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Potential of phosphatases for express assessment of self-purification at different types of pollution in running waters

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Abstract

The potential of an express enzymological indicator – phosphatase activity index (PAI) – for assessment of different types of pollution and self-purification potential in running waters was evaluated for three river subcatchments, representative for different ecological situations and impacts. According to the values of correlation coefficients, a significant positive correlation existed among PAI and total microbial count, organic loading and phosphate concentrations. The enzyme activity is useful tool for early identification of risks from point discharge of different wastewaters (treated or non-treated). The role of PAI as an indicator is significant at case of disinfection after treatment of wastewaters in treatment plant – the aquatic microbial community at the discharge point is suppressed but not at a functional level. The results allow classical microbiological and chemical parameters (state variables) to be related directly to the dynamics of the transformation processes by functional variable – PAI.

Key words: extracellular enzyme, functional indicator, organic and nutrient loading, phosphatase activity, running waters

INTRODUCTION

The organic loading and nutrient-related pollution of inland waters are global ecological problems with high risk level for non-achievement of criteria for good ecological state of surface waters according to environmental legislation (Sutton *et al.* 2013; Bouraoui *et al.* 2014). Despite that the field of pollution indication is well-developed for surface waters and variety of indicators is widely used, there is a deficit in early identification of risk situations by function indicators directly related to the pollutants fate and main transformation processes in the ecosystem. The imperative step for prevention, control and effective management of pollution is an application of system of early warning indicators for assessment of type and degree of organic and nutrient loading, and further revealing the self-purification capacity of ecosystem.

The existing network of different transformation processes of pollutants is an essential element for natural improvement of the quality of waters and maintenance of a healthy ecosystem. A leading biotic factor in these processes is the realization of biodegradation, mineralization and nutrient cycling from aquatic microbial assemblages (Hill *et al.* 2012). Due to their wide polyenzymatic profile and flexible adaptation to different physicochemical conditions and substrates, microorganisms respond quickly to organic/nutrient loading fluctuation, primary by changing the level of their enzyme activities (Dao 2011). The focus on enzyme activities as suitable indicators for the level of pollution and self-purification comes from the fact that heterotrophic microorganisms produce a

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wide range of extracellular enzymes to cleave their target substrates outside the cell to sizes small enough for uptake (Ziervogel *et al.* 2012; Arnosti *et al.* 2014). These complex enzyme activities of microbial populations determine the functionality and give the information for the mechanisms and rate of metabolic processes on ecosystem level. Extracellular phosphatases (enzymes that hydrolyze phosphate groups from a wide variety of organic substrates) are a significant component of most algae and bacteria and play an essential role in the recycling of P-containing organic matter. Phosphatases seem to be inducible catabolic ectoenzymes, as are most other hydrolytic ectoenzymes of aquatic microorganisms (Hoppe 2003). Activity of these extracellular enzymes are therefore a good measure for the initial step of carbon cycling in natural microbial assemblages (Arnosti 2011), and are used as indicators of heterotrophic microbial function in contaminated different types of waters, soils and sediments (Duarte *et al.* 2012; Sanchez-Hernandez *et al.* 2017). Phosphatase activities are considered to exist in every aquatic systems, soils and sediments as integral parts of complex enzyme profile and give information about effect of nutrients, organics and pollutants concentration on microbial activities (Chróst & Siuda 2002; Arnosti 2003; Wilczek *et al.* 2005). Bacterial extracellular enzymatic activity is regulated at the ecosystem level by environmental factors and at the microenvironment level by enzyme-substrate interactions (Cunha *et al.* 2010).

The previous studies found and validated the positive correlation between activity of phosphatase enzymes and chlorophyll concentration, viable bacterial count, total phosphate concentration, inorganic phosphate concentration, and temperature. The phosphatase activities are applicable to estimation of organic pollution, phosphorus limitation, eutrophication processes (Matavulj *et al.* 1990; Chróst 1991; Boavida *et al.* 1997; Hill *et al.* 2012) and hazardous pollution in soils and sediments (Todorova *et al.* 2016; Sanchez-Hernandez *et al.* 2017). But the potential of this indicator was not exploited from the aspect of dynamics and effectiveness of self-purification processes. The aim of this study is to apply and evaluate the complex enzyme indicator – phosphatase activity index (PAI) – for assessment of functional microbial response and self-purification effectiveness at different types of pollution in running waters.

MATERIALS AND METHODS

Experimental design and description of selected case studies

For assessment and verification of the potential of phosphatase activities as a functional indicator, the research strategy was extended on large spatial scale and investigations were carried out at three different river subcatchments representative for different ecological situations, types of pollution and impact. The river sectors belong to Iskar River and Ogosta River catchments (both from Danube basin). Study areas are shown on Figure 1.

The first river subcatchment studied (UI) was the upper part of Iskar River and its tributaries before Iskar Dam. This part of the river experiences low level of anthropogenic impact and good ecological/chemical state of surface waters. Only the Palakariya River tributary had a possible risk for pollution with organics and nutrients (Todorova & Topalova 2014). One point source of pollution – discharge of municipal wastewater treatment plant (WWTP) of a small town was registered in this sector.

The second studied sector (MI) was in the middle part of the Iskar River with a high level of different impacts – hydromorphological deterioration and pollution with organics, nutrients and xenobiotics (poor ecological state). This part of the river is a long-term receptor of treated and non-treated urban and industrial sewage from the Sofia area (capital of Bulgaria) and the pollution results in contamination of waters and sediments. In the same time, the construction of the Middle Iskar Cascade of 9 small hydro power plants (SHHP) with barrages and power stations has been started since

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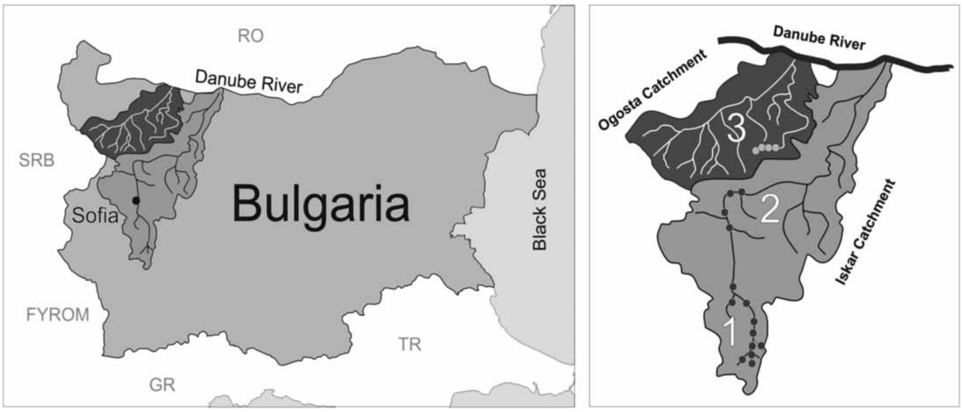

Figure 1 | Location of studied river subcatchments: 1 –Upper Iskar (UI), 2 – Middle Iskar (MI), 3 – Leva River (L).

the 2000's. Flow regulation combined with other anthropogenic impacts deepen the environmental and technological problems in the lotic ecosystem (Todorova *et al.* 2014; Todorova *et al.* 2016).

The third river sector was part of the Leva River (L) near to town of Vratsa with specific point pollution from the dairy industry, combined with discharge of non-treated municipal wastewaters from nearby villages and intensive agriculture activities (moderate ecological state in the whole sector and very poor in the zone of discharge) (Schneider & Topalova 2009).

The assessment of significant environmental risks categorizes the Upper Iskar subcatchment as 'not in risk' and as 'potential risk'; the river sectors of Middle Iskar and Leva River are 'in risk' from organics, nutrients and hazardous substances.

The sampling strategy with periods, sampling sites, number of samples and a brief description of sites is given in Table 1.

The analyses of three groups of indicators were included in the experimental design:

(1) Water quality parameters for assessment of organics and nutrients loading at sampling sites – gives information about the 'substrates and products' of biotransformation processes;
(2) Total microbial count (TMC) as the most frequently used monitoring parameter for the structural and functional state of microbial assemblage – information for the 'biotic factor' of self-purification;
(3) Index of phosphatase activities measured as a total activity – indicator for the functioning of the microbial community and the realization of transformation processes on the enzyme level.

Sampling and analytic procedures

Water samples were collected, transferred into bottles or sterile containers, stored (at 4°C) and processed within 4 hours. The physicochemical parameters (temperature, oxygen concentration, and pH) of water have been analyzed *in situ* immediately after sampling with portable Oxy-meter Handylab Ox1/set and pH-meter Handylab pH11/set (Schott Instruments, Germany).

After transfer to the laboratory, the samples were analyzed in triplicate for determination of COD (Chemical Oxygen Demand – dichromatic EPA 410.4/ISO 6060 method). The ammonium, nitrate and phosphate concentrations were measured after filtering of the samples (pore size 0.45 μm) and by colorimetric methods according to the BNS-EN-ISO standards. The TMC (CFU mL^{-1}) was determined by the use of count-plate technique on Nutrient agar for 24–48 h at 35°C.

PAI was determined as an average value of activities of acidic, neutral and alkaline phosphatases. The method was based on transformation of p-nitrophenolphosphate (Matavulj & Flint 1987).

The data were means of three or more repeats with associated standard deviations. A Spearman correlation was used to investigate the relationships between the enzyme activity and other indicators.

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Table 1 | Sampling details for the three case studies: Upper Iskar (UI), Middle Iskar (MI) and Leva River (L)

Case study	Sampling period	Sampling scale	Sampling sites; number of samples	Codes and descriptions of sites
Upper Iskar (UI)	Winter and summer low flow periods of 2004	Large – subcatchment	10 sites (7 at main stream and 3 at tributaries); 20 samples	UI1 – Beli Iskar River upstream of village Beli Iskar UI2 – Beli Iskar River downstream of village Beli Iskar UI3a – Cherni Iskar River below Govedarci UI3 – at confluence of Beli and Cherni Iskar UI4a – Borovishka Bistrica River UI4 – below Samokov and its WWTP UI5 – near the village of Dragoshinovo UI6 – before inflow of Palakariya River UI7a – Palakariya River UI7 – before Iskar Dam
Middle Iskar (MI)	Summer low and spring high flow periods of 2013–2014	Medium - 33 km sector	4 sites; 16 samples	MI1 – at Prokopanik below Sofia, before the Middle Iskar cascade and Svoge MI2 – at Cerovo below Svoge and SHPP Cerovo MI3 – at Lakatnik, before SHPP Lakatnik MI4 – at Gabrovnitza, below the cascade
Leva River	Summer low flow of 2008	Small < 1 km	5 sites; 5 samples	L1 – before the dairy wastewaters discharge L1a – in the area of water mixing L2 – 5 m after discharge L3 – 50 m after discharge L4 – 100 m after discharge

RESULTS AND DISCUSSION

Case study of Upper Iskar subcatchment

The organic content and phosphate concentrations in the surface waters of Upper Iskar subcatchment were the highest in site UI4 for both seasons (Figure 2). This site was situated immediately downstream the WWTP discharge point. The high nitrogen concentrations, especially ammonium, also indicated typical pollution from municipal wastewater (Table 2). But the microbial count at this site was low – less than 1,000 CFU mL^{-1} comparable to a mountain zone. The microbial community in UI4 site was affected by the disinfection of treated wastewaters in WWTP – near the discharge the bacteria were inhibited by the disinfectants. In the next site (only 500 m downstream) the microbial count was the highest during both seasons – the structural microbial parameters reacted to the high nutrient and organic content in the surface waters but with a replacement downstream. At site UI5 the dissolved oxygen was lower and corresponded to the high microbial activity and intensive aerobic transformation processes. But the PAI had a different dynamics that strictly followed the variations of organic substrate and phosphate concentrations – the highest values, 39.957 ± 12.034 and 30.358 ± 17.550 μM pNPP min^{-1} L^{-1}, were measured at the discharge of the WWTP during the winter and summer low flow seasons (Figure 3). The biota responded to high organic content primarily on the functional level with an increase of extracellular enzyme activities. This express self-purification reaction of hydroecosystem on the enzyme level was attendant with the slow change of the microbial count and a delayed response on the population level. At the discharge of the

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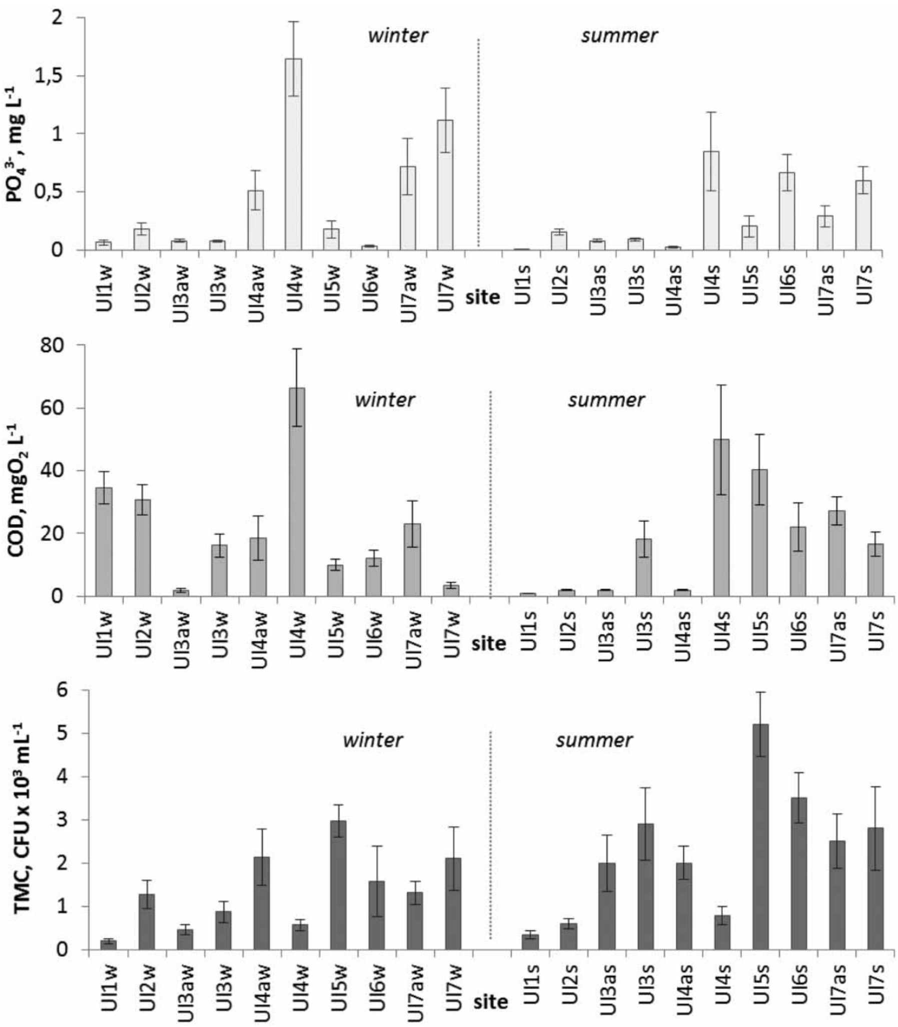

Figure 2 | Dynamics of phosphate concentration, organic content (COD) and total microbial count (TMC) in waters of the Upper Iskar subcatchment.

Table 2 | Basic chemical parameters of waters in the Upper Iskar subcatchment

Site	***UI1***	***UI2***	***UI3a***	***UI3***	***UI4a***	***UI4***	***UI5***	***UI6***	***UI7a***	***UI7***
pH										
Winter	6.98	7.07	7.40	7.22	7.60	7.50	7.57	7.99	7.56	7.61
Summer	7.15	7.28	7.45	7.49	7.96	7.07	7.53	7.32	7.62	7.78
Oxygen, mL^{-1}										
Winter	11.21	11.40	11.51	11.87	11.79	11.63	8.23	11.50	11.43	11.21
Summer	8.33	7.56	8.25	8.61	8.04	8.20	8.13	7.82	8.75	8.40
$N\text{-}NO_3$, mL^{-1}										
Winter	0.299	1.801	1.419	1.394	0.986	1.928	1.394	1.126	1.572	1.884
Summer	0.031	0.050	0.025	0.031	0.038	0.064	0.063	0.089	0.076	0.095
$N\text{-}NH_4$, mL^{-1}										
Winter	0.107	0.143	0.143	0.173	0.891	2.988	0.450	0.373	0.430	0.432
Summer	0.017	0.083	0.120	0.191	0.222	2.004	0.300	0.463	0.162	0.258

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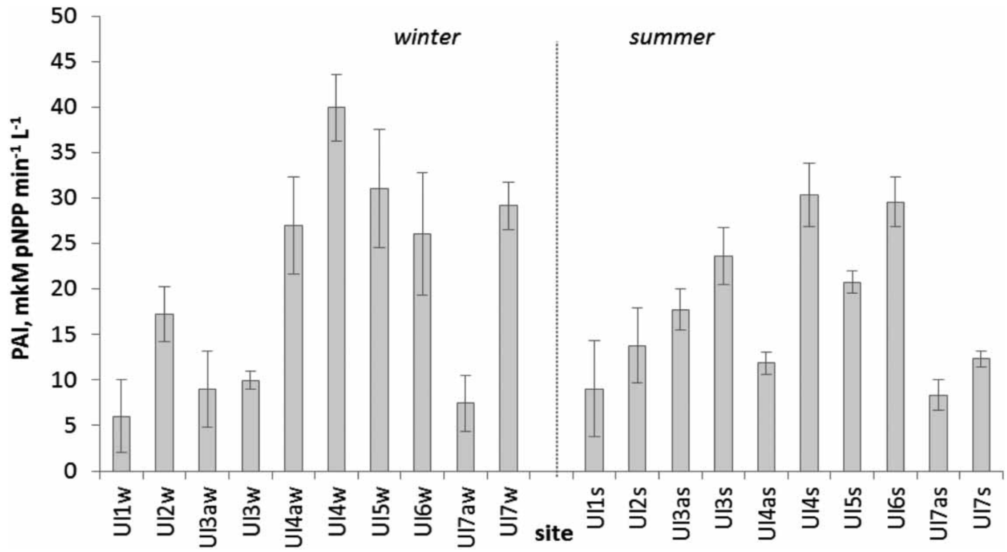

Figure 3 | Phosphatase Activity Index (PAI) in waters of the Upper Iskar subcatchment.

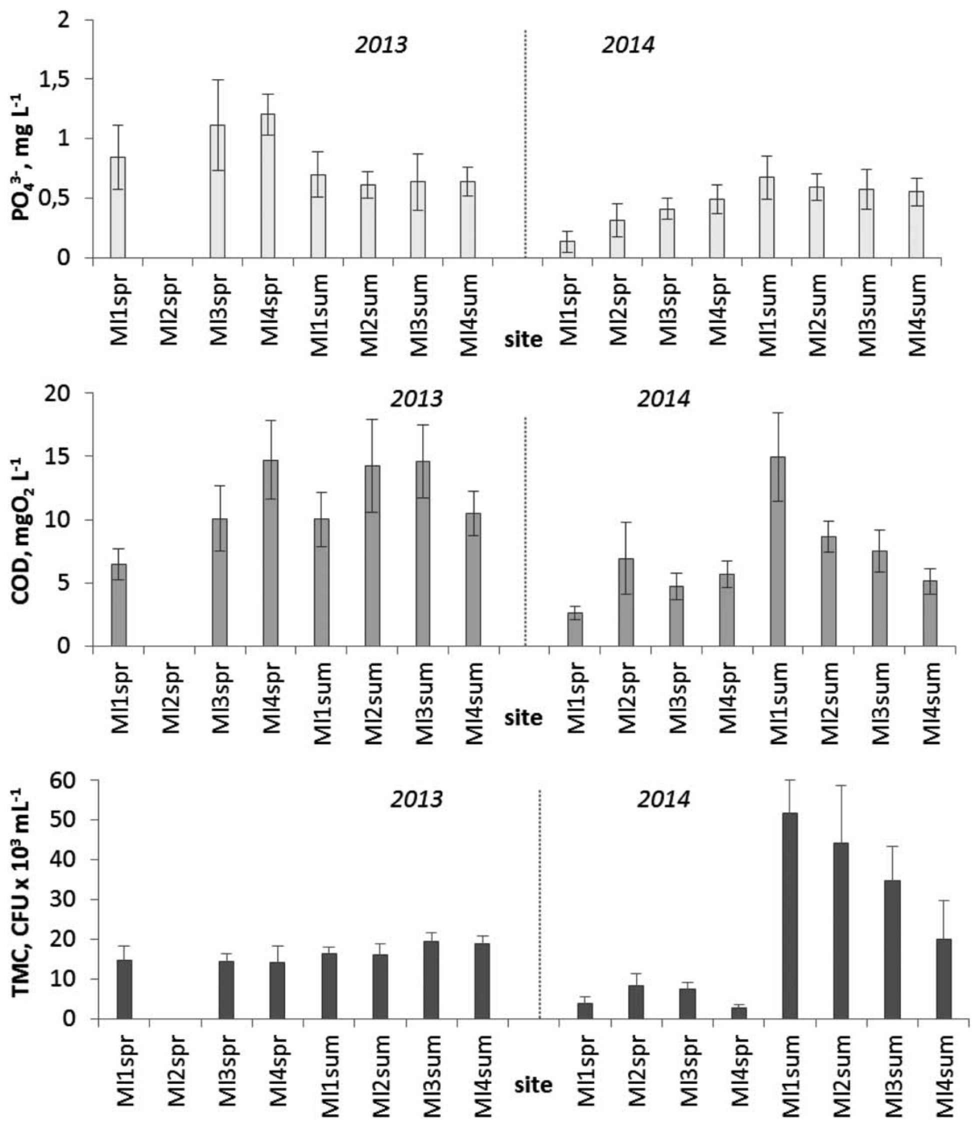

Figure 4 | Dynamics of phosphate concentration, organic content (COD) and total microbial count (TMC) in waters of the Middle Iskar cascade.

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WWTP the less adaptive microorganisms were eliminated from the community structure while the remaining exhibited high biotransformation potential and an intensified enzyme profile. These high adaptive bacteria realized the first reaction of biocenose and their effective functioning eliminated the initial high organics concentration. The PAI was a better indicator than microbial count for this type of risk situation – discharge of treated disinfected wastewaters in a clean ecosystem.

Case study of Middle Iskar cascade

The organic content of surface waters in Middle Iskar cascade measured as COD was low (under 15 mg O_2 L^{-1}) without large spatial and seasonal variations (Figure 4). This part of the river was also affected by the discharge of one large WWTP but the effect of treated wastewaters was not clearly presented because of the 30 km distance. In the river sector there are many other nonpoint sources of pollution – villages without sewages, intensive agriculture and this mixed type of pollution was reflected in the nitrogen and phosphorus dynamics (Table 3 and Figure 4). The measured concentrations were high and indicated a serious nutrient loading on surface waters. The microbial community was also presented with high abundance from 3,000 to 50,000 CFU mL^{-1} (Figure 4). The high microbial count was potentially related to intensive bacterial activity and high rates of main transformation processes. At the same time, the construction of the SHPP cascade based on modification of stream hydraulics and dam compartmentalization of the river channel, created an additional system for retention of nutrients and fast utilization of easy biodegradable organic substrates.

In this type of pollution, the PAI was well presented – 12.46 to 51.17 μM pNPP min^{-1} L^{-1} and this showed intensive transformation processes of P-containing organics and good self-purification capacity of the ecosystem (Figure 5). The spatial variations of PAI followed the change of microbial count and phosphate concentration.

Table 3 | Basic chemical parameters of waters in the Middle Iskar River sector

Site	MI1	MI2	MI3	MI4
pH				
Spring'13	7.45	nd	7.68	7.61
Summer'13	7.72	7.73	7.97	8.21
Spring'14	7.47	7.34	7.71	7.86
Summer'14	7.38	7.65	7.76	7.60
Oxygen, mL^{-1}				
Spring'13	7.45	no data (nd)	9.50	9.34
Summer'13	7.50	7.06	9.93	9.94
Spring'14	7.20	7.31	7.84	7.20
Summer'14	8.70	11.52	10.32	9.40
N-NO_3, mL^{-1}				
Spring'13	0.922	nd	2.195	3.080
Summer'13	2.737	3.055	4.538	4.958
Spring'14	1.142	2.423	3.006	3.493
Summer'14	3.508	3.042	3.144	2.860
N-NH_4, mL^{-1}				
Spring'13	1.143	nd	0.461	0.362
Summer'13	4.184	3.153	1.363	0.796
Spring'14	0.222	0.656	0.336	0.276
Summer'14	1.223	0.800	0.529	0.324

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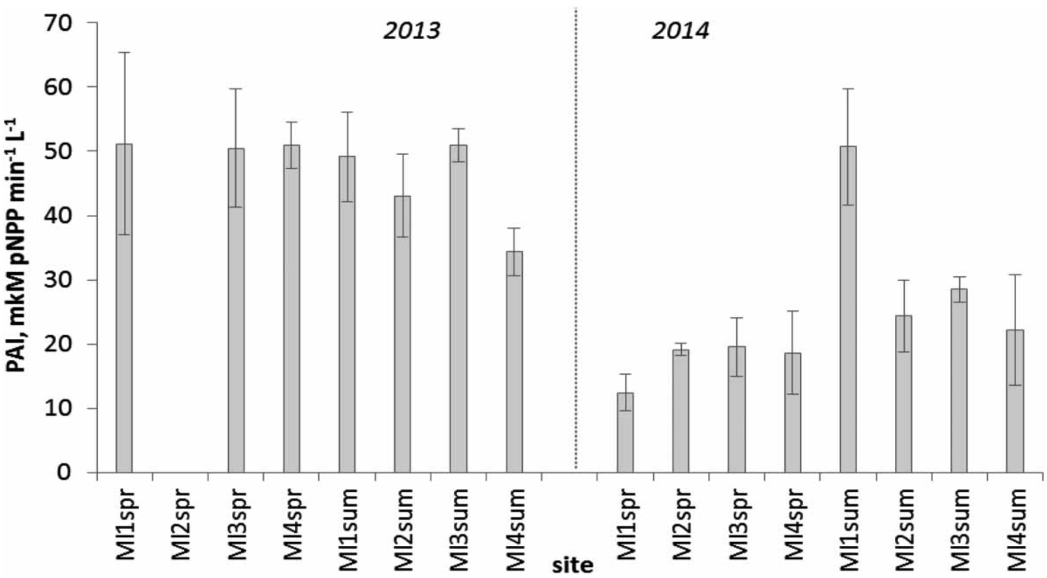

Figure 5 | Phosphatase Activity Index (PAI) in waters of Middle Iskar cascade.

Case study of the Leva River

The third case study – a sector of the Leva River was affected by the discharge of untreated dairy wastewaters. At the first unaffected sampling site, the bacterial abundance, phosphates and organics were very low – indicating a good ecological state of the ecosystem and clean surface waters. At the point of discharge (site L1a), the chemical and microbial parameters had typical dynamics for this type of pollution and the values of COD, nutrients, microbial count increased very extremely (Table 4, Figure 6). TMC values were more than 1,000 times higher, COD – 30 times, phosphates – 5 times compared to the values at site L1. The PAI also increased more than 1,000 times and the value in surface waters of site L1a was 3,390.27 $\pm$ 106.01 μM pNPP min^{-1} L^{-1} (Figure 7). Only 5 m further downstream from the wastewater discharge, the all studied parameters decreased sharply. This indicates a rapid elimination and utilization of organic pollution, adequate functional response of ecosystem and high effectiveness of self-purification processes.

Table 4 | Basic chemical parameters of waters in the Leva River

	L1	L1a	L2	L3	L4
pH	7.78	6.98	7.65	6.98	7.56
Oxygen, mL^{-1}	7.8	6.7	7.5	5.7	7.9
$N\text{-}NO_3$, mL^{-1}	0.852	0.531	0.768	0.596	0.382
$N\text{-}NH_4$, mL^{-1}	0.148	2.731	0.525	1.071	0.422

Relations between pollution indicators

Phosphatases are essential ectoenzymes for realization of P cycling in aquatic environment, take part in mineralization of organic P and release phosphates for macrophytes and algae. This functional role determines the direct relationships of the enzyme activity with the concentration of organics (substrate) and phosphates in the system (product from the enzyme reaction). The analysis of relationships between parameters was done for any particular case and for the all data set (Table 5). PAI correlates positively with high statistical significance with TMC, organic loading and

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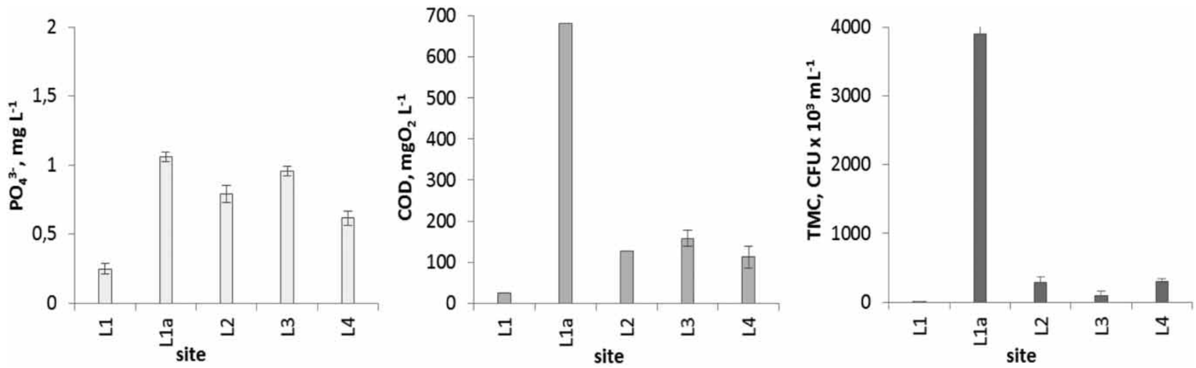

Figure 6 | Dynamics of phosphate concentration, organic content (COD) and total microbial count (TMC) in the Leva River.

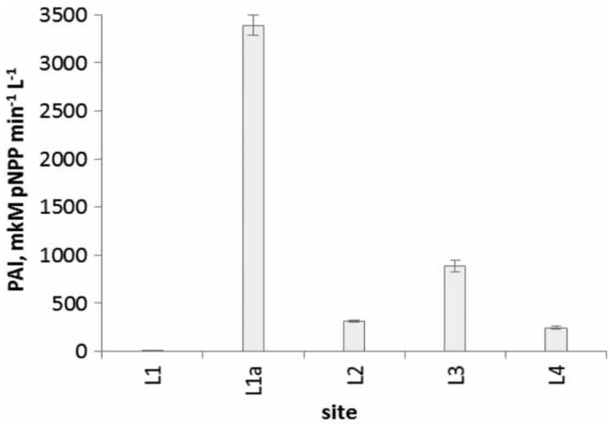

Figure 7 | Phosphatase Activity Index (PAI) in the Leva River.

Table 5 | Spearman correlation matrix between parameters (values are different from 0 with significance level = 0.05)

	PAI – Upper Iskar	PAI – Middle Iskar	PAI – Leva Iskar	Total data set
TMC	0.638	0.616	0.969	0.973
COD	0.684	0.788	0.993	0.985
PO_4^{3-}	0.790	0.697	0.725	0.642

phosphates concentration in all cases studied. The correlation coefficients for the entire data set are rPAI/TMC = 0.973; rPAI/COD = 0.985; rPAI/PO4 = 0.624 respectively. The positive correlation with phosphates is stronger in the Upper Iskar subcatchment – r = 0.790. Berman & Moses (1972) specified three available sources of phosphates in surface waters: (1) ortho-phosphates released from ectoenzymes; (2) intracellular microbial reserves; and (3) inorganic phosphates incoming in waters from pollution. The first source is the most important in clean waters with phosphorus limitation as in the case of the mountainous upper parts of the rivers. Many authors determined an inverse or no correlation between phosphatase activity and phosphates but in lakes and seas where the dominant producers of extracellular phosphatases are algae and the enzyme synthesis is a response to low orthophosphate in the environment and phosphorus competition (Nausch 1998; Wetzel 2001). For the dominant bacterial metabolism phosphatases is produced in response to organic carbon limitation rather than phosphate limitation (Chróst 1991).

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CONCLUSIONS

The indicative potential of phosphatases was widely used in past two decades for estimation of eutrophication processes, nutrient limitation, organic pollution in small ponds, fishponds, lakes, soils, streams. The analyses of these enzyme activities have been part of complex assessment of biotic integrity in aquatic ecosystems based on periphyton assemblages and data have been collected for many US streams (Hill *et al.* 2003). In Europe, the indicator has been applied successfully for assessment of water quality and as an additional parameter for categorization of surface waters in some parts of Danube's watershed. Our study expands the possible use of phosphatases as functional indicator with high potential for differentiation of type and degree of organic pollution and for self-purification effectiveness. This enzyme activity is useful tool for early identification of risks from point discharge of different wastewaters (treated or non-treated) and for additional assessment of organic and nutrient loading in running waters. The role of PAI as an indicator is significant for the case of disinfection after treatment of wastewaters in WWTP the aquatic microbial community at discharge points is suppressed but not on the functional level. The results allow classical microbiological and chemical parameters (state variables) to be related directly to the dynamics of the transformation processes by sensitive enzyme indicator PAI (functional variable).

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