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Short research contribution

Piotr SKÓRCZEWSKI, Zbigniew Jan MUDRYK*

Department of Experimental Biology, Pomeranian Academy,
Arciszewskiego 22 B, 76-200 Słupsk, Poland
*e-mail: mudryk@apsl.edu.pl (*corresponding author*)

CULTURABLE PROTEOLYTIC BACTERIA AND THE ACTIVITY OF LEUCINE AMINOPEPTIDASE IN RIVER ECOSYSTEM

ABSTRACT: The study determined the numbers and distribution of culturable heterotrophic and proteolytic bacteria and the level of leucine aminopeptidase activity in the water of the Słupia River within the town of Słupsk (Northern Poland). River Słupia is 138.6 km long and flowing into the Baltic Sea. The average water discharge of the river is 15.5 $m^3 s^{-1}$ and its slope is about 1.3% which gives it a mountainous character. The numbers of culturable heterotrophic bacteria was determined on iron-peptone agar (IPA) medium and the numbers of culturable proteolytic bacteria was assayed in IPA medium enriched with gelatin. Potential leucine aminopeptidase activity was carried out with the use of fluorescently labelled model substrate MCA-leucine.

The numbers of heterotrophic bacteria ranged between 0.33 to 183.0 × 10^3 cells cm^{-3} (maximum in spring, minimum in winter); the abundance of culturable proteolytic bacteria ranged 0.50 to 31.8 × 10^3 cells cm^{-3} and the maximum was noted in spring, the lowest in summer. The activity of extracellular leucine aminopeptidase ranged from 2.34 to 6.87 μM MCA dm^{-3} h^{-1} and the highest value was noted in spring while the lowest were noted in winter and summer. The values of bacteriological parameters and of leucine aminopepidase activity tended to be higher below the sewage treatment plant. The bacteriological parameters for River Słupia were compared with the relevant data for other river ecosystems.

KEY WORDS: river, culturable proteolytic bacteria, leucine aminopeptidase

Rivers are dynamic ecosystems which carry a significant load of dissolved organic matter (DOM) and particulate organic matter (POM) from both natural and antropogenic sources (Bellos *et al.* 2004). A large proportion of these organic compounds are proteins which are the most important, easily utilizable carbon, nitrogen and energy sources for aquatic heterotrophic bacteria (Wehr *et al.* 1999, Kiersztyn *et al.* 2002). However, bacterial membranes cannot generally be permeated by substrates with a molecular weight higher than approximately 650 Da (Keith and Arnosti 2001). Therefore, prior to being taken up by microbial cells, proteins as well as high molecular weight biopolymers must be hydrolysed by extracellular peptidases into mono- or oligomers, (mainly amino acids, oligopeptides and small peptides) outside the cell (Chróst 1991, Wilczek *et al.* 2004). In this form they can be actively transported across bacterial cell membranes, assimilated by bacteria and used in biosynthesis of cellular structures or respiration processes (Ainsworth and Goulder 1998, Worm *et al.* 2000).

The objective of the present study was to determine the numbers and distribution of culturable (number of colony forming units, CFU) heterotrophic and proteolytic bacteria as well as the activity of extracellular leucine aminopeptidase.

Authors of the present study are fully aware of fact that estimates of bacterial abundance based on CFU method represent only minor fraction of bacteria inhabiting aquatic ecosystem. According to Mokbel and Yamakanamardi (2008) approximately about 1% of the total bacteria can grow on the best artificial media.

This is why during last years in water microbiology several methods and techniques (epi-fluorescence microscopy, laser scanning microscopy, flow cytometry, ATP measurement, PCR-ELISA) to determine the total bacteria number were developed. However because numbers of heterotrophic bacteria determined by CFU method is the major microbial indicator of the organic loading in aquatic ecosystems this method is still used in water microbiology (Mokbel and Yamakanamardi 2008, Siebel *et al.* 2008).

The study was carried out in the coastal Słupia River (northern Poland), flowing into the Baltic Sea. It is 138.6 km long with the surface of the river hydrological basin of about 1623 km^2. Słupia River catchment area is strongly influenced by human activity (agricultural, agrochemical, heavy soil erosion, urbanisation, industrial, deforestation, flow regulation, municipal and industry sewage, hydrotechnical). The average water discharge of the river is 15.5 m^3 s^{-1} and its slope is about 1.3% which gives it a mountainous character. Mean values of selected chemical parameters in water of the River Słupia were following: BOD: 1.9–4.9 mg O_2 dm^{-3}, 0.30–1.31 mg N–NO_3 dm^{-3}, 0.86–1.92 mg N-total dm^{-3}, 0.039–0.088 mg P-PO_4 dm^{-3}, 0.08–0.18 mg P-total dm^{-3} (Moczulska *et al.* 2006). The River Słupia is inhabited by the diverse ichtiofauna, mainly by migratory fish: trout, salmon, grayling, river trout and rainbow trout.

The study area covered a 10 km section of the Słupia River within the limits of the town Słupsk. Water samples were taken at monthly intervals from January to December 2005 at three sites: site 1 was located at the 24th km of the river course, below the sewage treatment plant; site 2 was located at the 31st km of the river course, in the northern part of Słupsk town; site 3 was located at the 34th km of the river course, in the southern part of the town, approximately 250 m above the fish-pass. Water samples were taken from the middle of the river at a depth of about 20 cm directly into sterile glass bottles. The collected samples of water were transported to the laboratory in an ice container at the temperature of about 7°C within 2–3 hrs.

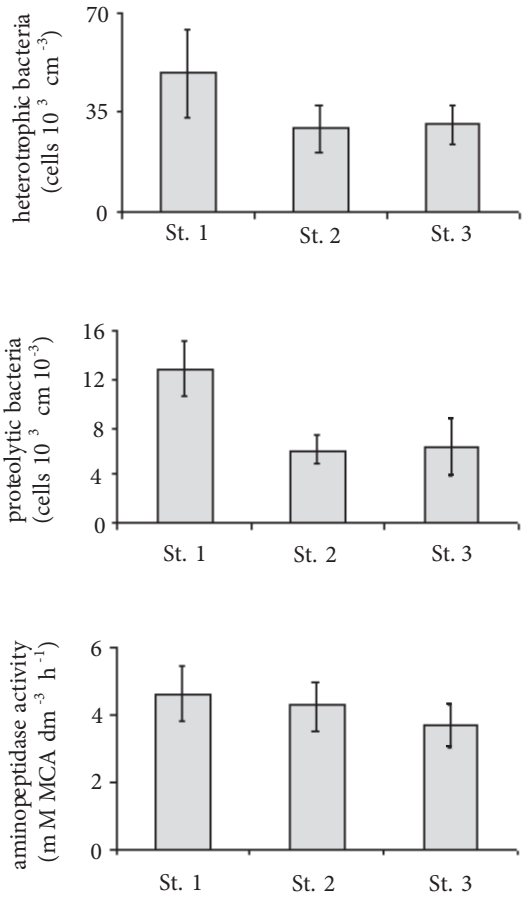

Fig. 1. Numbers of heterotrophic and proteolytic bacteria and leucine aminopeptidse activity in three sites of River Słupia (average from the pooled data of all months). Vertical bars represent standard errors of the mean n = 12. Station 1 – represents the river below sewage treatment plant; station 2 – located in the northern part of Słupsk town; station 3 – situated above the fish-pass.

In order to determine the number of culturable heterotrophic bacteria (CHB),

Table 1. Seasonal dynamics of the number of culturable heterotrophic bacteria (CHB), culturable proteolytic bacteria (CPB) (CFU × 10^3 cm^{-3}) and leucine aminopeptidase activity (LAP) (μM MCA $dm^{-3}h^{-1}$) in water of the River Słupia.

Parameter	Season	Mean	Range	SD
CHB	winter	27.72	0.33–81.67	26.91
	spring	51.02	3.33–109.17	37.67
	summer	35.41	10.50–183.00	55.92
	autumn	31.24	2.67–58.33	22.39
CPB	winter	9.07 (33)[a]	2.33–20.00	6.14
	spring	10.37 (20)	1.83–25.00	8.53
	summer	6.09 (17)	2.67–10.00	2.87
	autumn	8.02 (26)	0.50–31.83	10.82
LAP	winter	2.34	0.67–4.41	1.44
	spring	6.87	3.12–9.01	2.05
	summer	2.67	0.82–5.41	1.34
	autumn	4.79	2.16–8.29	2.21

[a] percentage of proteolytic bacteria in relation to the number of heterotrophic bacteria

the water of collected samples was diluted (10^{-2}–10^{-5}) and the diluted samples were inoculated by the spread method in five replicates on iron-peptone agar (IPA) medium (Ferrer *et al.* 1963). After 6 days of incubation at 20°C, bacterial colonies were counted, and the results expressed as CFU per 1 cm^3 of sample.

The number of culturable proteolytic bacteria (CPB) was assayed in IPA medium enriched with of 20.0 g gelatin per dm^3 (Mudryk and Donderski 1997). After 6 days of incubation at 20°C, clear zone formation around colonies of the bacteria, detected with Frazier`s reagent, indicated the presence of proteolytic bacteria. The results expressed as CFU per 1 cm^3 of sample.

Quantitative measurements of potential extracellular leucine aminopeptidase (LAP) (EC 3.4.1.1) activity were carried out with the use of fluorescently labelled model substrate MCA-leucine (L-leucine-4 methylcoumarinyl-7amide) (Sigma) according to Kierszstyn *et al.* (2002). Fluorescence of the samples was measured in a spectrofluorometer (Hitachi T-2500). Excitation/emission wavelengths were centred at 345/425 nm.

The numbers of culturable heterotrophic bacteria (CHB) in the studied section in the Słupia River ranged from 0.33 to 183.0 × 10^3 cells cm^{-3} (Table 1). Maximum CHB (51.0 × 10^3 cells cm^{-3}) was noted in spring and minimum (27.7 × 10^3 cells cm^{-3}) in winter. The highest average number of heterotrophic bacteria was determined at site 1 located below the sewage treatment plant (Fig. 1).

The numbers of culturable proteolytic bacteria (CPB) during the annual study cycle oscillated between 0.50 and 31.8 × 10^3 cells cm^{-3}. (Table 1). The maximum in this physiological group of bacteria was noted in spring (10.4 × 10^3 cells cm^{-3}) and the minimum in summer (6.1 × 10^3 cells cm^{-3}). Bacteria capable of hydrolysing proteins accounted for 17 to 33% of the culturable number of heterotrophic bacteria. The numbers of proteolytic bacteria at site 1 located below the sewage treatment plant were two times higher than at other study sites (Fig. 1).

The maximum activity of LAP (extracellular leucine aminopeptidase) was observed in the spring (6.87 μM MCA dm^{-3} h^{-1}) and the minimum (2.34–2.67 μM MCA dm^{-3} h^{-1}) of the activity of those enzymes was noted in the winter and summer. The highest average activity of extracellular leucine aminopeptidase was determined below the sewage treatment plant (site 1) (Fig. 1).

The numbers of culturable heterotrophic bacteria in the water of the Słupia River oscillated between 0.3 and 183 × 10^3 cells cm^{-3}. This range was consistent with the range reported in earlier studies for the water of the River Słupia obtained nearly 25 years ago by Mudryk (1981). Considerably higher numbers of heterotrophic bacteria (66.4–1426.9 × 10^3 cells cm^{-3}) was determined by Małecka and Donderski (2006) in the water of the River Brda (the left-bank tributary of the River Vistula) and by Niewolak (1998) in the water of the River Czarna Hańcza (the largest

river in Wigry National Park) (0.3–55050.0 × 10^3 cells cm^{-3}).

Bacteria capable of hydrolysing proteins constituted a dominant physiological group of microflora in water basins amounting to as much as 20 to 97% of the culturable heterotrophic bacteria numbers (Mudryk and Donderski 1997, Worm *et al.* 2000). The data on the abundance of proteolytic bacteria in our study are comparable (19–38%) with the values previously reported from this area (Mudryk 1981).

In rivers the enzymatic breakdown of proteins was clearly dominated by the activity of aminopeptidases (Chappell and Goulder 1995 and Wilczek *et al.* 2004). In the water of the River Słupia the potential activity of leucine aminopeptidase varied from 0.67 to 9.01 μM MCA dm^{-3} h^{-1}. The range of the activity of leucine aminopeptidase in the River Słupia was higher than the ranges reported in other rivers of comparable size and trophic character (Chappell and Goulder 1995, Brown and Goulder 1999, Ainsworth and Goulder 2000). According to Foreman *et al.* (1998) aminopeptidase activity in water basins depends to a great extent on the concentration of proteins. Wilczek *et al.* (2004) pointed out to a high activity of aminopeptidase in highly eutrophicated water basins in which concentrations of proteins are high. This is probably the reason why a high potential activity of leucine aminopeptidase comparison in other rivers, was also noted in the water of the eutrophicated the Słupia River.

In natural waters, bacteria are considered to be the major microbial source of different extracellular proteinases mainly of extracellular leucine aminopeptidase (Chróst 1991, Worm *et al.* 2000). According to Rosso and Azam (1987) the highest level activity of proteolytic enzymes occurred when number of protein hydrolysing bacteria were increasing. In the water of the River Słupia the coupled increase in leucine aminopeptidase activity and the number of proteolytic bacteria (site 1) was also demonstrated.

In the water of the River Słupia the values of both bacteriological parameters and leucine aminopepidase activity, tended to be higher in below the sewage treatment plant (site 1). Increased numbers of culturable bacteria and leucine aminopeptidase activity due to sewage works has been shown also in English rivers (Chappell and Goulder 1994, Brown and Goulder 1999). According to Kang and Goulder (1996) and Ainsworth and Goulder (2000) enrichment by sewage-works effluent sometimes causes an increase in bacteria cell size and in the proportion of metabolically active cells as well as an increase in the abundance of surface associated bacteria.

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