



Article

Influence of Experimental Eutrophication on Macrozoobenthos in Tufa-Depositing System of Plitvice Lakes National Park, Croatia

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Abstract: The process of tufa deposition created the Plitvice Lakes, a unique freshwater cascade system of 16 lakes separated by tufa barriers. This complex karst hydrosystem reacts very sensitively to even small changes, and eutrophication can directly and indirectly affect tufa formation. With the purpose of determining the influence of nitrogen (N) and phosphorus (P) on periphyton's chlorophyll *a* concentration, tufa deposition, and macrozoobenthos, we used nutrient-diffusing substrates. The in situ experiment combined the effects of seasons, stronger/weaker tufa deposition, and the presence/absence of macrophytes. The season was the dominant factor influencing hydrology, physicochemical factors, tufa deposition, and the effects of eutrophication. Phosphorus was the limiting factor for periphyton developing on artificial substrates, as evidenced by the highest chlorophyll *a* level on P and N+P substrates. Lower tufa deposition supported a higher chlorophyll *a* level, while macrophytes reduced the chlorophyll *a* concentration and tufa deposition, possibly through effects on the flow and via root respiration. The effects of nutrients on tufa deposition were not recorded. P and N+P treatment increased macrozoobenthos abundance only in some seasons. Trophic groups of macrozoobenthos responded to the addition of P and N+P in the form of higher proportions of grazers and detritivores; however, the response of macrozoobenthos was generally weaker than that of autotrophs.

Keywords: chlorophyll *a*; tufa deposition; nitrogen; phosphorus; macrozoobenthos; functional feeding groups



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1. Introduction

In karstic freshwaters, calcite sediments in the form of tufa are defined as freshwater calcareous deposits formed mainly in the late Quaternary as a product of precipitated calcium carbonate at near-ambient temperatures, with remnants of micro- and macrophytes, invertebrates, and bacteria [1]. Rivers depositing calcite can develop into systems with barriers that transform former river valleys into numerous fluvial lakes in the form of a cascading barrage system, as in Spain (Ruidera Lakes Natural Park) or Great Britain (North Derbyshire) [2]. Calcareous lakes have been shown to be very sensitive to nutrient enrichment, as elevated phosphorus concentrations can halt the process of calcite precipitation [3].

Anthropogenic activities near water bodies lead to cultural eutrophication, which causes a faster process of eutrophication in a shorter time compared to naturally occurring changes. The impact of eutrophication can be seen in the increase in algal biomass [4–6], the increase in meso- and hypertrophic species [7], the decrease in characteristic macrophyte species [8], and the decrease in species diversity at all trophic levels [9]. Many organisms are

involved in the tufa deposition process by providing substrates for calcite nucleation [10], but sometimes also by stimulating precipitation through their metabolic activity [11]. Thus, eutrophication can have both a direct effect on tufa deposition and an indirect effect on the so-called tufa-forming communities. Knowledge of these effects is therefore crucial for the preservation of sensitive tufa-forming systems.

The Plitvice Lakes are a unique freshwater lake system in Croatia. Declared a national park in 1949 and included in the UNESCO World Heritage List, the lakes are formed as a cascading barrage system. Based on the long-term precipitation and air temperature data, the area of the Plitvice Lakes has a prevailing perhumid climate. By comparing the standard climate in the period 1961–1990 with 1991–2019, ref. [12] found an increase in air temperature over the years and a decrease in precipitation in summer, which is a possible consequence of climate change. The system of 16 lakes is fed by surface streams and numerous springs, while underground, water exchange also plays an important role, due to limestone bedrock [12]. The fragility of the hydrosystem and the tufa-deposition process could be threatened by the observed decrease in the amount of water flowing through the Plitvice Lakes since the 1950s [13]. Despite the level of protection of the national park, the area is a well-known tourist destination. In addition to the tourist trails that have an effect on the shores of the lakes, there are the risks of accidental pollution and the impact of nearby roads [14]. The possible influence of eutrophication, i.e., the enrichment of the water body with higher phosphorus and nitrogen concentrations, has been noted for the Plitvice Lakes in several previous studies [15,16]. The composition of macrophytes in calcareous lakes has been shown to change with eutrophication from charophytes to angiosperms [8,17]. Researchers pointed out the lack of studies on responses to eutrophication in marl lakes [17]. Changes in vegetation cover due to eutrophication have also been noted in tufa systems, and in the Plitvice Lakes, the characteristic moss cover is being displaced by vascular plants [18]. These changes have the potential to lead to barrier instability and cause the dissolution of calcite via root respiration [19].

Nutrient-diffusing substrates (NDSs), first used in the 1940s [20], provide an acceptable method for exploring the spatial and temporal patterns of limiting nutrient effects. They have typically been used for periphyton studies in freshwater ecosystems [21,22]. NDSs have rarely been used to study the influence on zoobenthos [23,24], also showing that trophic cascades can be strongly influenced by heterogenous biotic factors [25].

Macroinvertebrates are an important component of freshwater ecosystems and fulfil a number of ecological functions. Depending on their taxa, they belong to different feeding groups, making them important components of food webs. Macroinvertebrate food sources are periphyton, coarse and fine particulate organic matter, and animals [26]. Due to their sensitivity to environmental changes and our comprehensive understanding of their ecology, macroinvertebrates are often used as bioindicators, particularly in the assessment of ecological status, as under the EU Water Framework Directive [27]. The barriers of the Plitvice Lakes are dominated by functional groups of collectors, which can be linked to the abundance of organic matter in the bryophytes and the rich seston [28]. By analyzing more broadly defined trophic groups of macroinvertebrates, ref. [29] has shown that there has recently been a trophic-level shift in English rivers.

Periphyton is the main food source for grazers, and the effect of the increased development of the autotrophic component of periphyton due to experimental eutrophication should be seen in a larger proportion of this trophic group. While some studies have shown the positive effects of experimental eutrophication on invertebrate grazers [23,24], others reported its weak effects on heterotrophs, possibly due to the presence of other food sources [22] or other dominant stressors [30].

The objective of this study was to observe in situ the seasonal effects of experimental eutrophication using NDSs at several levels: (a) on calcite deposition, (b) on the productivity of the autotrophic component of the periphyton, and (c) on macrozoobenthos. Given the well-developed macrophyte vegetation on the tufa barriers of the Plitvice Lakes and its potential influence on nutrient dynamics and tufa deposition, the experiment was conducted at sites with and without macrophytes.

2. Materials and Methods

2.1. Study Area

The Plitvice Lakes National Park is located in the central part of the Dinaric Karst (Figure 1). A total of 80% of the park's area is covered with beech–fir forests, while freshwater ecosystems cover about 1% of the area. The system of 16 lakes, divided into upper and lower lakes, has an estimated volume of 22.95 million m³ of water [31]. The lakes are separated by tufa barriers and form a cascade system that descends from 636 m a.s.l. to 503 m a.s.l. The area of the upper lakes is built upon a geological base of Triassic dolomites, while the area of the lower lakes lies on Cretaceous limestones. Several intermittent and perennial streams flow into the lakes and at the end of the lake system, the river Korana begins to flow. The research was carried out with the permission of the Ministry of the Environment and Nature Protection (permission number: UP/I-612-07/14-33/54).

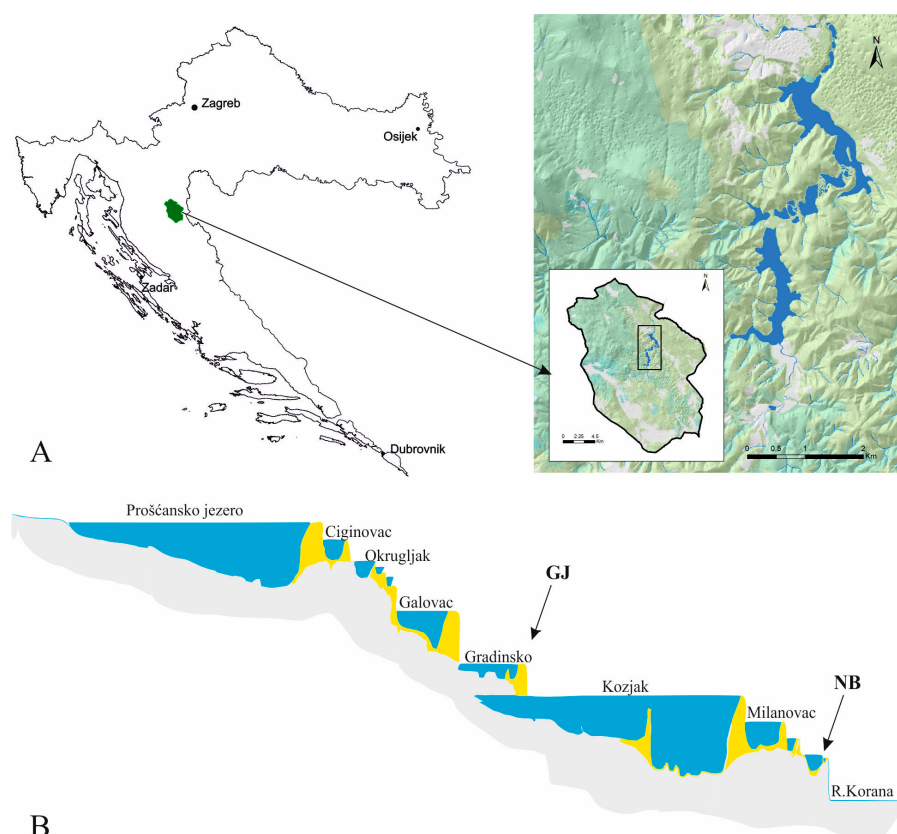


Figure 1. Position of Plitvice Lakes National Park in Croatia, with the section of the park and the freshwater ecosystem of lakes and tributaries (A). Section of the barrage lake system with marked sampling sites: GJ (Gradinsko jezero lake outlet) and NB (Novakovića brod lake outlet) (B).

2.2. Nutrient-Diffusing Substrate Design and Placement

The Plexiglas plates (NDS plates) served as a carrier for fifteen 120 mL plastic containers covered with plastic net (mesh size 3 mm) and secured with a modified lid, resulting in an exposed surface of 4.7 cm² of net (Figure 2). The nets served as a substrate for the colo-

nization of periphyton and macrozoobenthos, partly mimicking the natural tufa-covered moss substrate. Additionally, the nets protected the agar from being disturbed by larger organisms, such as decapod crustaceans and fish. Three containers per plate represented 0-control, 1-control, N treatment, P treatment, and N+P treatment. Empty containers were 0-control, while 1-control containers were filled with 1% agar (Biolife, Milano, Italy) dissolved in distilled water. The N treatment (containing 0.5 M N-NO_3^-) was prepared with NaNO_3 (BDH Prolabo, Leicestershire, UK) in the 1% agar, and the P treatment (containing 0.05 M P-PO_4^{3-}) was prepared with KH_2PO_4 (BDH Prolabo, Leicestershire, UK) in 1% agar. The N+P treatment contained both components at the aforementioned concentrations in 1% agar. The concentrations and design were chosen following previous studies [21,22,25]. The aim was to ensure nutrient enrichment, taking into account the nitrate and phosphate levels in the water of the Plitvice Lakes [32].

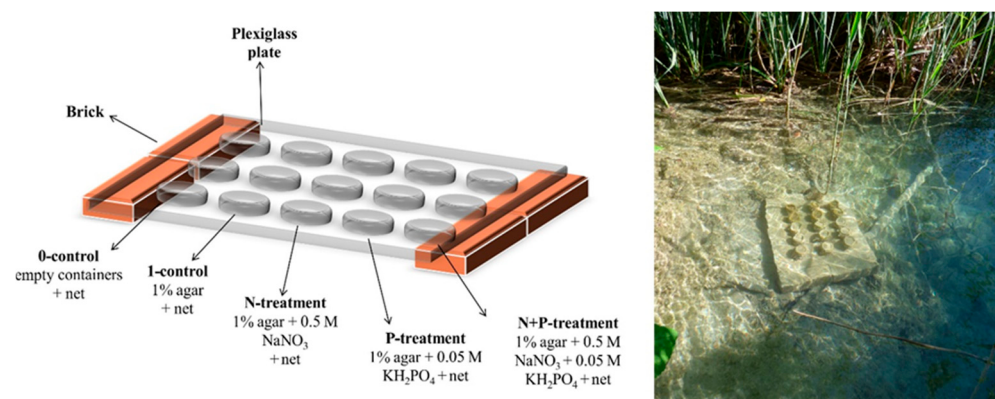


Figure 2. Design of NDS plate with holes for plastic containers (left) and plate in one of the microhabitats of Novakovića brod lake outlet (NB-tufa) in summer 2015 (right).

Plates with nutrient-diffusing substrate containers were placed on tufa barriers of an upper (outlet of Gradinsko jezero lake—GJ) and lower lake (outlet of Novakovića brod lake—NB) in the Plitvice Lakes National Park (Figure 1). Three replicates of NDS plates with 15 containers each (Figure 2) were placed in each of the microhabitats (tufa barriers without macrophytes: GJm– and NBm– and tufa barriers with macrophytes: GJm+ and NBm+), i.e., 6 at each barrier and 12 in total (Appendix A.1). Microhabitats were selected based on the measured water velocity, with the intention that the measured values would be hydrologically similar (water velocity ranged from 12.3 to 20.5 cm s^{-1} at the start of the study in January 2015). Microhabitats with macrovegetation included willows (*Salix* spp.), sawgrass (*Cladium mariscus*), butterbur (*Petasites* spp.), and reeds (*Phragmites* sp.).

The field research was conducted from February to November 2015 and included all four seasons. The NDS plates were placed in the water in four microhabitats each season, i.e., a newly prepared set of NDS plates was placed for each season and left for six weeks before collection. After removal, a simple diffusion test was conducted to determine if the NDSs were still releasing nutrients. In general, the concentrations of nutrients released had no impact on the environment, and with regard to the lotic biotope system of the tufa barriers and their hydrological conditions, this type of in situ research was localized and did not represent a significant nutrient impact.

2.3. Measurement of Physicochemical Water Parameters and Chlorophyll *a* Analysis

The physicochemical water parameters (temperature, pH, electrical conductivity) were measured with a digital field multimeter (WTW, Bremen, Germany) at the beginning of each seasonal series, once in the middle of exposure, and at NDS removal. Dissolved oxygen, total hardness, orthophosphates, and nitrates analyses were performed according to [33], while the

total nitrogen (TN) and total phosphorus (TP) were analyzed using Hach Lange tests (Hach Lange GMBH, Berlin, Germany). Water samples were collected in the microhabitats GJm−, GJm+, NBm−, and NBm+. The water velocity was measured at each of the replicates at the edge of the brick, which was approximately 10 cm from the bottom. Samples (nets and water) were collected in all seasons at the same time of day in clean and pre-labelled containers or bottles. A field form was used to record the field data. All measurements of the physicochemical parameters were performed in triplicate to ensure the accuracy of the results. The instruments used for the chemical analyses were regularly calibrated according to the manuals provided with each instrument. Chlorophyll *a* analyses were performed on the collected nets. For each control or treatment, we analyzed 4 replicates randomly sampled from 3 replicate NDS plates (4 microhabitats × 4 replicates × 5 treatments = 80 samples in each season). The periphyton was analyzed directly from the nets, i.e., each of the harvested nets was transferred to a test tube containing 96% ethanol and further analyzed according to the method of [34]. Each of the harvested nets was transferred to a test tube containing 10 mL of 96% ethanol and heated on the flame for 1 min. We then performed microfiltration through the 0.45 µm filter into test tubes and added 10 mL of 96% ethanol. The spectrophotometric absorbance was measured in two steps: the first at 665 nm and the second at the same wavelength, but with the addition of 1 drop of HCl. The chlorophyll *a* (Chl *a*) concentration was calculated as follows:

$$\text{Chl } a [\mu\text{g}/\text{cm}^2] = 29.6 \times (A_{665} - A_{665+\text{HCl}}) \times V (\text{mL}) / a (\text{cm}^2) \times d (\text{cm}) \quad (1)$$

where *V* = volume of the extracted sample, *a* = net area (17.34 cm²), and *d* = cross-section of the cuvette.

2.4. Macrozoobenthos and Tufa Deposition Analysis

For macrozoobenthos analyses, four replicates of plastic nets were harvested from each control or treatment NDS container and preserved in 70% ethanol (4 microhabitats × 4 replicates × 5 treatments = 80 samples in each season), followed by isolation and the determination of organisms. Isolated organisms were determined to be species-, genus-, or family-level [35–42]. Functional feeding groups were determined according to [43]. The remaining nets were used for calcium carbonate analyses. For this purpose, the nets were dried at 104 °C for 4 h, cooled in a desiccator, and then weighed. The samples were dissolved with 16% HCl, dried again, and weighed after cooling. The concentration of precipitated calcium carbonate was calculated from the difference between the dried material before and after dissolution in HCl.

2.5. Data Analysis

Statistical analyses were performed in Statistica 13.3. Distribution tests (Shapiro–Wilk *W* and Kolmogorov–Smirnov tests) were performed to check whether the data sets followed a normal distribution. When necessary, the data were transformed using square root, fourth root, and logarithmic transformations. These transformations helped to mitigate the effects of outlier values so that they were not excluded from the analyses. Factorial ANOVA was conducted to identify statistically significant differences in the water velocity, physicochemical parameters, chlorophyll *a* concentration, macrozoobenthos abundance, number of taxa, and number of functional feeding groups among locations (barriers), microlocations, seasons, and treatments. We applied the Tukey HSD post hoc test. Nonparametric analyses using the Kruskal–Wallis test were performed when a normal distribution of data was not obtained. Multidimensional scaling (MDS) analyses were used for the two-dimensional representation of macrozoobenthos similarity (performed in PRIMER 6). The data for the analysis were square-root-transformed, and Bray–Curtis similarity was used.

3. Results

3.1. Water Velocity and Physicochemical Parameters

The water velocity was highest in microhabitat GJm−, with the peak value in spring 2015 (46.7 cm s^{-1}), while the lowest values were measured in microhabitat NBm+ in summer 2015 (Figure 3). There were statistically significant differences between seasons (ANOVA, $F_{3,129} = 20.362$, $p < 0.001$), with winter and spring showing statistically higher water velocities than summer and autumn ($p < 0.001$). The microhabitats also showed statistically significant differences in water velocity (ANOVA, $F_{3,129} = 7.148$, $p < 0.001$), with NBm+ having a statistically significantly lower water velocity compared to NBm− and GJm− (Tukey HSD, $p < 0.05$ and $p < 0.001$, respectively). There was no statistically significant difference for the combination of factors season x microhabitat ($F_{9,128} = 1.267$, $p > 0.05$).

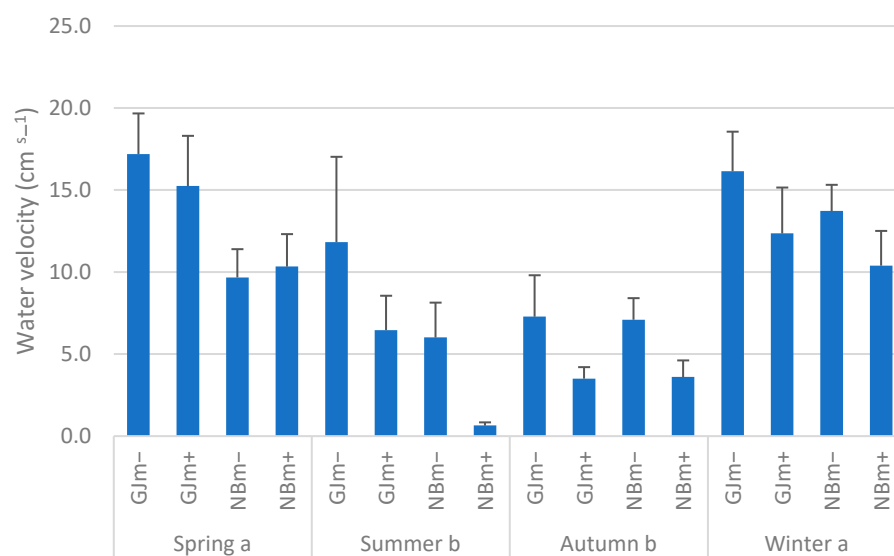


Figure 3. Average water velocity (+SE) above nutrient-diffusing substrata during the in situ experiment in four microhabitats (GJ—Gradinsko jezero lake, NB—Novakovića brod lake, m—without macrophytes, and m+—with macrophytes) in different seasons. Different letters after seasons indicate significant differences according to post hoc Tukey HSD test following factorial ANOVA.

The temperature showed seasonal variations, with elevated values in the summer months (the highest value was 22.9°C), and the seasons differed statistically significantly in temperature (Appendix A.2). The dissolved oxygen levels followed the seasonal changes in temperature, with values decreasing in summer compared to the other seasons. Electrical conductivity also followed seasonal variations, with values lower in summer (approximately $325 \mu\text{S cm}^{-1}$) than in other seasons (approximately $350 \mu\text{S cm}^{-1}$). The GJ sites had higher conductivity than the NB sites. The pH values were elevated at barrier NB compared to barrier GJ. The concentrations of total hardness in water were lower in the summer months than in autumn and winter. The nitrate concentrations were lowest at GJm+, and seasonal changes were observed, with a decreasing trend towards the warmer season and an increase from September onwards. Concentrations of orthophosphates were generally low, with higher values measured in late summer than in the other months of the year. There were no significant differences in either physicochemical parameter between the microhabitats.

3.2. Tufa Deposition and Chlorophyll a on Nutrient-Diffusing Substrata

Tufa deposition, expressed as calcium carbonate deposited on plastic nets of NDSs, was statistically significantly higher during spring than in other seasons (ANOVA, $F_{3,74} = 10.711$, $p < 0.001$) (Figure 4). There were significant differences between microhabitats ($F_{1,74} = 4.5$

$p < 0.05$), with NBm— having statistically significantly higher values of deposited tufa compared to all other microhabitats (post hoc Tukey HSD test, $p < 0.05$), and NBm+ differing from GJm+ ($p < 0.01$). Nutrients from NDSs had no statistically significant effect on tufa deposition.

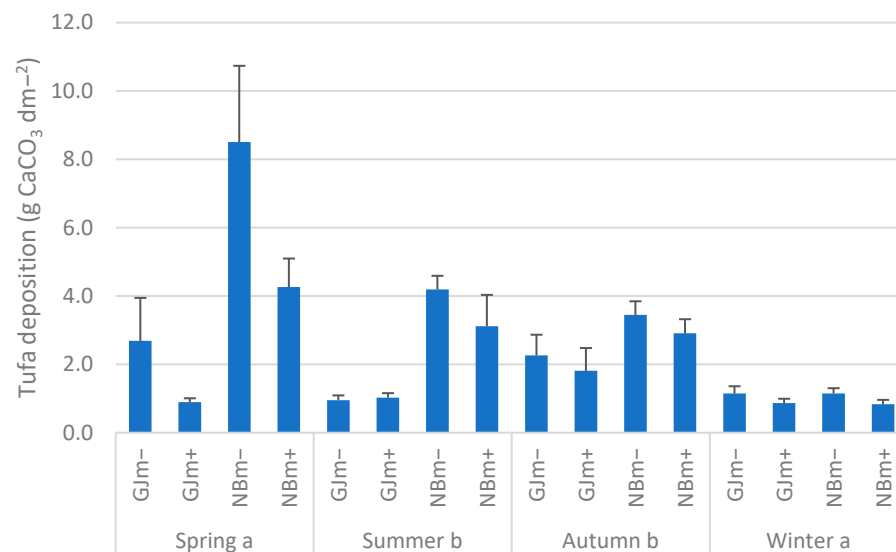


Figure 4. Average tufa deposition (+SE) above nutrient-diffusing substrata during the in situ experiment in four microhabitats (GJ—Gradinsko jezero lake, NB—Novakovića brod lake, m—without macrophytes, and m+—with macrophytes) in different seasons. Different letters after seasons indicate significant differences according to post hoc Tukey HSD test following factorial ANOVA.

Statistically significant differences were observed in chlorophyll *a* concentrations between microhabitats, seasons, and treatments, with seasons and treatments showing the strongest effect. The combinations of factors were also statistically significant, with the exception of microhabitats and treatments and the combination of all three factors (Table 1). The highest chlorophyll *a* concentrations were measured in winter, with an average value of $6.0 \mu\text{g cm}^{-2}$, followed by summer and autumn, with about $3.5 \mu\text{g cm}^{-2}$, while the lowest average value was measured in spring ($2.5 \mu\text{g cm}^{-2}$). Winter was statistically significantly different from all other seasons in chlorophyll *a* concentration (post hoc Tukey HSD, $p < 0.001$), and spring was different from summer ($p < 0.05$). A higher chlorophyll *a* concentration was observed at the GJ barrier (microhabitat GJm— had the highest values) than at the NB barrier, while at both barriers, the microhabitats without macrovegetation had up to two times higher values than those with macrovegetation, with the exception of the autumn series (Figure 5).

Table 1. Three-way ANOVA results for chlorophyll *a* concentration on nutrient-diffusing substrata after six weeks of exposure in different seasons and microhabitats in Plitvice Lakes National Park. Bold *p*-values indicate significant differences.

Effect	d.f.	F	<i>p</i>
Season	3, 204	51.21	0.00000
Microhabitat	3, 204	8.80	0.00002
Treatment	4, 204	36.23	0.00000
Season*Microhabitat	9, 204	5.93	0.00000
Season*Treatment	12, 204	1.86	0.04010
Microhabitat*Treatment	12, 204	1.36	0.18855
Season*Microhabitat*Treatment	36, 204	1.19	0.21977

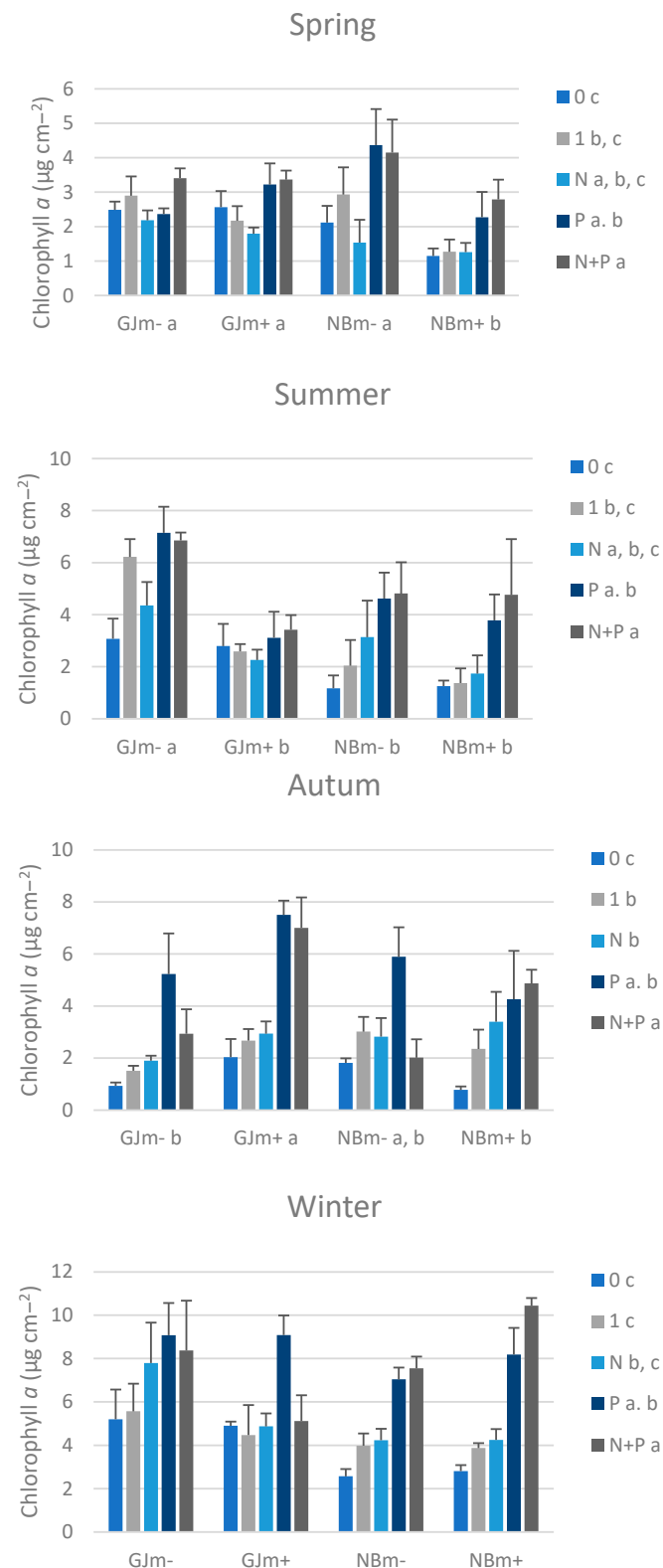


Figure 5. Mean ($\pm$ SE) chlorophyll *a* concentrations on nutrient-diffusing substrata in different seasons in microhabitats in Plitvice Lakes (GJ—Gradinsko jezero lake, NB—Novakovića brod lake, m—without macrophytes, m+—with macrophytes, 0—control—empty containers, 1—control—agar-filled containers, N—containers with 0.5 M N-NO_3^- in agar, P—containers with 0.05 M P-PO_4^{3-} in agar, and N+P—containers with both 0.5 M N-NO_3^- and 0.05 M P-PO_4^{3-} in agar). Different letters after seasons indicate significant differences according to post hoc Tukey HSD test following factorial ANOVA. The y-axes have different scales for the seasons.

The influence of nutrients on the chlorophyll *a* concentration was clearly visible in certain seasons and in certain microhabitats, for example, in autumn and winter (Figure 5). The treatments had a statistically significant effect on all seasons tested separately, with the P and P+N treatments having the highest values, while the controls (0 and 1) had the lowest values, which were often not statistically significantly different from the chlorophyll *a* concentration in the N treatment (Figure 5).

3.3. Macrozoobenthos on Nutrient-Diffusing Substrata

The macrozoobenthos taxa that colonized NDS plastic nets belonged to 13 groups and 83 taxa (Appendix A.3). The most diverse group was the Trichoptera (32 taxa). The abundance of macrozoobenthos was high for Diptera (e.g., 8294 Ind. dm⁻² in GJm+), while the lowest abundance was observed for Turbellaria (23.1 Ind. dm⁻², only in NBm+) and Ostracoda (5.8 Ind. dm⁻², only in NBm-). The highest abundance was found for the chironomid subfamilies Tanytarsini, Tanypodinae, and Chironomini (Appendix A.3).

Statistically significant differences in macrozoobenthos abundance were observed between seasons, microhabitats, and treatments and their combinations, with the same pattern as for chlorophyll *a* (see Tables 1 and 2). The GJ microhabitats mostly showed a statistically higher macrozoobenthos abundance than the NB microhabitats (Figure 6). The highest abundance was observed in spring (210.1 Ind. dm⁻²), and the lowest in winter (32.9 Ind. dm⁻²). All seasons differed from each other according to the post hoc Tukey HSD test ($p < 0.001$ for all combinations and $p < 0.05$ for summer $\neq$ autumn).

Table 2. Three-way ANOVA results for macrozoobenthos abundance on nutrient-diffusing substrata after six weeks of exposure in different seasons and microhabitats in Plitvice Lakes National Park. Bold *p*-values indicate significant differences.

Effect	d.f.	F	<i>p</i>
Season	3, 228	84.47	0.00000
Microhabitat	3, 228	50.70	0.00000
Treatment	4, 228	12.42	0.00000
Season*Microhabitat	9, 228	15.53	0.00000
Season*Treatment	12, 228	2.50	0.00430
Microhabitat*Treatment	12, 228	1.52	0.11845
Season*Microhabitat*Treatment	36, 228	0.87	0.68741

Differences in the average abundance of macrozoobenthos were observed between treatments, with higher values for the N+P and P treatments and the lowest for the 0-control. However, in summer, there was no statistically significant difference between treatments, and in the other seasons, only the 0-control had statistically significantly lower values, and N+P statistically higher compared to the other treatments, probably due to high variance within groups and the influence of other factors.

The functional feeding groups (FFGs) of macroinvertebrates on the NDSs included shredders (SHRs), grazers (GRAs), active filter feeders (AFILs), passive filter feeders (PFILs), detritivore collectors (DETs), predators (PREs), and others (OTHs). The proportion of FFGs was analyzed for all factors together (Figure 7). PFILs and DETs had a higher proportion of GJ microhabitats compared to NB. At both barriers, the microhabitats with macrophytes had a higher proportion of AFILs than microhabitats without macrophytes. When comparing the seasonal proportions of FFGs, winter is characterized by a higher proportion of PFILs compared to other seasons, while autumn has a higher proportion of SHRs and DETs. The proportion of GRAs was slightly higher in spring. A higher

proportion of DETs was observed in the N, P, and N+P treatments compared to the other groups (Figure 7). Statistically significant differences were found between treatments for the proportion of GRAs ($F_{4,297} = 8.6$, $p < 0.001$) and the proportion of DETs ($F_{4,286} = 11.3$, $p < 0.001$), while there were no significant differences between treatments for other groups.

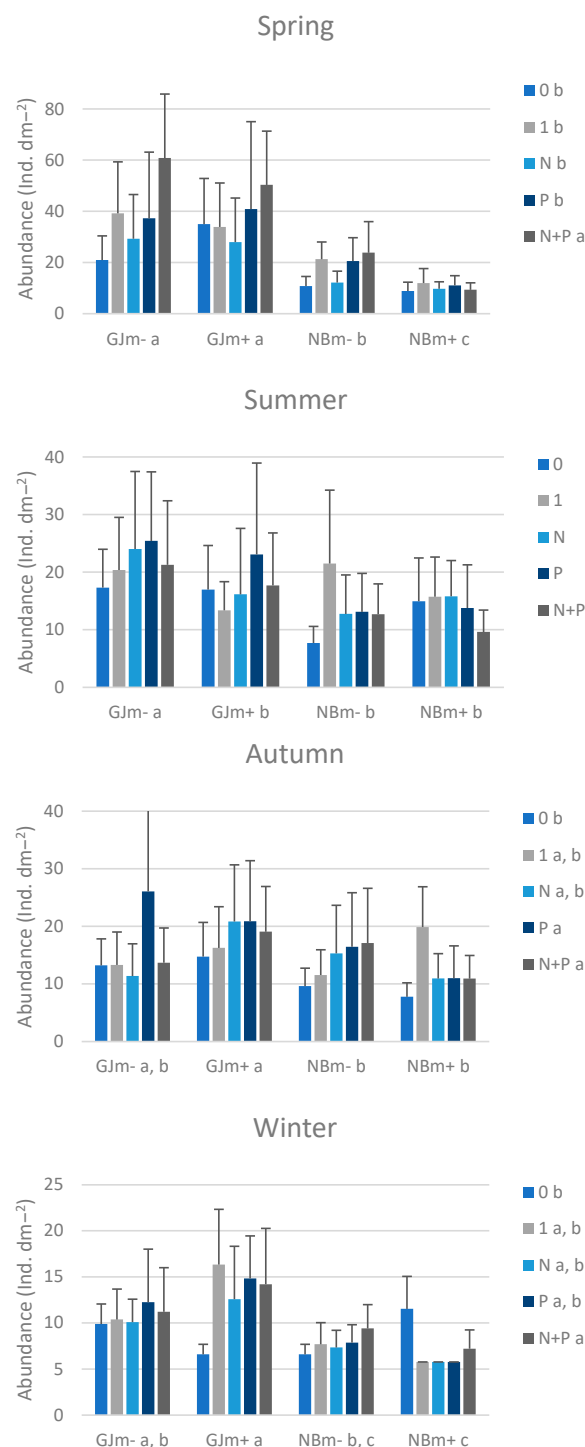


Figure 6. Mean ($\pm$ SE) macrozoobenthos abundance on nutrient-diffusing substrata in different seasons in microhabitats in Plitvice Lakes (GJ—Gradinsko jezero lake, NB—Novakovića brod lake, m—without macrophytes, m+—with macrophytes, 0—control—empty containers, 1—control—agar-filled containers, N—containers with 0.5 M N-NO_3^- in agar, P—containers with 0.05 M P-PO_4^{3-} in agar, and N+P—containers with both 0.5 M N-NO_3^- and 0.05 M P-PO_4^{3-} in agar). Different letters after seasons indicate significant difference according to post hoc Tukey HSD test following factorial ANOVA. The y-axes have different scales for the seasons.

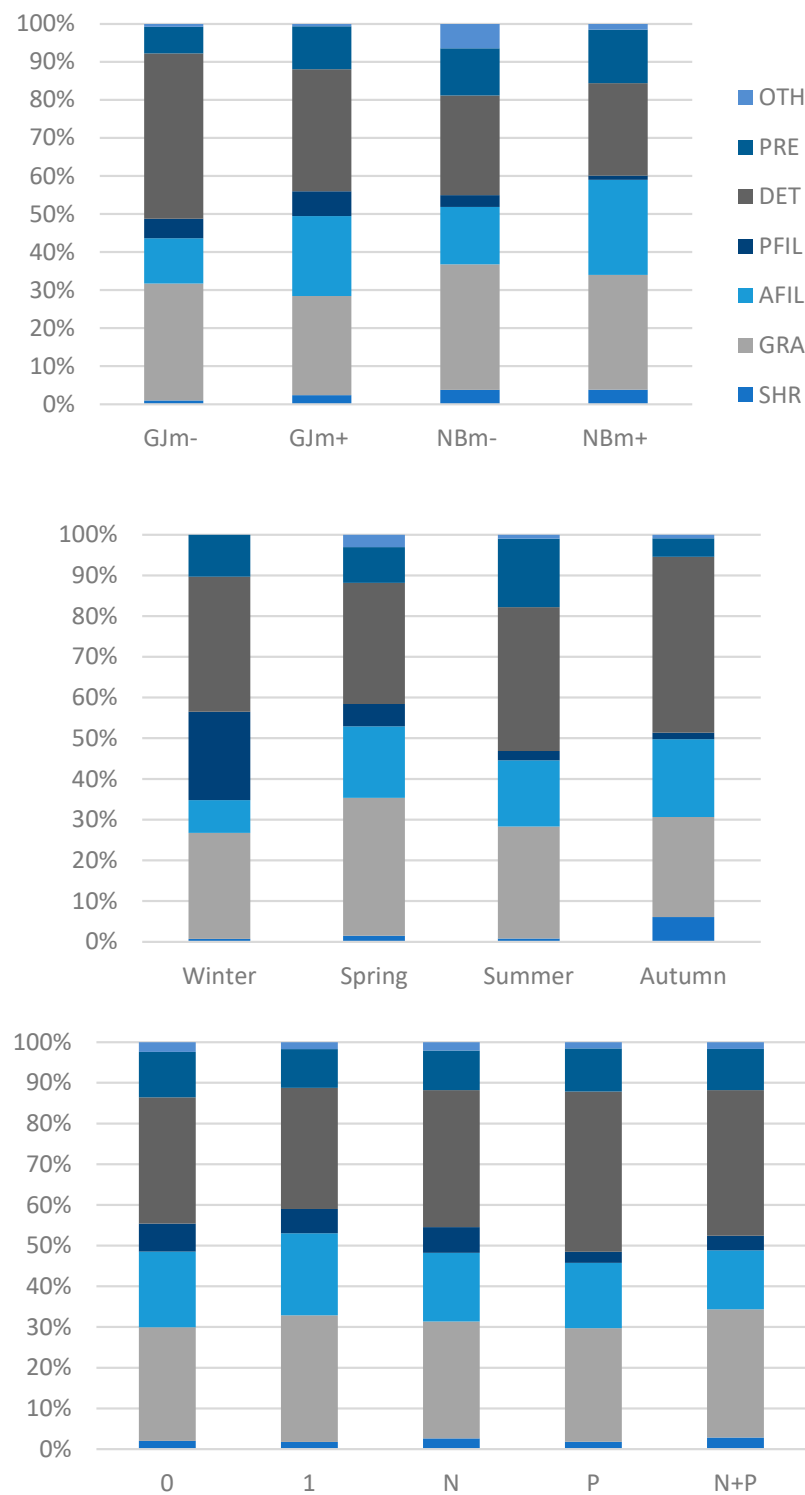


Figure 7. Share of functional feeding groups by microhabitats, seasons, and treatments (GJ—Gradinsko jezero lake, NB—Novakovića brod lake, m—without macrophytes, m+—with macrophytes, SHRs—shredders, GRAs—grazers, AFILs—active filter feeders, PFILs—passive filter feeders, DETs—detritivore collectors, PREs—predators, and OTHs—others).

The multidimensional scaling analysis (MDS) of macrozoobenthos between treatments for all seasons showed a higher homogeneity within the macroinvertebrate assemblages in spring and autumn, while a lower similarity was observed for the winter and summer assemblages (Figure 8). The winter macroinvertebrate assemblage differed the most from the other seasons, possibly due to the lowest abundance.

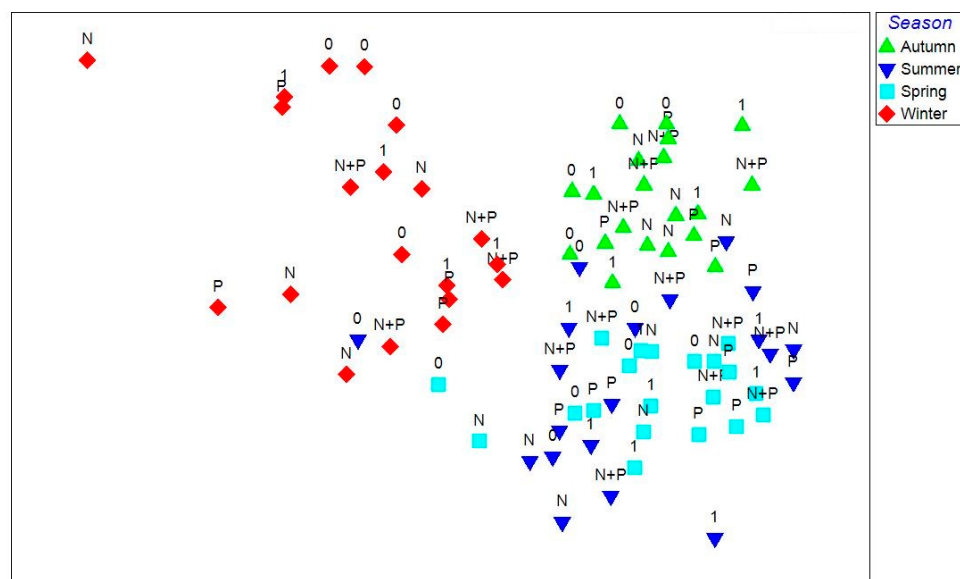


Figure 8. Multidimensional scaling analyses (MDS) of macrozoobenthos in different seasons and treatments (0—control—empty containers, 1—control—agar-filled containers, N—containers with 0.5 M N-NO_3^- in agar, P—containers with 0.05 M P-PO_4^{3-} in agar, and N+P—containers with both 0.5 M N-NO_3^- and 0.05 M P-PO_4^{3-} in agar). The data for the analysis were square-root-transformed, and Bray–Curtis similarity was used; stress value was 0.22.

4. Discussion

Our results show great heterogeneity in the microhabitats of the Plitvice Lakes, with a strong influence of the season in this complex hydrosystem of barrage lakes created via tufa deposition. The seasonality in this hydrosystem manifests in differences in hydrology, physicochemical factors, tufa deposition, and in the effects of eutrophication on the autotrophic and heterotrophic components of the communities, as shown in this study.

Seasonal differences in the water velocity confirm previous results in this hydrosystem [44]. Macrophytes generally slowed down the water velocity at tufa barriers, which is in contrast to the results of [18]. The temperature and oxygen levels expressed characteristic seasonal dynamics, with values comparable to those of previous studies [45–47]. The strong influence of the season on electrical conductivity, total hardness, and pH is the result of the strong precipitation of calcium carbonate during the warmer season [45,47,48]. In addition, there is a downstream decrease in calcium carbonate-bound parameters due to the greater precipitation of tufa in the lower lakes, as previously reported [49,50]. The influence of hydrology on travertine formation and hydrochemical evolution varies from system to system, as has been shown for systems in Australia [51].

The observed nutrient concentrations (nitrates and orthophosphates) differed significantly between the seasons, probably influenced by the plankton in the lakes and the mixing processes [52]. The relatively low nutrient concentrations in this hydrosystem [14,53] could be related to the increased carbonate concentrations and phosphorus immobilization due to calcite co-precipitation processes [8,17]. The analysis of long-term data has shown that nutrient concentrations in the Plitvice Lakes are not increasing, and that the system remains in an oligotrophic state [32]. More frequent extreme events such as prolonged low precipitation in the summer months [12] can lead to changes that can alter the physicochemical factors and disrupt the ecological balance. Constant monitoring is therefore necessary to ensure the preservation of this unique system.

The amount of tufa on the NDSs statistically significantly differed between the barriers, and calcium carbonate precipitation was higher on the NB barrier, which is consistent with previous studies [22,45,53]. Macrovegetation appears to have had a negative effect on

precipitation, which is a possible consequence of slower water flow, measured in our study among macrophytes. The deposition rate of calcite in lotic biotopes (streams) is almost four times as high as in lentic waters [54]. Root respiration may also have an influence, by lowering the pH value, increasing the carbon dioxide partial pressure, and thus causing a considerable dissolution of calcite [19]. In contrast to [22], added nutrients showed no effect on tufa deposition. It is possible that the effects of the seasons and the different water velocities among microhabitats had a stronger influence on the tufa deposition than the added nutrients.

Above all, our results showed a recognizable response of periphytic autotrophs to the addition of nutrients, which was particularly evident in the higher concentration of chlorophyll *a* on NDSs with P and N+P. The strongest response to nutrient enrichment in autumn and winter can be explained by the low phosphate concentrations in the surrounding water. Our results contradict some previous studies [55,56], which showed the strongest response to NDS enrichment in summer. Nutrient availability can explain about 40% of the variation in autotroph biomass in different streams, and N and P nutrients can be considered limiting factors [57], as also shown for periphyton in the Plitvice Lakes [22]. In general, low concentrations of measured orthophosphates in Plitvice Lake lotic biotopes definitely refer to phosphorus being the nutrient-limiting factor. This can be demonstrated by the ratio of the chlorophyll *a* concentration to P and C NDSs, which was about 2.2, while this ratio was 1.2 for N/C. The results obtained are consistent with a meta-analysis involving more than 200 studies [58], which states that the limiting factor increases the biomass of autotrophs by a factor of about two. It should be noted that maintaining low concentrations of orthophosphates is essential for the persistence of the fundamental phenomenon of this national park, namely the tufa deposition process [3,59].

Chlorophyll *a* concentrations also differed significantly between seasons and microhabitats. Increased values in winter and decreased values in spring can be observed as an unusual seasonal dynamic in this barrage system, as ref. [60] reported a maximum of algal biomass in spring. The observed differences between the microhabitats confirm the possible detrimental effect of the high tufa deposition on the periphyton [22], while the differences between the microhabitats with macrophytes indicate the effect of shading. The reduced biomass of benthic algae has been linked to the influence of riparian vegetation as a shade provider [61,62].

The abundance of macrozoobenthos differed between the microhabitats (increased at GJ and at the tufa barrier without macrovegetation). The precipitation of calcium carbonate represents stress for periphytic protozoans [22], so it probably also affects the macrozoobenthos, explaining the higher abundance at the GJ compared to the NB barrier. Sedimentation reduced the abundance and species richness of macroinvertebrates in a mesocosm experiment [30]. As previously reported, a reduction in the amount of organic matter was observed downstream in the Plitvice Lakes [63], possibly influencing macrozoobenthos. The same was hypothesized by [64], who reported a downstream decrease in taxa abundance along the longitudinal profile of the studied barrage lake system of the Plitvice Lakes. The abundances recorded in our study were lower than those recorded for the natural substrate in this system [28,65], which indicates the certain level of inadequacy of the artificial substrate for the macrozoobenthos. Pronounced seasonal differences were observed, confirming previous studies showing an increased macrozoobenthos density in spring and a decreased density in winter [26]. The response of macrozoobenthos to the addition of nutrients was recorded, which followed the pattern of primary producers, although it was not observed in all seasons. However, a stronger response was observed compared to [22], who reported only a weak effect of NDSs on protozoa and micrometazoans.

In this study, we observed an increased macrozoobenthos density in microhabitats without macrovegetation, and this can be explained by increased chlorophyll *a* in the same microhabitats, which was further confirmed after sorting the functional feeding groups. At the GJ barrier, in contrast to the NB barrier, an increased density of grazers (GRAs) and detritivore collectors (DETs) was observed, while the proportion of the same groups was increased at the tufa barriers without macrovegetation. Grazers are considered to be macroinvertebrates that favour algae and attached microflora for feeding, while detritivore collectors feed on algae or decaying organic matter [66]. As previously mentioned, the chlorophyll *a* concentration was also elevated at the GJ barrier, consistent with the elevated GRA and DET groups, indicating that these groups had the acquired food source within the primary production. The most common organisms in this study include Oligochaeta and Diptera (Chironomidae, Tanypodinae, and Tanytarsini). According to [43], these organisms are categorized as detritivore collectors (Oligochaeta and Tanypodinae) and grazers (Chironomidae and Tanytarsini). Oligochaeta and Chironomidae also dominated at the barriers in natural filamentous algae-covered substrates in the Plitvice Lakes [65], while ref. [67] reported that Simuliidae and Chironomidae were most abundant among the dipterans at the tufa barriers of the upper lakes in contrast to the lower lakes. The functional feeding group DETs had a greater proportion on substrates with N, P, and N+P treatments. GRAs and DETs showed statistically significant differences among treatments, in contrast to the other groups, again confirming the trophic relationship with primary production. The trend of the increasing abundance of grazers on enriched substrates can be linked to a trophic shift, as recently described by [29] for English rivers. A positive response to the addition of phosphorus was found in Trichoptera larvae (grazers) with increased periphyton biomass [23], while ref. [24] reported the positive response of Ephemeroptera and chironomid larvae following the addition of nutrients, which followed an increase in the periphyton biomass.

The response of biota to experimental eutrophication in this hydrosystem is not simple and is influenced by many factors, mainly by the season, but also by tufa deposition and the presence of macrophytes. The observed patterns show great complexity, characteristic of this natural system, and can help in the further management of the national park.

5. Conclusions

The nutrient-diffusing substrates, used more frequently for research on the possible influence of increased nutrient concentrations on autotrophs, proved to be suitable for research on experimental eutrophication effects on macrozoobenthos. As the Plitvice Lakes are a protected area, this approach allows for the assessment of eutrophication effects without major impact on the underlying processes. The added nutrients had a strong influence on the chlorophyll *a* concentrations, with pronounced seasonal differences. Tufa deposition differed between microhabitats, particularly barriers, and also influenced communities. Functional feeding groups of macrozoobenthos responded to the addition of P and N+P; however, the response of macrozoobenthos was generally weaker compared to that of autotrophs. The heterogeneity of this hydrosystem is also reflected in the response to eutrophication, and the concentrations studied did not significantly alter tufa deposition.

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Abbreviations

The following abbreviations are used in this manuscript:

NDS	Nutrient-diffusing substrates
GJm–	Gradinsko jezero tufa barriers without macrophytes
GJm+	Gradinsko jezero tufa barriers with macrophytes
NBm–	Novakovića brod tufa barriers without macrophytes
NBm+	Novakovića brod tufa barriers with macrophytes
TN	Total nitrogen
TP	Total phosphorus
Chl <i>a</i>	Chlorophyll <i>a</i>
MDS	Multidimensional scaling
FFGs	Functional feeding groups
SHRs	Shredders
GRAs	Grazers
AFILs	Active filter feeders
PFILs	Passive filter feeders
DETs	Detrivore collectors
PREs	Predators
OTHs	Others

Appendix A

Appendix A.1

Table A1. Locations, microhabitats, and replicates with coordinates of where the in situ experiment using nutrient-diffusing substrata was conducted within NP Plitvice Lakes (m—without macrophytes and m+—with macrophytes).

Location	Microhabitats	Replicates	Coordinates
Novakovića brod lake outlet (NB)	NBm–	A	15°36'35.633" E 44°54'6.442" N
		B	15°36'35.452" E 44°54'6.649" N
		C	15°36'35.521" E 44°54'6.919" N
	NBm+	D	15°36'38.055" E 44°54'7.96" N
		E	15°36'37.339" E 44°54'7.989" N
		F	15°36'38.445" E 44°54'8.019" N
Gradinsko jezero lake outlet (GJ)	GJm–	G	15°36'47.803" E 44°52'46.472" N
		H	15°36'48.164" E 44°52'46.469" N
		I	15°36'48.496" E 44°52'46.458" N
	GJm+	J	15°36'48.69" E 44°52'46.057" N
		K	15°36'48.973" E 44°52'46.11" N
		L	15°36'49.208" E 44°52'46.262" N

Appendix A.2

Table A2. Physicochemical parameters of water (mean ± SD) and ANOVA results.

	Spring	Summer	Autumn	Winter	ANOVA Results
Temperature (°C)	13.22 ± 3.11	20.24 ± 1.96	11.74 ± 2.40	4.34–1.18	F _{3,42} = 99.4 p < 0.001
Dissolved oxygen (mg O ₂ L ^{−1})	10.69 ± 0.81	9.53 ± 0.45	10.49 ± 0.57	12.27 ± 0.54	F _{3,42} = 42.3 p < 0.001
Electrical conductivity (μS cm ^{−1})	352.42 ± 5.70	325.42 ± 7.42	348.42 ± 9.64	367.08 ± 5.12	F _{3,42} = 85.6 p < 0.001
pH	8.35 ± 0.06	8.17 ± 0.05	8.25 ± 0.07	8.35 ± 0.12	F _{3,42} = 23.5 p < 0.001
Total hardness (mg CaCO ₃ L ^{−1})	228.10 ± 5.18	211.90 ± 5.56	227.91 ± 11.02.	236.02 ± 1.56	F _{3,26} = 17.5 p < 0.001
Nitrates (mg N L ^{−1})	0.65 ± 0.07	0.47 ± 0.07	0.55 ± 0.08	0.73 ± 0.02	F _{3,26} = 23.9 p < 0.001
Orthophosphates (mg P L ^{−1})	0.02 ± 0.02	0.02 ± 0.01	0.01 ± 0.00	0.00 ± 0.00	F _{3,26} = 5.1 p < 0.01
Total nitrogen (mg TN L ^{−1})	1.35 ± 0.99	0.77 ± 0.37	0.70 ± 0.14	0.89 ± 0.46	N.S.
Total phosphorus (mg TP L ^{−1})	0.15 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	0.14 ± 0.06	F _{3,26} = 26.1 p < 0.001

Appendix A.3

Table A3. Total number of individuals on nutrient-diffusing substrata placed in different microhabitats during in situ experiment at Plitvice Lakes (GJ—Gradinsko jezero lake, NB—Novakovića brod lake, m—without macrophytes, and m+—with macrophytes).

Taxa	NBm−	NBm+	GJm−	GJm+
Turbellaria				
Turbellaria non det.		4		
Gastropoda				
<i>Bithynia tentaculata</i> (Linnaeus, 1758)			3	
<i>Holandriana holandrii</i> (C. Pfeiffer, 1828)	3			
Oligochaeta				
Oligochaeta non det.	45	63	524	304
Hydrachnidia				
Hydrachnidia non det.	10	4	4	5
Ostracoda				
Ostracoda non det.	1			
Cladocera				
<i>Alona</i> sp.	50	83	22	45
Copepoda				
Copepoda non det.	6	28	1	5
Ephemeroptera				
Baetidae juv.		7	39	5
<i>Baetis alpinus</i> (Pictet, 1843)	1	2	2	
<i>Baetis</i> sp.	7	1	4	1
<i>Centroptilum luteolum</i> (Muller, 1776)	8		41	24
<i>Centroptilum</i> sp.		3	3	5
<i>Ephemera danica</i> (Muller, 1764)	1		1	1
Ephemeridae juv.	1	2		
<i>Ephemerella</i> sp.	1	1	19	5
Ephemeroptera non det.	25	15	12	12
<i>Habroleptoides</i> sp.			4	
Leptophlebiidae juv.				1
<i>Paraleptophlebia</i> sp.	3	3	28	10
Plecoptera				
<i>Amphinemura</i> sp.			1	
<i>Isoperla</i> sp.	12	1	9	12
<i>Leuctra</i> sp.	4	1		

Table A3. Cont.

Taxa	NBm–	NBm+	GJm–	GJm+
Nemouridae juv.			3	
<i>Nemurella</i> sp.	80	16	4	53
<i>Perloides</i> sp.	2	1		
Perlodidae juv.	12	7	6	12
Perloidea juv.		1	5	3
Plecoptera non det.	67	21	48	116
<i>Protonemura</i> sp.	1		11	
Odonata				
Corduliidae ili Libellulidae		2	2	2
Coleoptera				
Coleoptera non det.				1
Coleoptera pupae	1			
Dryopidae			1	
Dryopidae imago		1	1	1
Elmidae	6	16	67	3
Elmidae imago				1
<i>Elodes</i> sp.	13			2
Trichoptera				
<i>Agraylea multipunctata</i> (Curtis, 1834)				1
<i>Apatania</i> sp.			1	
<i>Athripsodes</i> sp.	1	2		
Beraeidae		9		
Ecnomidae juv.	4			
<i>Hydropsyche angustipennis</i> (Curtis, 1834)	6	1	4	
<i>Hydropsyche incognita</i> (T. Pitsch, 1993)			1	
<i>Hydropsyche instabilis</i> (Curtis, 1834)			9	
<i>Hydropsyche</i> sp.	1		11	
Hydropsychidae juv.	1			
<i>Hydroptila occulta</i> (Eaton, 1873)		1		2
<i>Hydroptila</i> sp.	69			
Hydroptilidae juv.	2	1	1	2
Hydroptilidae pupae	2			
Limnephilidae non det.			1	
<i>Limnephilus</i> sp.		1		
<i>Orthotrichia</i> sp.			8	3
<i>Oxyethira flavicornis</i> (F.J. Pictet, 1834)			5	3
Philopotamidae juv.	3	1	3	1
<i>Philopotamus</i> sp.				2
<i>Philopotamus variegatus</i> (Scopoli, 1763)	1	1		
<i>Plectrocnemia conspersa</i> (Curtis, 1834)	1			
Polycentropodidae juv.		1	2	1
<i>Polycentropus flavomaculatus</i> (Pictet, 1834)	1			
<i>Polycentropus</i> sp.			1	3
<i>Rhyacophila</i> sp.			1	6
<i>Sericostoma</i> sp.	1	18		1
<i>Stactobia</i> sp.	102		25	59
Trichoptera non det.		1		
Trichoptera pupae	5			
<i>Wormaldia occipitalis</i> (Pictet, 1834)	1		1	4
<i>Wormaldia</i> sp.	5	3	1	
Diptera				
Athericidae		5		
Ceratopogonidae	7	1		
Chironomidae	196	82	652	207
Chironomidae pupae		1	4	3
Chironominae	1	3	5	8
Chironomini	43	21	94	195
Diptera pupae	3	4	26	57
Empididae	16	13	15	83
Limoniidae		1		
<i>Simulium</i> sp.	127	4		125
Tanypodinae	108	99	179	156
Tanytarsini	186	155	451	596
Total	1253	712	2366	2147

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