Int. J. Environ. Res., 5(4):891-900, Autumn 2011
ISSN: 1735-6865

Water Quality Assessment in Lakes of Vojvodina

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Received 10 Dec. 2010; Revised 12 May 2011; Accepted 19 May 2011

ABSTRACT: This study focused on use of bacterial extracellular enzyme activities as biochemical parameters, along with the microbiological and physicochemical characteristics, in a comprehensive assessment of water quality of four lake ecosystems in the Province of Vojvodina (northern Serbia): Provala, Ludas, Zobnatica and Palic. Water samples were collected in June and October, 2008. For assessment of microbiological water quality, heterotrophic plate count, total and fecal coliform count, as well as total bacterial count was determined. Based on microbiological parameters, the water ecosystems were appropriately classified. The following extracellular enzyme activities were determined: alkaline phosphatase, β-D-glucosidase and acetate esterase using fluorogenic 4-methylumbelliferone labeled substrates. The results showed that Provala Lake had the best water quality taking in consideration all of the parameters, followed by Zobnatica, while Ludas and Palic had a significant level of organic water pollution both in June and October. The results indicate that further similar studies should include both microbiological and biochemical analyses, in order to obtain more relevant data on water quality.

Key words: Lake water, Alkaline phosphatase activity, β-D-glucosidase activity, Acetate esterase activity, aerobic heterotrophic bacteria, Total coliform count, Total bacterial count

INTRODUCTION

The growth of heterotrophic bacteria is affected by organic matter. More than 95% of organic matter in subsurface aquatic habitats consists of high molecular weight compounds which cannot be directly exploited by bacteria. In order to provide low molecular weight organic compounds that could be uptaken into the cells, heterotrophic microorganisms produce extracellular enzymes (Münster and Chróst, 1990). Microbial enzymes are therefore, directly related to the concentrations and ratios of limiting nutrients, and may be sensitive indicators of organic pollution in aquatic ecosystems. According to many authors (Boetius, 1995, Jackson *et al.*, 1995, Mallet and Debroas, 1999), enzyme assays can provide powerful tools for studying organic matter presence and degradation in aquatic ecosystems. Activities of many hydrolytic extracellular enzymes can be measured with high sensitivity, using fluorogenic model substrates, which are available for the most of natural compounds (Hoppe, 1993). We studied the extracellular enzymatic activities, physicochemical and microbiological parameters in surface water of the lake ecosystems Provala, Ludas, Zobnatica and Palic of the Province of Vojvodina (northern Serbia) in June and October, 2008 in order to assess water quality with special attention paid to determine applicability of both microbiological and biochemical parameters for water quality assessment.

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MATERIALS & METHODS

Water samples were taken from four lakes of the Province of Vojvodina (northern Serbia): Provala Lake, Ludas Lake (two sampling sites), Zobnatica and Palic lakes on June 1, and October 1, 2008. The location and coordinates of sampling sites are shown in Fig. 1. The geographic coordinates are: 45°24' N, 019°05' E for Provala, 46°06' N, 019°49' E for Ludas 1, 46°03' N, 019°49' E for Ludas 2, 46°05' N, 019°45' E for Palic and 45°50' N, 019°38' E for Zobnatica Lake. The North latitudes and East longitudes were the same in June and October. Palic and Ludas lakes are protected areas. Palic Lake is proclaimed as a protected area and a Park

of Nature, while Ludas Lake is a special natural preserve and, since 1977, designated as a swamp area of international significance by the Ramsar Convention. Lake water samples were collected at depth of 1.5 m. The samples were kept and transported to the laboratory in cooled containers and analysed within 24h. For determination of total bacerial count (TBC) water samples were fixed with formaldehyde (2% final concentration) after the sampling. The basic parameters of water quality oxygen, temperature, electrical conductivity and pH were determed using oxymeter and conductivity meter (Hanna HI-9142 and HI-933000, Hanna Instruments, USA), as well as pH meter (WTW InoLab-4, Germany). The total suspended solids (TSS), chemical oxygen demand (COD), biochemical oxygen demand (BOD), total oraganic carbon (TOC) and surfactante (SUR) were measured at sampling site after the sampling using multiparameter PASTEL-UV Portable Analyzer "SECOMAM" (Nova Analytics Corporation, USA). Total nitrogen (TN), nitrate (NO_3^-), total and inorganic phosphorus (P_{tot}, PO_4^{3-}) were measured in laboratory within 24h. TN was determined according to the method of Kjeldahl in unfiltered samples (CNR-IRSA, 1994). $NO_3^{"}$ was determined spectrophotometrically in filtered samples according to Cataldo (Cataldo *et al.,* 1975). Ammonia was determined by treating the lakewater samples in an alkaline citrate medium with sodium hypochlorite and phenol in the presence of sodium nitroprusside that acted as a catalyser (APHA, 1992). The blue indophenol colour formed with ammonia was measured spectrophotometrically at 640nm. P_{tot} was determined by antimony method in unfiltered samples (Genchi, 1990) as well as PO_4^{3-} in filtered samples, using the same method. Organic phosphorus (P_{org}) was calculated by $P_{org} = P_{tot} - PO_4^{3-}$. For this analysis the spectrophotometer Jenway 6505 UV/Vis, UK was used. In all samples, counts of aerobic heterotrophic bacteria, fecal and total coliforms were determined on solid media using the spread plate method. The aerobic heterotrophic bacteria (HPC) were grown on Nutrient agar (Torlak, Serbia) using R2A medium (Merck, Germany). The media were incubated at 26°C for 5-7 days. Total and fecal coliforms were counted on Chromocult Coliform Agar (Merck, Germany) after 48 hours of incubation at 37°C. The waters were classified according to Kohl (1975) on the basis of HPC while coliforms were used for classifying water classification was according to Kavka *et al.* (2006). Bacterial counts in samples were determined by epifluorescence microscopy according to Raymond *et al.* (1994). Sample aliquots were filtered under vacuum onto Sudan Black prestained 0.2 µm pore size Nuclepore polycarbonate membrane filter with cellulose-acetate supporter filter. TBC was enumerated by direct counting of acridine orange stained cells (0.01% w:v, 5 min) using Olympus CX41 epifluorescent microscope with coresponding filter. The bacterial cells were counted on at least 10 fields. The cell count was averaged and the results were expressed per milliliter of water samples. Extracellular enzyme activities were determined in triplicate samples using fluorogenic model substrate 4-methylumbelliferone (MUF) according to Hoppe (Hoppe, 1983). Appropriate MUF-supstrates were added to final concentrations: 500 µmol/L MUF-glucose, 250 µmol/L for MUF-phosphate and 100 µmol/L MUF-acetat, according to determined enzyme activities: β-D-glucosidase, alkaline phosphatase and acetate esterase. The reaction mixtures were incubated for 8 hours in dark at room temperature. The reaction was interrupted by boiling for 5 min. The pH of mixtures was adjusted to 10.5 by adding 10:1 of ammonium glycine buffer (0.1 M) before fluorescence measurement. The fluorescence of 4-MUF, hydrolysed from the model substrates was measured, using 96-well microtiter plate and automated microtiter plate fluorometer (Fluoroscan Ascent FL, USA) with exitation and emission filters of 355 and 460 nm. Its quantity determined fluorometrically indicated the level of extracellular enzyme activity in the sample. Standard curve was constructed using an appropriate range of known concentrations. The enzyme activities were expressed as total activity (nmol/h/dm^3) and as specific enzyme activities, calculated per bacterial cell (amol/h/cell).

The correlation coefficients (r) and the statistical significance of the relationships (p-values) were calculated using Statistica 9.1 (StatSoft, USA). The data were analysed using two-way analyses of variance (ANOVA) followed by *Tukey's HSD* test (Hinkle, 1994).

RESULTS & DISCUSSION

Water samples were analysed for the physicochemical characteristics at each sampling site and the results are shown in Table 1. The total phosphorous (P_{tot}) values in the water samples during 2008 varied between 0.00-2625.5 µg P dm^{-3} while organic phosphorous varied between 0.00-1748.8 µg P/dm^3 (Table 1). In June, all water samples showed higher organic than inorganic phosphorus fraction, with the exception of Ludas 1 and Palic, where inorganic fraction was 3.57 and 2 fold higher than P_{org}, respectively. In October, higher inorganic than organic phosphorus level was detected in Palic (1.3 fold higher) and in Ludas 2 (1.5 fold higher). The results of microbiological parameters are presented in Table 3 and Fig. 4. Using R2A medium, bacterial count varied from 3.11+0.18 CFU/mL (Zobnatica, June) to 4.76±0.14 CFU/mL (Palic, October). Total coliforms were detected in the majority of the samples. In June, the highest count was detected

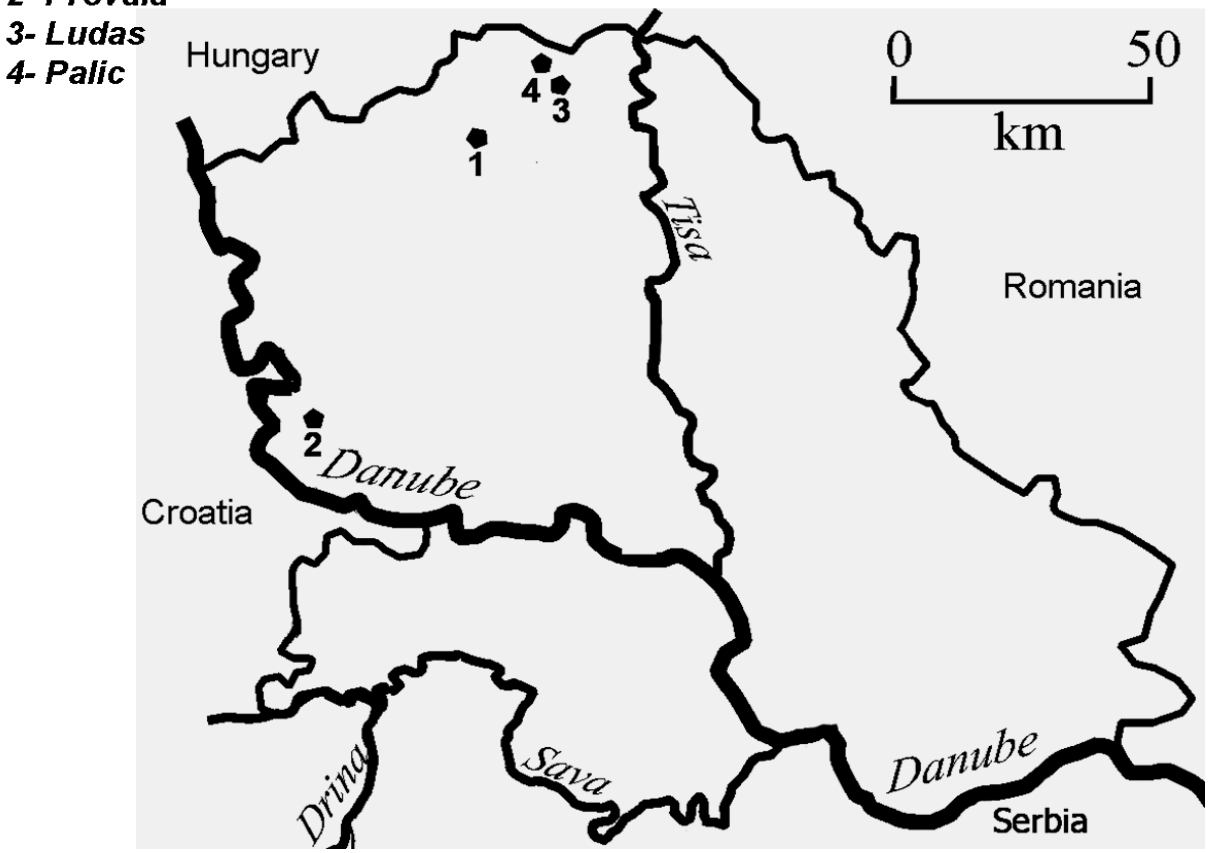

Fig. 1. Location of the sampling sites of Provala, Ludas 1, Ludas 2, Zobnatica and Palic lakes in the Province of Vojvodina (northern Serbia)

Table 1. Main physicochemical parameters of water quality at sampling sites: Provala, Ludas 1, Ludas 2, Zobnatica and Palic in June and October, 2008

Parameter	Sampling site				
	Provala	Ludas 1	Ludas 2	Zobnatica	Palic
	June - October	June - October	June - October	June - October	June - October
NH_4^+ [mg/dm^3]	0.3 - 0.1	1.8 - 1.2	1.1 - 0.7	0.9 - 0.5	2.0 - 1.1
NO_3^- [mg/dm^3]	0.7 - 1.2	1.7 - 3.7	1.1 - 1.8	1.0 - 3.3	1.8 - 3.9
NO_2^- [µg/dm^3]	30.0 - 17.2	107.2 - 80.1	87.2 - 62.9	25.7 - 31.5	58.6 - 115.8
DO [mg/dm^3]	4.8 - 3.3	6.1 - 6.4	0.2 - 3.1	3.9 - 3.4	1.88 - 4.5
O_2 [%]	62.3 - 57.8	76.0 - 91.7	2.8 - 46.5	47.5 - 52.2	24.3 - 71.5
Temp. [°C]	28.0 - 17.1	26.3 - 15.4	23.2 - 14.9	25.2 - 15.2	27.5 - 15.0
pH	9.2 - 8.9	9.5 - 9.8	8.1 - 8.5	8.6 - 9.1	8.3 - 9.6
EC_w [µS/cm]	380.0 - 156.8	896.0 - 1078.0	1300.0 - 1368.0	1210.0 - 1190.0	1111.0 - 939.0
P_{tot} [µg P/dm^3]	ND - 88.2	1175.0 - 148.4	270.8 - 43.0	32.1 - 32.3	2625.5 - 144.1
PO_4^{3-} [µg P/dm^3]	ND - 17.2	918.0 - 53.8	110.2/25.8	13.8/10.8	1748.8/81.7
P_{org} [µg P/dm^3]	ND - 71.0	257.0 - 94.6	160.6 – 17.2	18.3 - 21.5	876.7 - 62.4
TOC [mg/dm^3]	5.1 - 3.3	16.2 - 15.6	- / -	8.4 - 7.3	13.9 - 10.0
TSS [mg/dm^3]	<2.5 - 5.7	54.5 - 29.4	- / -	<2.5 - <2.5	<2.5 - 15.4
SUR [mg/dm^3]	3.3 - 0.6	6.8 - 5.6	- / -	3.7 - 1.0	8.1 - 2.8
COD [mg/dm^3]	11.5 - 9.9	42.5 - 42.0	- / -	21.4 - 22.0	33.0 - 28.0
BOD [mg/dm^3]	7.0 - 4.8	22.8 - 22.2	- / -	11.9 - 10.8	17.0 - 14.4

All the values are expressed as mean, number of examinations n=3

in Ludas 2 (6.43±0.39 CFU in 100 mL) and in October in Ludas 1 (4.48±0.48 CFU in 100 mL). No fecal coliforms were detected in one milliliter of the samples. Total bacterial count in June was on the order of 10^7. In October it decreased to the order of 10^6 (Table 3). The highest bacterial density in June was detected in Ludas 1 and Zobnatica ($3.98x10^7$ and $3.91x10^7$, respectively), and the lowest in Palic and Provala ($1.32x10^7$ and $1.41x10^7$, respectively). In October, the bacterial density showed variation from $1.81x10^6$ (Ludas 1) to $5.33x10^6$ (Ludas 2).

Alkaline phosphatase activity in June significantly differed depending on the locality, with the highest activity in Ludas 2 (Fig. 2A, Table 2). In relation to the season, in localities Provala and Zobnatica a significant increase in phosphatase activity was recorded in October, while in Palic and Ludas 2 there was a decrease. The results of phosphatase activity in Ludas 1 are not shown due to the lack of samples. The results of specific phosphatase activity also varied depending on the locality and season (Fig. 2B, Table 2). Thus, the specific phosphatase activity was significantly lower in Provala than in Zobnatica, while their activities were lower in comparison with Palic and Ludas 2 (no significant difference was observed). In comparison with June, a significant increase of specific phosphatase activities was detected at all of the sites in October. The highest β-D-glucosidase activity in June was recorded in Ludas 1, being significantly higher than in Ludas 2. Both of these localities showed significantly higher levels in relation to Palic and Provala, whose activities were approximate (Fig. 4A, Table 2). The results for glucosidase activity in Zobnatica are not shown due to the lack of samples. In October, compared to June, there was a significant increase of β-D-glucosidase activity in Provala and Palic and a decrease in Ludas 1 and Ludas 2. The specific β-D-glucosidase activity significantly increased in October at all of the sites (Fig. 4B, Table 2). Acetat esterase activity was different at all sites in June, with the highest activity recorded in Provala (Fig. 3A, Table 2). In terms of specific acetate esterase activity, the highest levels were detected in Provala, while significantly lower ones were recorded in Palic. Ludas 1, 2 and Zobnatica showed similar activities, which proved to be significantly lower in comparison with Palic (Fig. 3B, Table 2). In October the total and specific esterase activity increased significantly at all sites (and between them), the exeption being Provala for total activity, which appeared to be significantly lower (Fig. 3A and B, Table 2).

The composition and avaiability of organic matters, which are mainly derived from allochthonous rather than from autochthonous matters (Findlay, 1991), are the major factors which influence the development and activity of the microbial heterotrophic communities. Heterotrophic bacteria are the key trophic level in natural organic matter decomposition, nutrient cycling, and structure of the food web (Chróst and Siuda, 2002, Münster, 1999). Therefore the studies of the microbial mobilization, transformation and utilization of a variety of natural organic matters are one of the most important areas of the aquatic microbial ecophysiology (Chróst, 1991).

The results of microbiological analyses indicated that all of the examined ecosystems showed a significant variation of water quality. Generally, better water quality was detected during October in all of the examined lakes. According to HPC, in June water of the four lakes belonged to I-II and II-III class, while in October the water was classified from I to II-III class. Taking into consideration total coliform counts, the water quality varied from II to V class. The detected total coliforms, which naturally occur and multiply in waters (De Zuane, 1997), without fecal coliforms, indicate the absence of water fecal pollution. It is interesting to notice a generally higher count of bacteria on R2A medium than on Nutrient agar reported in certain studies of various waters types (Massa *et al.*, 1998), as the R2A medium is a low nutritive and more suitable for autochthonous water bacteria. However, our results showed that the bacterial counts obtained on Nutrient agar and R2A medium did not statistically differ, neither in June nor in October ($p= 0.601$ and $p= 0.08$, respectively). Such results may indicate that most of the bacteria detected on Nutrient agar are autochthonous, and that they dominate in the ecosystems. Generally, the water of Provala had the best quality, followed by Zobnatica and Palic, while Ludas 1 and 2 had the worst quality, considering all microbiological parameters. Physicochemical parameters of the lake water samples also indicate generally better water quality in October (Table 1). Considering the examined ecosystems, Palic and especially Ludas showed worse water quality than Zobnatica and Provala lakes. Similar findings for physicochemical parameters of the same localities were reported earlier by other autors (Simeunovic *et al.*, 2010).

Our results of estimated extracellular enzymatic activities showed that the hydrolysis activity increased in June as well as in October in the following order: β-D-glucosidase < alkaline phosphatase < acetat esterase, which complies with the results of Mudryk and Skórczewski (Mudryk and Skórczewski, 2004) regarding lake water ecosystems. According to Münster *et al.* (Münster and de Haan, 1998), among the most important microbial extracellular enzymes in surface waters are glucosidases and phosphatases,

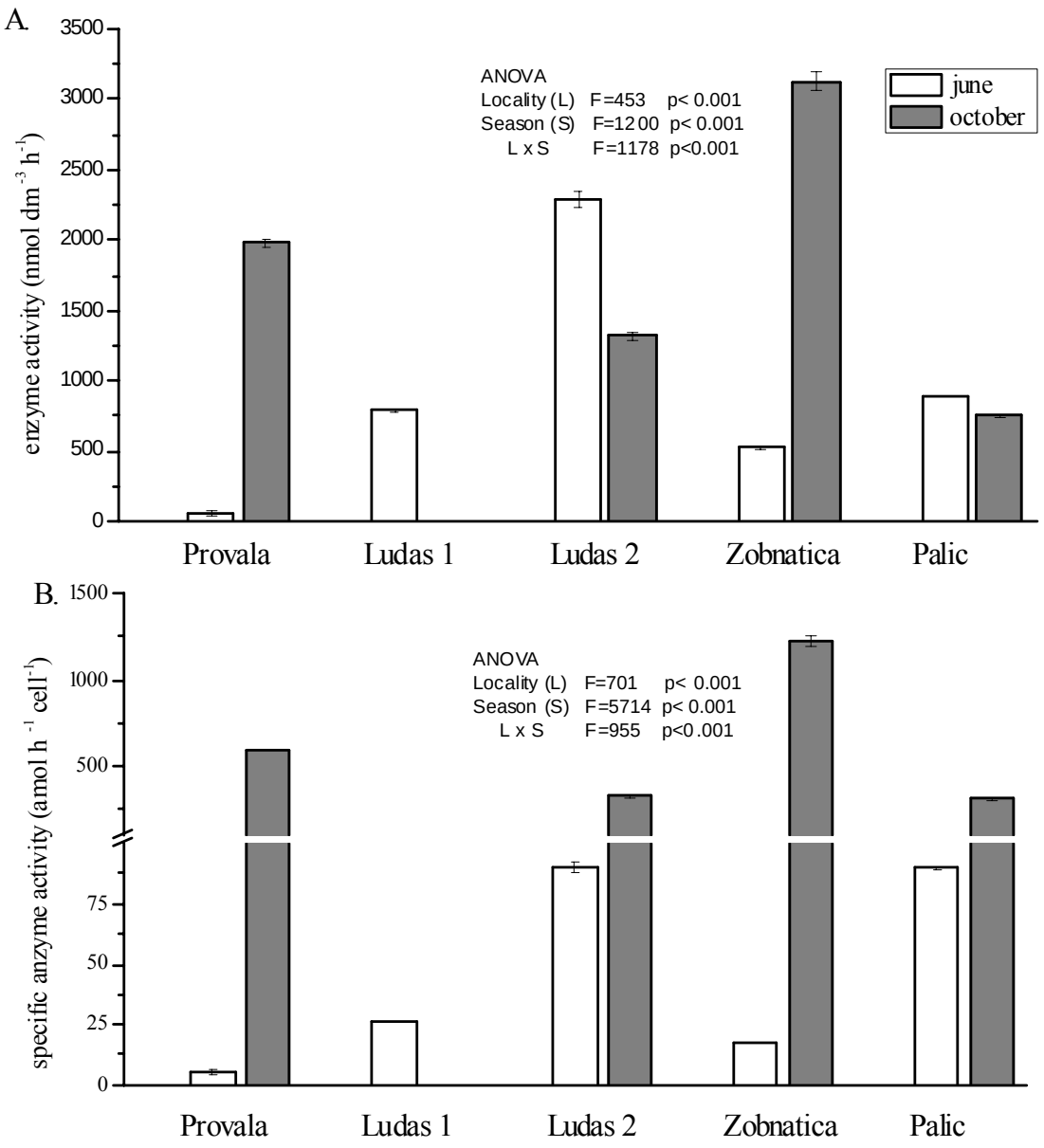

Fig. 2. Alkaline phophatase activity (A) and specific alkaline phosphatase activity (B) in localities Provala, Ludas 1, Ludas 2, Zobnatica and Palic in June and October, 2008. Differences between groups were tested by two-way analysis of variance (two-way ANOVA) with Tukey's HSD post hoc test, with locality (L) and season (S) as factors with lokality (L) and season (S) as factors

the former participating in depolymerisation of polysaccharides, and the latter in dephosphorylation of organic molecules. Esterases, on the other hand, can be used for the determination of general microbial extracellular enzyme activities due to their varying substrate specificity and biological functions.

In general, the lowest β-D-glucosidase activities in both seasons might be attributed to reduced availability of polymeric carbohydrates (cellulose and cellobiose) as a source of energy in these lake ecosystems. In early summer (June), a significantly higher level of β-D-glucosidase activity ($p < 0.001$) in Ludas Lake (localities Ludas 1 and 2) in relation to Palic and Provala was detected. According to Chróst and Overbeck (Chróst and Overbeck, 1990) it could indicate on phytoplankton bloom breakdown and water blooms in the lakes examined in the present study are documented during early summer (Simeunovic, 2010). A significant decrease in alkaline phosphatase activity recorded in localites Palic and Ludas 2 and increase in localities Provala and Zobnatica recorded in October is in accordance with the content of organic

Table 2. Results of statistical analyses two-way ANOVA with Tukey's HSD post hoc test, with locality (L) and season (S) as factors, for enzyme and specific enzyme activity of alkaline phosphatase, β-D-glucosidase and acetate esterase

Enzyme activity		Locality (L)	Season (S)	L x S
	df	3	1	3
Alkaline phosphatase	MS	1.65 x 10^6	4.36 x 10^6	4.3 x 10^6
	F	**453** ***	**1200****	**1178** ***
	df	3	1	3
β-D-glucosidase	MS	5665	1870	7824
	F	**111** ***	**36.7** ***	**153** ***
	df	4	1	4
Acetate esterase	MS	261 x 10^3	7.2 x 10^6	1.4 x 10^6
	F	**10.5** ***	**292** ***	**57.9** ***
Specific enzyme activity				
	df	3	1	3
Alkaline phosphatase	MS	235 x 10^3	1.9 x 10^6	320 x 10^3
	F	**701** ***	**5714** ***	**955** ***
	df	3	1	3
β-D-glucosidase	MS	1053	7135	1010
	F	**211** ***	**1429** ***	**202** ***
	df	4	1	4
Acetate esterase	MS	496 x 10^3	5.5 x 10^6	595 x 10^3
	F	**845** ***	**9346** ***	**1014** ***

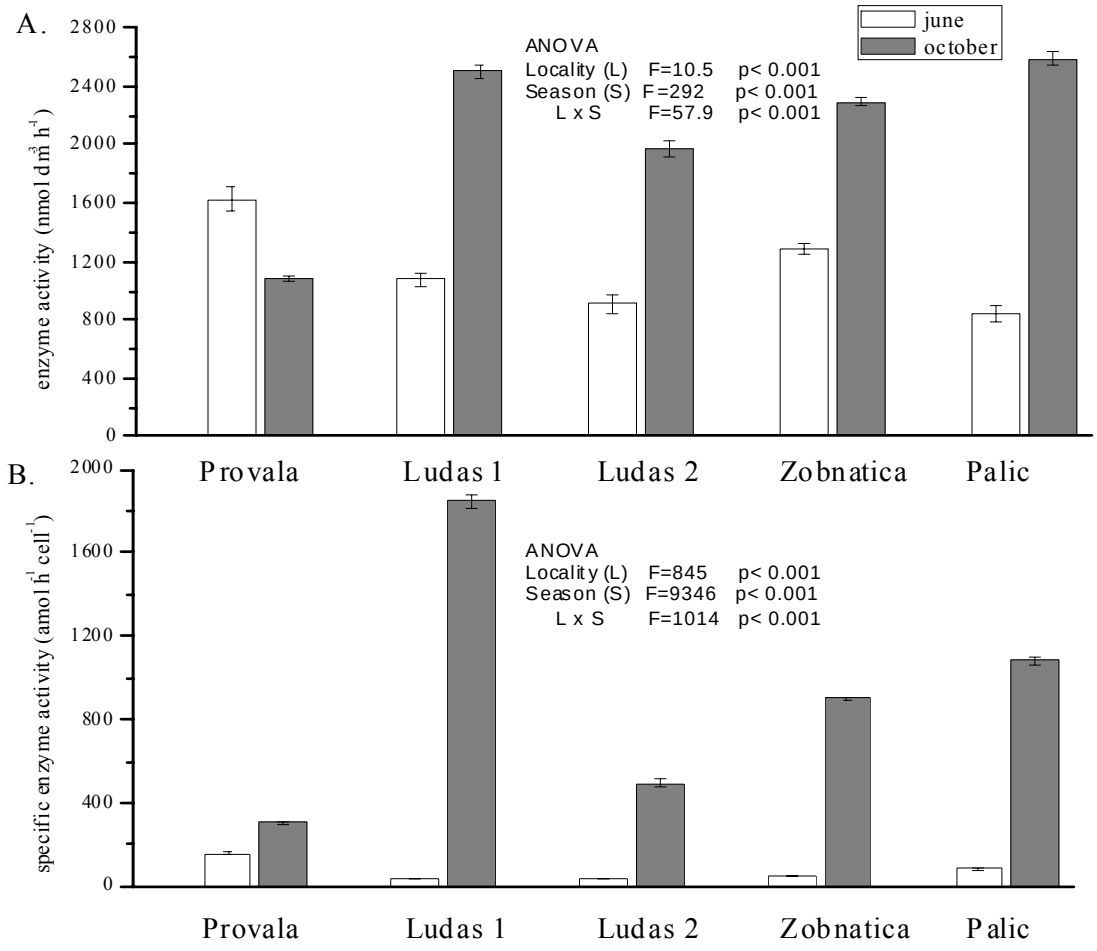

Fig. 3. Acetate esterase (A) and specific acetate esterase activity (B) in localities Provala, Ludas 1, Ludas 2, Zobnatica and Palic in June and October, 2008. Differences between groups were tested by two-way analysis of variance (two-way ANOVA) with Tukey's HSD post hoc test, with locality (L) and season (S) as factors with lokality (L) and season (S) as factors

Table 3. Total bacterial counts, total coliform counts and aerobic heterotrophic bacteria on R2A medium

Parameter		Samples				
		Provala	Ludas 1	Ludas 2	Zobnatica	Palic
Total bacterial count ($x10^6$/mL)	June	14.10±1.90	40.0±2.90	33.90±3.50	40.00±2.50	13.20±1.20
	October	4.45+0.63	3.20+0.63	1.81+0.58	5.33+0.65	3.40+0.32
Heterotrophic bacteria on R2A (CFU/mL)	June	3.38+0.37	4.53+0.32	4.55+0.36	3.11+0.18	4.33+0.20
	October	3.96±0.82	4.62±0.22	4.17±0.13	4.32±0.78	4.76±0.14
Total coliforms (CFU/100 mL) (water class)	June	3.00+0.15 II	4.48±0.86 III	6.43±0.39 V	3.91±2.5 II	4.39±0.39 III
	October	N.D.* -	4.48+0.48 III	4.00+0.00 II	4.00+00 II	N.D. -

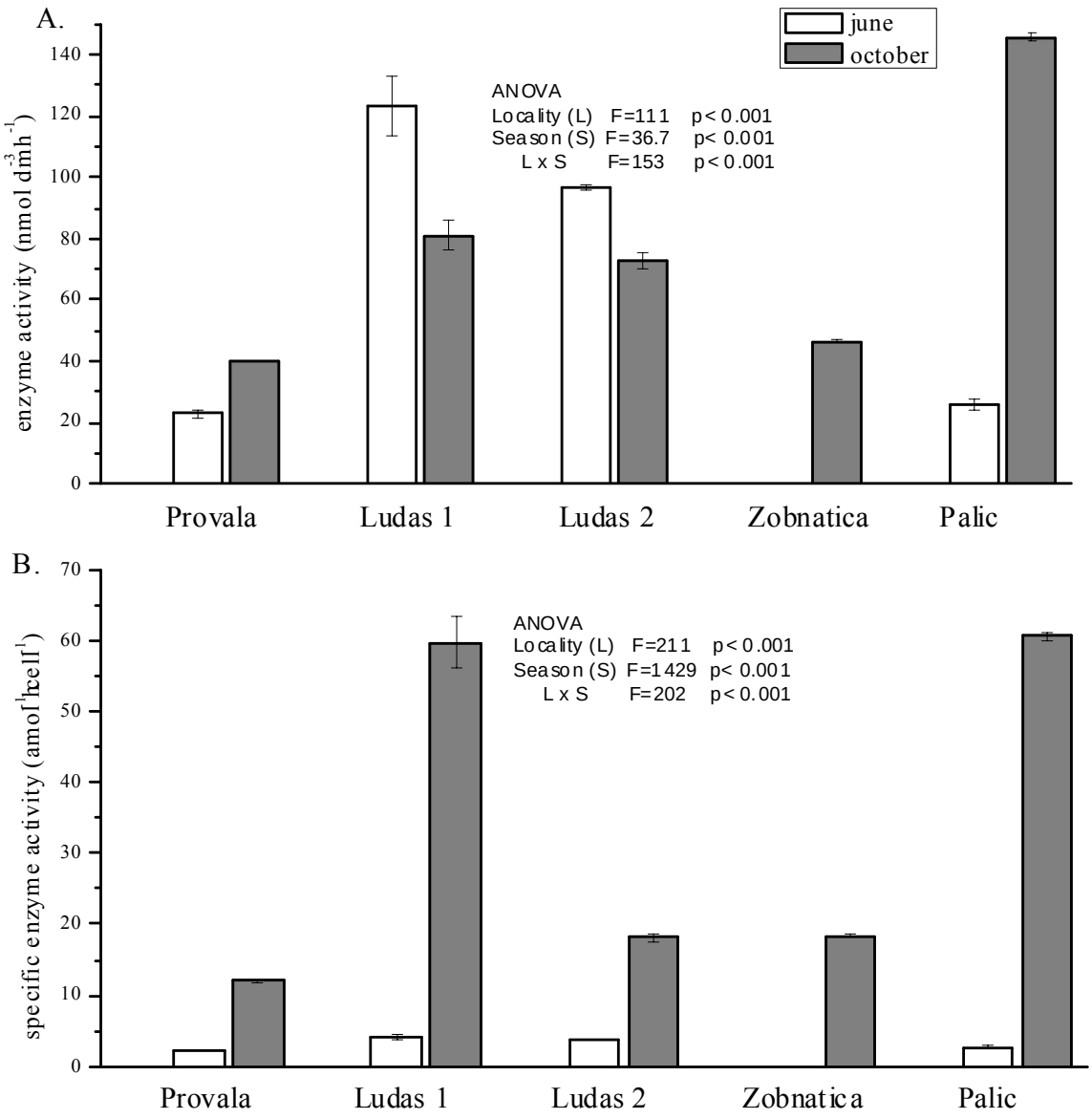

Fig. 4. β-D-glucosidase activity (A) and specific β-D-glucosidase activity (B) in localities Provala, Ludas 1, Ludas 2, Zobnatica and Palic in June and October, 2008. Differences between groups were tested by two-way analysis of variance (two-way ANOVA) with Tukey's HSD post hoc test, with locality (L) and season (S) as factors with lokality (L) and season (S) as factors

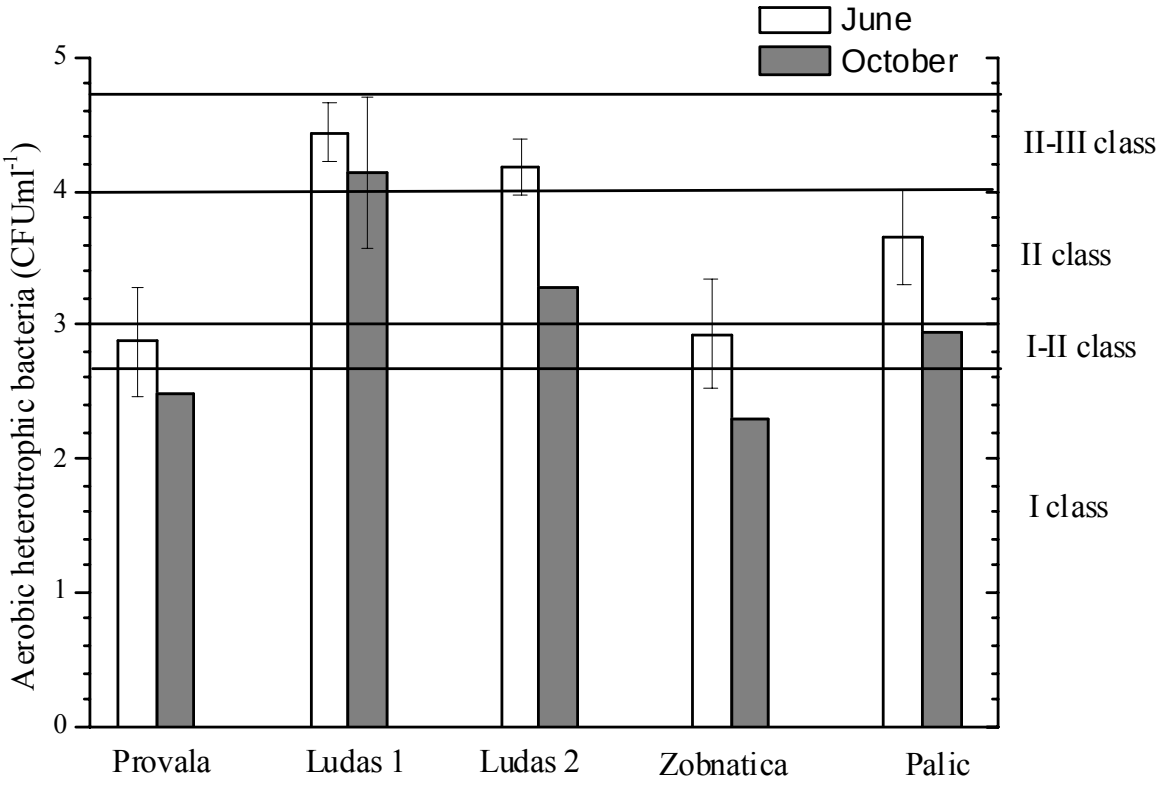

Fig. 5. Water categorization based on aerobic heterotrophic bacteria

phosphorus fraction in examined lake ecosystems (Fig. 2A, Table 1). The highest hydrolysis activity of acetate esterase from measured extracellular enzyme activities (Fig. 2, 3 and 4A) might be in correlation with the presence of broad substrate spectrum of acetate esterase in the lake ecosystems. The reason for significantly higher acetate esterase activities in October than in June at all localities, except for Provala ($p < 0.001$) (Fig. 3A), could be the presence of high level of surfactants in October (Table 1). SUR matters could affect microbial growth by increasing bioavilability of hydrophobic compounds and accelerating their degradation (Bardi *et al.,* 2000). The highest hydrolytic activity of acetate esterase in Palic and Ludas 1 in October was in correlation with TOC levels in water ecosystems. Significantly higher specific enzyme activities ($p < 0.001$) in October than in June (Table 2), among the other factors, could be related to the effect of temperature on enzyme synthesis and activity. This complies with the well known fact that low water temperature could induce greater enzyme production by certain bacteria (Quist and Stokes, 1969).

CONCLUSION

The correlation between aerobic heterotrophic bacteria, aerobic heterotrophic bacteria on R2A medium and total coliforms, on one hand, and enzyme activities of waters of the examined lakes on the other hand, did not prove to be statistically significant. For instance, linear correlation between β-glucosidase activity and bacterial count was established during filtration process at various sites in a groundwater recharge plant at the Ruhr River (Hendel *et al.,* 2001), but not in Danube water (Kavka *et al.,* 1996). The absence of the correlation in the present study could be the result of various factors (physical conditions, nutrient availability, presence of various compounds such as humic acids, biocides and toxins etc.) that influence on heterotrophic bacteria count, enzymatic production and/or activity, and thus on ecophysiology of the ecosystems (Boavida and Wetzel, 1998, Barcina *et al.,* 1997). In addition, some heterotrophic bacteria do not produce phosphatase, lipase and/or glycosidase, while other organisms, including metabolically active but not cultivable microorganisms can produce these enzymes (Barcina *et al.,* 1997). The complexity of the relationships among microbiological and bacteriological parameters indicates the necessity of performing both microbiological and biochemical analyses in order to obtain more relevant data on water quality(Fig.5). Such analysis provides information on sanitary quality of water in one hand, and gives insight into the processes that take part in examined ecosystems. Taking in consideration all examined

parameters, it is obvious that among four lakes of the Province of Vojvodina, Provala and Zobnatica lakes had the best water quality, while Ludas and Palic were overloaded with organic matter both in June and October, 2008.

ACKNOWLEDGEMENT

This work was supported by the grant from the *Ministry of Science* and Technological Developmen of the Republic of Serbia.

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