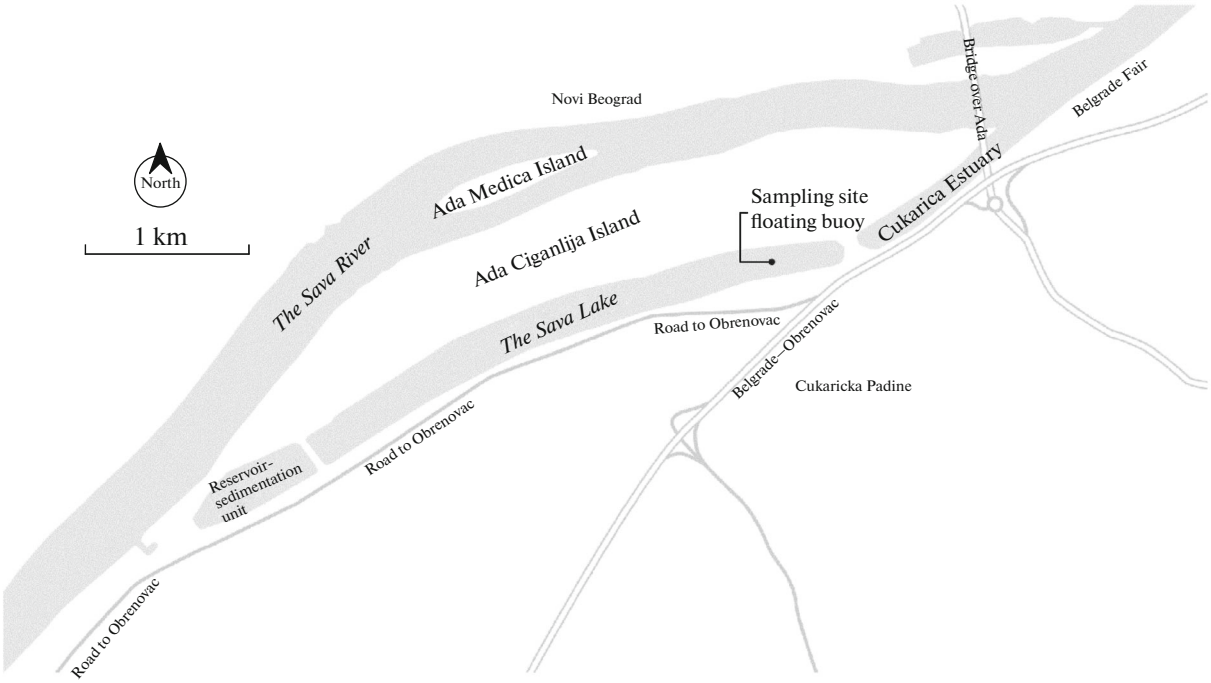

Fig. 1. Map of the Sava Lake showing the position of the lake, the surroundings and the location of the sampling site.

periphyton attributes are to be employed in biomonitoring studies.

In this study periphyton developed on four different types of artificial substrates, deployed on three depths in the limnetic, photic zone of the Sava Lake was investigated in terms of non-taxonomic attributes. The aim of our study was to explore if there is a significant effect of substrate type and incubation depth on periphyton phototrophic component development and the ratio of autotrophic and heterotrophic component of this complex community. We also investigated if the substrate and incubation depth choice will affect potential applicability of non-taxonomic periphyton characteristics in assessment of water quality.

MATERIALS AND METHODS

The Sava Lake (N 44°47′02.28″, E 20°23′25.64″; 73 m a.s.l.) is a reservoir that was formed in 1967 by damming the right branch of the Sava River 4km upstream from its confluence with the Danube, near the center of Belgrade (Fig. 1). The reservoir is about 4.4 km long and about 250 m wide, the average depth is 4.5 m and the maximum recorded depth is 12 m [14].

During the period from 11th July until 9th September, acrylic holders with artificial substrates for periphyton growth were submerged into the Sava Lake, at three depths: 0.5 m (depth 0.5 m), 0.8 m (depth 0.8 m) and 1.4 m (depth 1.4 m). Acrylic holders were specifically constructed for the purpose of the experiment, and during the experiment they were attached to a floating buoy anchored in northeastern part of the lake (Fig. 1). Glass, ceramic, willow and yew tree tiles were used as artificial substrates, all 2.6 × 7.6 cm in dimension, and orientation of tiles in water column was vertical. All tiles were deployed in the start of the experiment, and sampled weekly (altogether 8 sampling weeks). The first two sampling weeks were in July, the third, fourth, fifth and sixth week in August, and the seventh and eighth week in September. Every time, samplings were taken in triplicate for each type of substrate and from each depth.

Water transparency, water temperature and dissolved oxygen/saturation were measured *in situ* using a Secchi disk and a YSI ProODO Optical Dissolved Oxygen Instrument. Water samples for physical and chemical analyses were taken using a Ruttner's bottle, and transported to the laboratory in a mobile freezer. All chemical analyses were performed at the Institute of Public Health of Serbia using standard analytical methods [15].

Artificial substrates with developed periphyton were always collected during the period 9 am to 1pm and transported to the laboratory in separate plastic containers stored in a mobile freezer. In the laboratory the periphyton was scraped from each tile with the aid of a stainless steel razorblade, then suspended in 100 ml of tap water and homogenized with a hand blender; from each tile suspension, subsamples were taken for Chlorophyll *a* (Chl *a*), dry mass (DM) and ash free dry mass (AFDM) analyses.

Replicates were analyzed separately for biomass estimations. The measurements of Chl *a* were performed using the spectrophotometric method accord-

Table 1. The physical and chemical parameters of the Sava Lake

Parameter	Units	Min	Average	Max
Water temperature	°C	24.2	26.3	27.2
Transparency	M	2.6	3.0	3.5
Turbidity	NTU	0.96	1.34	3.17
pH		7.3	7.7	8.2
Dissolved oxygen	mgL^{-1}	9.1	10.3	12.8
Dissolved oxygen saturation	%	82	89	97
Conductivity	$\mu S\ cm^{-1}$	214	222	229
Silicon dioxide	$mg\ L^{-1}$	0.090	0.51	0.70
Ammonia	$mg\ L^{-1}$	0.016	0.046	0.069
Nitrites	$mg\ L^{-1}$	0.001	0.003	0.005
Nitrates	$mg\ L^{-1}$	0.002	0.068	0.292
Orthophosphates	$mg\ L^{-1}$	0.001	0.035	0.165
Total phosphorus	$mg\ L^{-1}$	0.009	0.052	0.198
Biological oxygen demand	$mg\ O_2\ L^{-1}$	1.8	3.1	6.1

ing to ISO 10260 [16], after extraction in warm ethanol. Growth rate of photosynthetic component of periphyton (Chl *a*) was calculated according to the Ahn et al. [17]. The AFDM and the DM were done according to the standard analytical methods [15]. In order to define the composition of the periphyton, the Autotrophic Index (AI) was calculated as a ratio of the AFDM to Chl *a* [15].

To explore the effect of depth and substrate type on autotrophic component biomass (Chl *a*), and the ratio of autotrophic and heterotrophic component of periphyton (AI), Kruskal Wallis non-parametric ANOVA was applied, and then Mann Whitney U test followed to detect between which groups differences are statistically significant. Non-parametric analyses were performed since normality (Shapiro Wilks test) and homogeneity of variance (Levene's test) were not met. Substrate type effect was tested on every incubation depth separately (Mann Whitney U test), and depth effect was tested on every type of substrate separately (Mann–Whitney *U* test). All analyses were performed using the statistical package Statistica 6.0 (Statsoft, Inc., Tulsa, OK, USA) with significance threshold of $p \le 0.05$ for all tests. Results of Mann Whitney test are presented without Bonfferoni corrections for p value, since this correction is highly conservative and increases the risk of Type II errors; additionally, the nature of field experiments implies generally low power to reject the null hypothesis [18].

The principal component analysis (PCA) was performed to illustrate the relationship between biomass growth rate based on Chl *a* from all three depths and all four substrates and measured environmental parameters. For project data, growth rate based on Chl *a*, from all depths and substrates was used as a measure. Water quality data were used as supplementary variables, as well as weeks and months of sampling. The statistical analyses were performed using CANOCO software for Windows, Version 5.0 [19].

RESULTS AND DISCUSSION

In Table 1, the physical and chemical parameters in the Sava Lake during the study period show the average, maximum and minimum values.

When substrate type effect on Chl *a* was considered, Kruskal Wallis ANOVA showed statistically significant differences between substrate types while Mann Whitney U test results at every incubation depth separately, are presented in Fig. 2a. Generally, the highest biomass accumulation at depth 0.5 m was detected on glass slides, and second preferred substrates for colonization of autotrophic organisms were willow tree tiles. It has been previously recorded that glass is the preferable substrate over wood for periphytic algae colonization [8], and our results are in accordance with conclusions of these researchers. Also, wood is proposed to be a valuable source of available forms of nutrients for autotrophic component development, primarily due to the heterotrophic activity in the adhering biofilm [11, 20], thus high values of Chl *a* detected on willow tiles could be the result of those characteristics. At depth 0.5m, biofilms developed on ceramic and yew tiles were very similar, although biofilm from yew tiles had higher concentrations of Chl *a* in earlier phases of colonization, and biofilm from ceramic later. Yew plants (*Taxus* spp.) are in general characterized as poisonous, due to toxic alkaloid taxins that are present in all parts of these plants except of berries (scarlet aril) [21]. Wood dura-

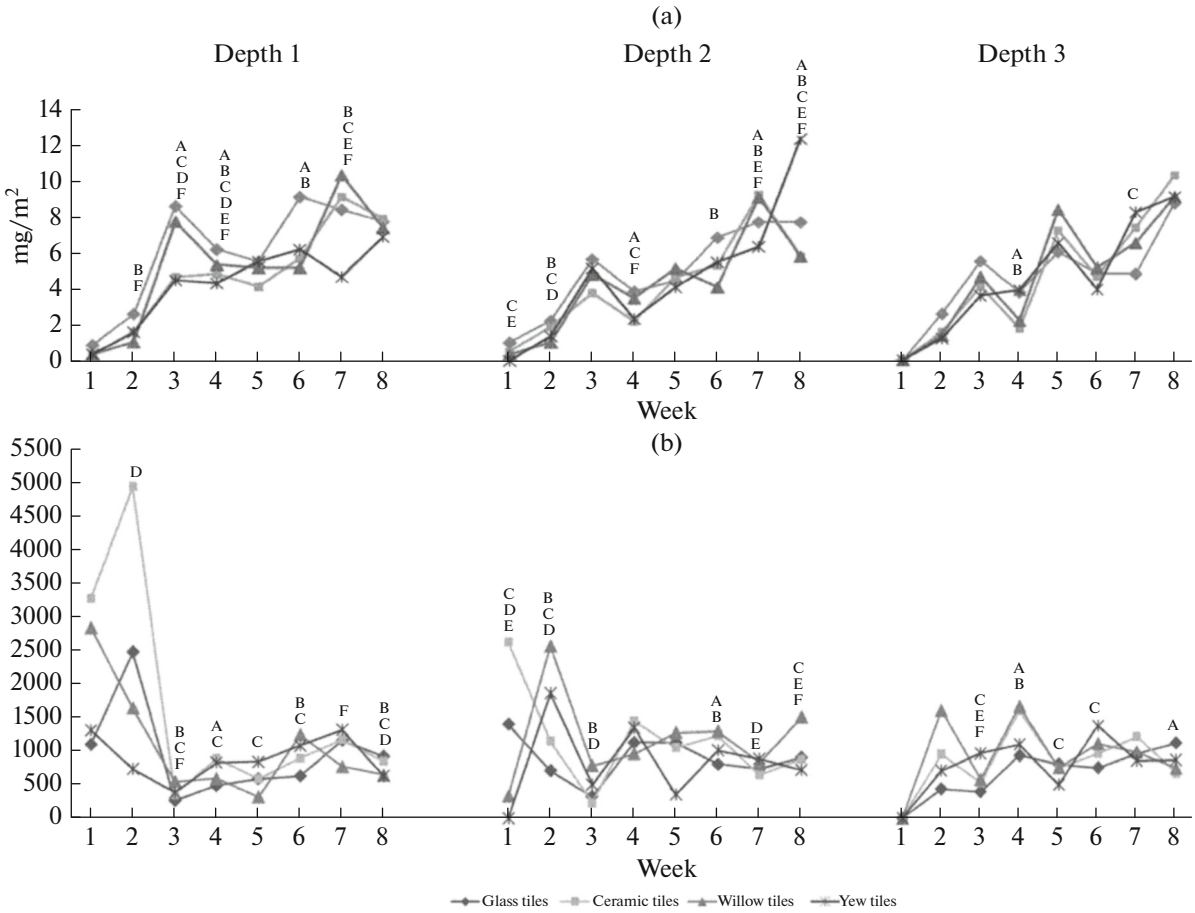

Fig. 2. The effect of substrate type on (a) autotrophic component biomass accumulation (Chl *a*) and (b) the ratio of autotrophic and heterotrophic component of periphyton (AI) at each depth of incubation (depth 0.5 m (depth 1), depth 0.8 m (depth 2) and depth 1.4 m (depth 3)). In each group (sampling week), when significant difference ($P \leq 0.05$) between substrates (glass (g), ceramic (c), willow (w) and yew (y)) according to the Mann Whitney U test was recorded, graphs was marked with A when difference was between g and c, B (g and w), C (g and y), D (c and w), E (c and y) and F (w and y).

bility, meaning also rot and decay resistance is related to ability to produce toxic compounds, which are consequently deposited in heartwood cell wall [22]. In that context, yews are classified as exceptionally high decay resistant, while willows are grouped with slightly or non-resistant woods [22]. When our results are observed, it is clear that in general periphytic algae grew better on willow than on yew tiles, which could be connected to the different decay resistance of these woods. At the depth 0.8 m, situation was similar to the depth 0.5 m, glass was preferred for colonization. At depth 1.4 m, biomass was quite uniform on all substrates, indicating that substrate type effect is impaired by increase of incubation depth.

When substrate type effect on AI was tested, Kruskal Wallis ANOVA also showed statistically significant differences between substrate types and Mann Whitney U test results at every incubation depth separately are presented in Fig. 2b. It is recorded that at all depths of incubation generally lowest AI values were detected on glass substrate. It is interesting to point out that both wooden substrates (willow and yew) were generally preferred by heterotrophic periphyton component (in the most of groups AI was significantly higher on wooden substrates in comparison to glass and ceramic). Zhang et al. [11] reported that wooden substrates are preferable by saprophytic bacteria and fungi decomposing submerged wood, thus our results could be pointed to that specific organisms growing better on wooden substrates making developed biofilm more heterotrophic in comparison to inert (not organic) substrates such as glass and ceramic. Ceramic had higher AI values in comparison to the wooden substrates on the very start of the colonization process and in the end of experiment, while in the middle AI values on ceramic was usually higher than glass and lower than wooden substrates. The least level of differences between AI values on tested substrates was detected at depth 1.4 m, which confirms that depth affects community structure of periphyton just as autotrophic biomass per se.

Influence of incubation depth on Chl *a* was lowest on yew tiles (Fig. 3A d), indicating that depth effect on autotrophic component development on yew tiles is diminished by strong substrate effect. On glass tiles

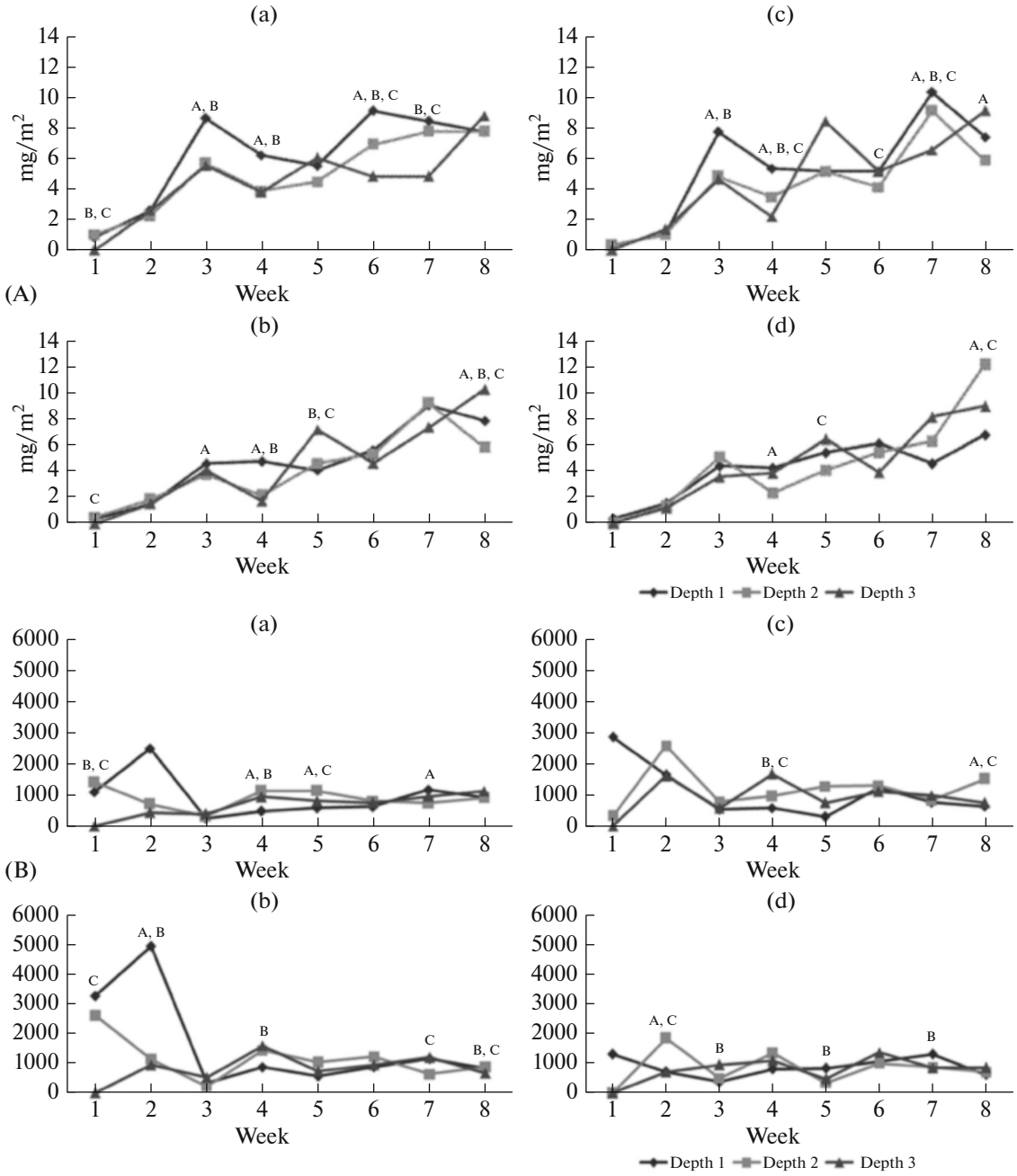

Fig. 3. Depth effect on (A) autotrophic component biomass accumulation (Chl *a*), and (B) ratio of autotrophic and heterotrophic component of periphyton (AI), on every type of substrate: (a) glass tiles, (b) ceramic tiles, (c) willow tiles and (d) yew tiles. In each group (sampling week), when significant difference ($P \leq 0.05$) between incubation depths (depth 0.5 m (depth 1), depth 0.8 m (depth 2) and depth 1.4m (depth 3)) according to the Mann Whitney U test was recorded, graphs was marked with A when difference was between depth 0.5 m and 0.8 m, B when difference was between depth 0.5 m and 1.4 m, and C when difference was between depth 0.8 m and 1.4 m.

(Fig. 3A, a) Chl *a* content was almost constantly the highest at depth 0.5m. On willow tiles (Fig. 3A, c) depth 0.5 m was also favored for colonization of autotrophic component. On ceramic (Fig. 3A, b) tiles Chl *a* values were more variable between incubation depths, but generally highest values of Chl *a* on ceramic were recorded at depth 1.4 m. Thus, it can be concluded that autotrophic component of periphytic biofilm developed on glass and willow substrates was the most sensitive to environmental characteristics associated with depth, among which temperature and light conditions are highlighted [23], even when these factors variations were very slight.

During the first two weeks of sampling, the Autotrophic Index (AI) was very high on each type of substrate (Fig. 3B a, b, c, d) due to the low values of both

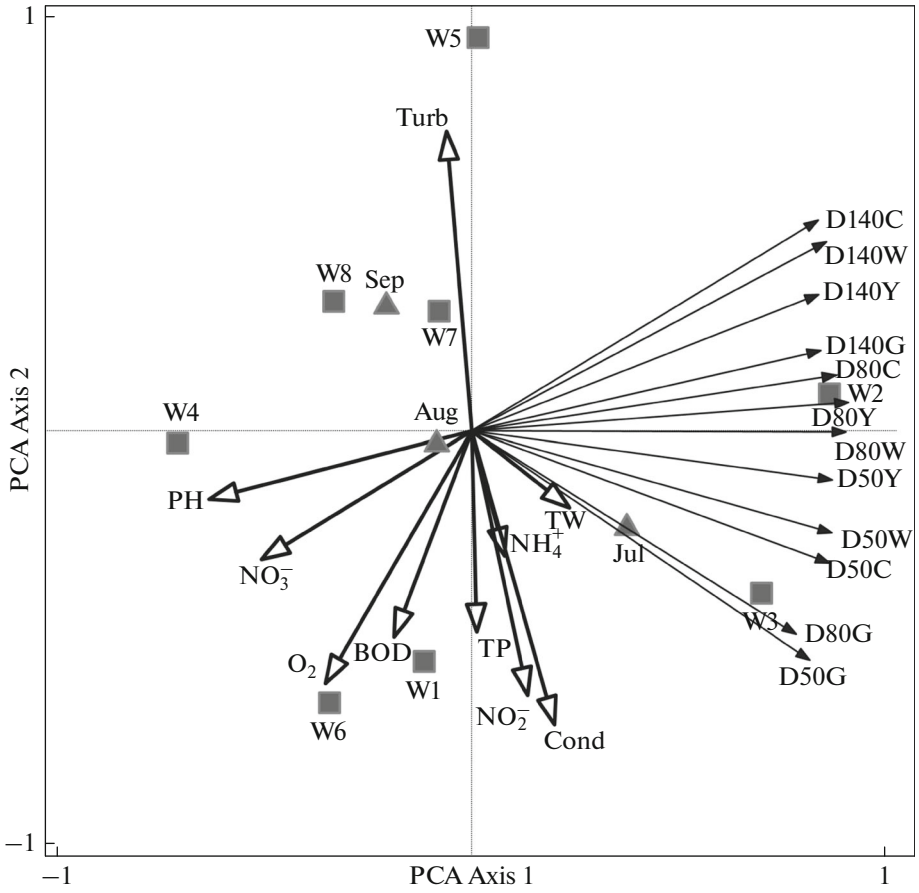

Fig. 4. The PCA biplot ordination on the basis of the biomass growth rate based on Chl *a* and measured environmental parameters included as supplementary variables. Biomass growth rate based on Chl *a* included data from all substrata and all depths: glass (depth 0.5 m—D50G, depth 0.8 m—D80G, depth 1.4 m—D140G), yew (depth 0.5 m—D50Y, depth 0.8 m—D80Y, depth 1.4 m—D140Y), willow (depth 0.5 m—D50W, depth 0.8 m—D80W, depth 1.4 m—D140W) and ceramic tiles (depth 0.5 m—D50C, depth 0.8 m—D80C, depth 1.4 m—D140C). Supplementary variables included physical and chemical water parameters: BOD—Biological oxygen demand, pH—pH value, NH_4^+—Ammonium ion, O_2—Oxygen, Cond—Conductivity, TP—Total phosphorus, Turb—Turbidity, TW—Water temperature, NO_3^-—Nitrates and NO_2^-—Nitrites. Sampling weeks (W1–W8) and sampling months (Jul–Sep) were also included.

the AFDM and Chl *a*, thus these unreasonably high AI values should be considered biased [24]. From the third week on, the AI values at depth 0.5 m were stabilized, with the values 260, 348, 536 and 378 on glass, ceramic, willow and yew tiles respectively, which continued to increase throughout the period indicating that community shifted toward heterotrophy. When period from 3rd week on is considered, autotrophic component had greater share in periphytic biofilm developed on glass slides (Fig. 3B a) closer to the surface than in deeper places, indicating that even slight depth difference is important factor for structuring periphyton on glass tiles. On the other substrates AI was more or less uniform between incubation depths. Generally, on all types of substrates autotrophic component was represented in greater share in earlier period of experiment (until week 6) at depths 0.5m and 0.8m, while at depth 1.4m development of autotrophic component was much slower and relatively higher share was achieved in later period (week 7 and 8) of experiment. Thus, periphytic community became more heterotrophic with depth increase, and this phenomenon was previously confirmed to be affected by the light regime [25]. The glass substrate appeared to be the most sensitive on depth effect when AI (periphyton community composition) is considered. Still according to the AI, periphyton community on all substrate types and at all depths was in general heterotrophic (AI > 400), except in week 3 when values were lower than 400 on all substrates at depth 0.5 m.

Since all substrates were incubated permanently, we found appropriate to demonstrate the relationship between biomass growth rate (considering Chl *a* as a proxy for biomass of autotrophic component of periphyton) and measured water-quality parameters using PCA (Fig. 4). Among all included supplemen-

tary variables, the highest correlation with the first PCA axis (negative one) showed pH (–0.6346) and nitrates (NO_3^-, –0.5083). The highest correlation with the second PCA show: positively – turbidity (0.7225), and negatively – conductivity (–0.7102), nitrites (NO_2^-, –0.6393), total phosphorous (TP, –0.4857) and dissolved oxygen (O_2, –0.6097). Together, the first two PCA axes explain a relatively high portion (84.21%) of the total variation in the data set. Thus, we can say that the first axis is primarily defined by gradients of pH and NO_3^- and second PCA axis with the gradients of nutrients (TP and NO_2^-), conductivity, O_2 and turbidity. Only biomass growth rates at the depth 0.5 m (D50) (from all substrates) and also depth 0.8 m (D80) for glass substrates showed positive correlation to the negative part of the second PCA axis that correspond to TP, ammonium and nitrites gradients. Although differences in periphyton characteristics among substrate types are noticeable in our study (previous sections), it seems that biomass growth rates are under primary control of water column nutrients, when light conditions are favorable and uniform. Our results indicate that depth 0.5 m should be most suitable for incubation and also glass substrates among others, when potential application in biomonitoring studies is assumed.

CONCLUSIONS

Substrate type and incubation depth were pronounced drivers of periphyton biomass development and biofilm composition. Glass was preferred by autotrophic component, while wooden substrates were preferred by heterotrophic component. Willow and yew as wooden substrates differed, and when Chl *a* content is considered yew biofilm was more similar to the one developed on ceramic, suggesting that alkaloids deposited in cell walls of this wood indirectly influence decomposition process and make this substrate more inert and more alike to ceramic than the other wooden substrate. Incubation depth had lowest effect, pronouncing substrate as prime factor when Chl *a* content on yew substrate was considered. In general, substrate type effect was diminished by increasing incubation depth.

Our results suggest that when non-taxonomic features are to be used in biomonitoring purposes, periphyton should be developed in shallow layer of water column (up to 50 cm). Although biofilm developed on wooden and ceramic substrates incubated at depth of 50 cm also showed a positive response to nutrient fluctuations (when biomass growth rate is considered), our results suggested that glass is the most inert substrate, allowing biofilm characteristics to be most tightly correlated with water quality parameters (even when incubated in deeper layers up to 80 cm), thus in biomonitoring studies substrate of the first choice should be glass.

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