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Microbial communities in a strongly anthropogenic affected stream

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Abstract: Microbiological indicators (total coliforms, faecal coliforms, *E. coli* and enterococci) and phylogenetic groups (domain *Archaea*, classes *Alpha-, Beta-, Gammaproteobacteria* and the *Cytophaga-Flavobacterium* group) detected by FISH were detected in profiles of a highly anthropogenically-affected stream (Luzická Nisa; Northern Bohemia, Czech Republic). This study aimed to assess the changes in the microbial communities of such a polluted stream, and possible relationships between "classic indicators" and the phylogenetic groups. One particular aim concerned a characterisation of the fluorescence *in situ* hybridization (FISH) method, the source of any uncertainty and its limit in terms of quality control (QA/QC). Of the phylogenetic groups studied, the *Proteobacteria* phylum was more abundant in comparison to the *Cytophaga-Flavobacterium* group or the *Archaea* domain. The profile Lucany (above the start of city urban areas) was very different from later downstream profiles, because of its very low faecal bacteria content, low counts of *Gammaproteobacteria,* and evident dominance of the *Cytophaga-Flavobacterium* group together with *Betaproteobacteria.* Later profiles did not show such large differences among themselves. The group of *Gammaproteobacteri*a was very common mainly in profiles with high amounts of untreated faecal pollution. The repeatability of counting bacteria by the FISH method was 14 % on average, an "uncertainty" similar to that of cultivation methods.

Key words: Anthropogenically-affected stream, microbiological indicators, FISH, *Proteobacteria, Archaea,* DAPI

Introduction

Bacterial communities can be studied by many different approaches, but the most common approach is indirect and based on the detection of indicator microorganisms, mainly represented by indicators of faecal pollution such as total coliforms, faecal coliforms, *E. coli* and intestinal enterococci, using standard cultivation methods given in European or National Standards. Another approach is based on culture-independent methods that can sort bacterial cells without the need for cultivation. This is useful as it has been estimated that only about 5 to 10% of all bacterial species are cultivable. For this classification of bacteria the molecules 16S and 23S rRNA are standard (Wagner et al. 2003). The domains *Archaea* and *Bacteria* have prokaryotic characteristics, and are presently divided into 26 phyla. The largest phylum is *Proteobacteria* with 5 classes (*Alpha-*, *Beta-*, *Gamma-*, *Delta-* and *Epsilonproteobacteria*). The new edition of Bergey´s Manual (2005) is based on this phylogenetic system. rRNA-targeted oligonucleotide probes are ideal for studying this complex microbial community and the bacterial cells can be detected by the method of fluorescence *in situ* hybridization (FISH).

Bacterial communities in rivers, detected by FISH, have been studied by several authors. The factors influencing the detection of bacterial cells using FISH were summarized by Bouvier & del Giorgio (2003). Cells detected with the Eubacterial probe EUB338 varied from 1 to 100% of the total bacterial count, and in freshwater 20 to 92%. The source of this large variability could have been due to the protocols of the methods used, or depended on the physiological conditions of the cells. Brűmmer et al. (2000) studied bacterial communities in sediments of the Elbe (medium level of pollution) and one of its tributaries, the massively polluted Spittelwasser. They showed the importance of *Betaproteobacteri*a, which formed the largest single group (31% on average) in the Spittelwasser at all times (in the Elbe, it was only 18%). Clear seasonal peaks of abundance were observed for *Planctomycete*s and the *Cytophaga-Flavobacterium* cluster. The ecology of the *Cytophaga-Flavobacterium* group,

which is especially proficient in degrading various biopolymers, has been described by Kirchman (2002). Physiological activity and the community structure of planctonic and biofilm microbial communities in an urban river were studied by Araya et al. (2003). FISH analysis revealed that the bacterial populations in both streams and biofilms were dominated by *Betaproteobacteria* and the *Cytophaga-Flavobacterium* group; *Eubacteria* represented 55-73% of total bacterial counts in plankton samples. Mlejnkova & Sovova (2010) studied the differences in microbial community structure with respect to water pollution levels and seasonal changes; again, *Betaproteobacteria* and the *Cytophaga-Flavobacterium* group predominated in the polluted surface water from South Moravia. Tirodimos et al. (2009) combined both cultivation and FISH analysis methods for their study of the bacterial community in samples from the river Aliakmon; here, *Firmicutes* rather than *Actinobacteria* dominated the Gram-positive group, and *Betaproteobacteria* and *Gammaproteobacteria* dominated the Gram-negative group, although an enrichment step used might have favoured the *Gammaproteobacteria* class. Seasonal and along-river variations were observed for Gram-negative bacteria, but not for *Firmicutes*. The quantitative detection of specific *Bacteriodales* by 16S rRNA-based assays is nowadays often used in microbial source tracking (MST) (Balasté & Blanch 2010, Kildare et al. 2007).

This study attempted to describe the level of faecal microbial indicators and communities (based on detection of phylogenetic groups by FISH) in the River Luzicka Nisa (part of the River Odra catchment, Northern Bohemia, Czech Republic; total length of the Czech part of the river being 53 km) around the city of Liberec. This stretch of the river is strongly affected by anthropogenic (mainly municipal) pollution. The river (stream) is rather small – having a flow rate (Q_{355}) of 0.479 $m^3 \cdot s^{-1}$ in the centre of Liberec - and the great effect of changes of flow on microbial pollution is clearly evident. The microbiological study was complemented by the measurement of some basic chemical indicators.

The study aimed to assess how important are the changes in the microbial communities in such a polluted stream, and whether there is any relationship between "classic indicators" and phylogenetic groups. A particular aim of this study concerned a characterisation of the fluorescence *in situ* hybridization (FISH) method, the source of uncertainties and their limits from the point of view of quality assurance and quality control (QA/QC).

Material and methods

Sampling

Sampling was performed during three years (2006-2008), four times per year, and grab samples (samples taken at one point in time at one sampling place) were taken. Microbiological analyses were completed within 18 hrs of sampling, and samples for microscopic examination (total counts of bacteria and FISH) were immediately fixed with sterile 38 % formaldehyde (50 µl/1 ml of sample) after sampling and stored at 4 °C until further processing. A list of sampling points is given in Table 1, and a map of the area shown in Figure 1.

Methods of examination

The following microbiological indicators of faecal pollution were determined: total coliforms (Endo agar, cultivation for 24 hrs at 36°C according to Czech Standard CSN 75 7837); faecal (thermotolerant) coliform bacteria (mFC agar, cultivation for 24 hours at 44°C according to Czech Standard CSN 75 7835); *Escherichia coli* (determined among faecal coliform bacteria according to the basis of activity of enzyme β-D-glucuronidase using a fluorogenic substrate, cultivation for 4 hours at 36°C according to Czech Standard CSN 75 7835); and intestinal enterococci (cultivation on Slanetz-Bartley agar for 48 hours at 36°C and a confirmation on bile aesculine agar with sodium azide according to Standard EN ISO 7899-2).

The chemical indicators were determined by Czech standard methods.

Before the FISH procedure the samples were filtered through a polycarbonate filter (pore size 0.2 µm) and washed with deionised water (Sekar et al. 2003). Filtration was performed with equipment for vacuum filtration with hand-operated vacuum generation (work press –17 kPa, diameter of filter area 2.5 cm). The filter was cut into small sections.

Microorganisms from the domain *Bacteria* - classes *Alpha-*, *Beta-*, and *Gammaproteobacteria*, the group of *Cytophaga-Flavobacterium* and microorganisms from the domain *Archaea* were detected by the method of fluorescence *in situ* hybridization (FISH). Microscopic examination was performed using an Olympus BX41 fluorescence microscope equipped with a DP70 camera. A filter set for DAPI (358 nm excitation, 463 nm emission) and for Cy3 (549 nm excitation, 562 nm emission) was used. The probes used are given in Table 2. All probes were labelled by fluorochrome Cy3 on the 5´- end, and competitors were not labelled. Sequences were taken from the ProbeBase of Vienna Ecology Centre database, Faculty of Life Sciences at University of Vienna - Department of Microbial Ecology (Loy et al. 2007). Probes for the detection of *Archaea* were applied in the ratio of 1:1. The FISH procedure was performed according to Amann (1995) and Nielsen et al. (2009).

Hybridisation was performed on black epoxy-resin-coated glass with 6 or 8 wells (Marienfield, Germany). Filter sections with samples were dehydrated in an ascending ethanol series (3 min each, 50%, 80% and 100% ethanol and placed on single wells.) Hybridisation was carried out in a humid chamber for 2 h at 46°C in 8 µl hybridisation buffer containing 0.9 M sodium chloride, 0.01% (w/v) SDS, 10 mM Tris-HCl (pH 8.0), formamide (final concentration 35%) and 5 ng of probe. After hybridisation, the filter sections with sample were washed in pre-warmed washing buffer for 20 min at 48°C. Composition of the washing buffer was: 20 mM Tris-HCl (pH 8.0), 0.01% (w/v) SDS and 0.08 M sodium chloride. To remove salts and the rest of the probe the samples were rinsed with deionised water. After air drying in the dark, samples were prepared for DAPI staining.

DAPI (Sigma-Aldrich) staining was performed with a working solution of 1 µg/ml concentration, 15 µl of which was applied to the filter sections. Samples on the filter were incubated for 5 minutes at room temperature in the dark and washed with deionised water. After drying in the dark and embedding the filters with Citifluor AF1 (Citifluor Ltd., UK) they were prepared for microscopy examination.

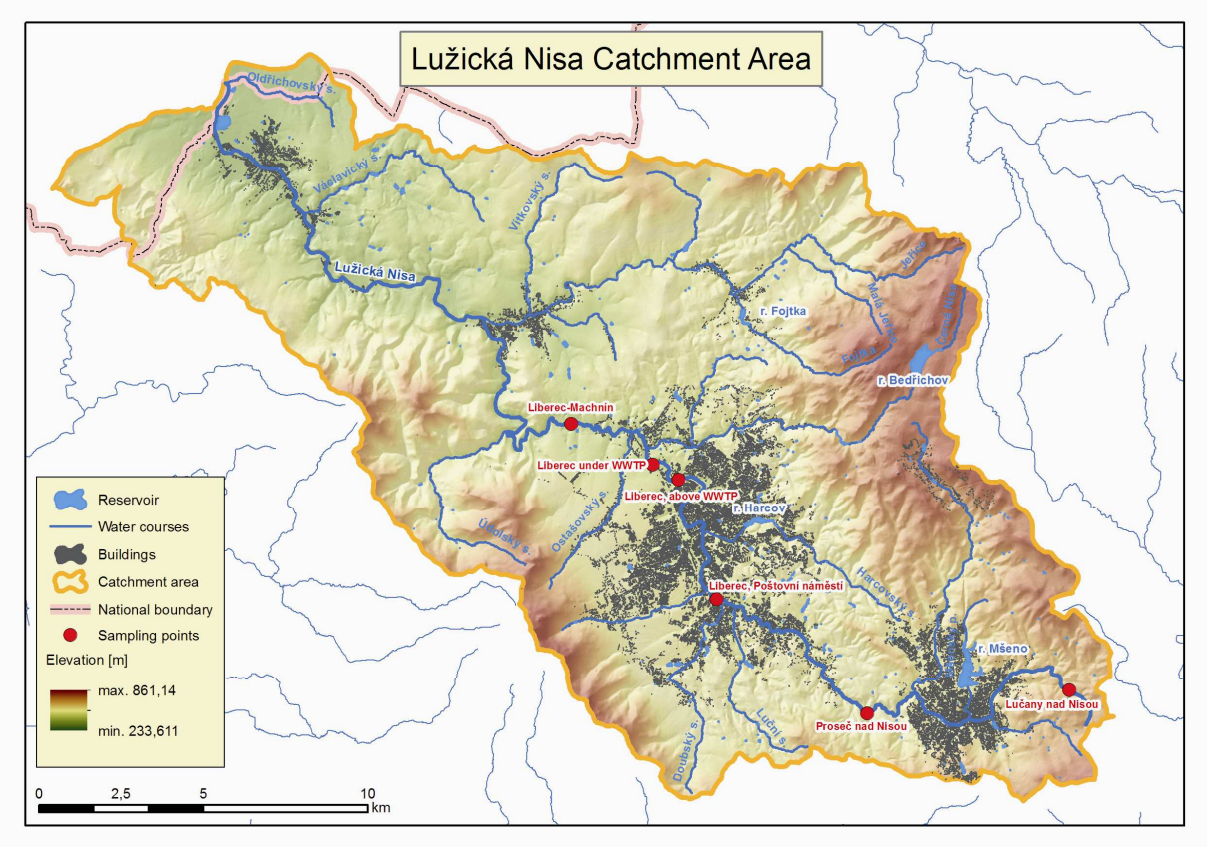

Fig 1: Map of the sub-catchment of Luzicka Nisa (part of the River Odra catchment, Northern Bohemia, Czech Republic; total length of Czech part of river - 53 km) [made by Tomas Fojtik]

Results and Discussion

Our results confirmed good water quality at the sampling point Lucany (above the first built-up urban areas). The high level of microbial pollution, significantly dwarfing the values permissible under Czech regulations (according to 23/2011 Collection of laws – for Czech Republic, i.e. Percentile 90 2500 cfu/100 ml – 3.4 $\log_{10}$ for *E. coli*, 4000 cfu/100 ml – 3.6 $\log_{10}$ for faecal coliforms and 2000 cfu/100 ml – 3.3 $\log_{10}$ for enterococci) was almost evident after the first few kilometres of the stream (in the city of Jablonec nad Nisou). In the

next couple of profiles, mainly in the built-up area of the city of Liberec (after 15-20 km of stream), the values of faecal pollution indicators exceeded permissible values almost ten times (total coliforms on average 400,000 cfu/100 ml – 5.6 $\log_{10}$, faecal coliforms 65,600 cfu/ml – 4.8 $\log_{10}$, *E. coli* 43,600 cfu/100 ml – 4.6 $\log_{10}$ and enterococci 22,000 cfu/100ml – 4.3 $\log_{10}$). The next increases in microbial pollution were detected below the waste-water treatment plant (WWTP) Liberec and then, after a further 4 km at Machnin, levels of faecal indicators became more favourable. Among the worst results from the chemical indicators was that of ammonia nitrogen being found: values exceeding the permissible limit were detected in the profile downstream of the city of Jablonec nad Nisou. On the other hand, amounts of organic compounds (dichromate value results; COD) were suitable even in the profile at Machnin, downstream of the city of Liberec (according to 23/2011 Collection of laws, i.e. average value for N_{Amon} 0.23 mg/l, COD_{Cr} 26 mg/l, BOD_5 3.8 mg/l and total P 0.15 mg/l). Summaries of the results of the microbiological and chemical analyses are given in Figures 2 to 4. The sub-catchment lies in a mountainous region, and problems with poor connections in the sewage network still occur in certain parts. Also found in this stream were changes in the microbial communities , depending on the flow rate. The counts of faecal coliforms and actual stream flow in the profile at Prosec are given in Figure 5. For the profile at Prosec counts of faecal coliforms were directly proportional to actual flow.

Total bacterial counts (DAPI) presented an abundance of 10^7 /ml on average but did not exhibit such great changes throughout the whole longitudinal stream profile as did the indicators of faecal pollution (see also Baudisova et al. 2008). Of the phylogenetic groups studied, the *Proteobacteria* phylum was more abundant in comparison to the *Cytophaga-Flavobacterium* group or the *Archaea* domain. *Proteobacteria* were detected at an abundance of 10^6 /ml on average, whereas *Cytophaga-Flavobacterium* and *Archaea* were at an abundance of 10^4 to 10^5 /ml. The most abundant group in the profile sampled before the city built-up areas start (Lucany) was the *Cytophaga-Flavobacterium* group, together with *Betaproteobacteria*, both of which are typical for surface waters (Mlejnkova & Sovova, 2010). Only a small proportion of *Gammaproteobacteria* (which includes all species of *Enterobacteriaceae*) was detected in this profile, which corresponded to very low faecal pollution. The *Gammaproteobacteri*a group was mainly very common in those profiles with high faecal pollution that was untreated. Figure 6 shows the resulting predominance of the relative proportions of *Alpha-* and *Betaproteobacteria* below the effluent from Liberec WWTP (the proportion of *Alpha-proteobacteria* around 20% is similar to that detected by Mlejnkova & Sovova 2010 in municipal wastewaters). However, the situation just above the WWTP was different, the prevalence of the relative proportions of *Gammaproteobacteria* and *Cytophaga-Flavobacterium* being very evident. *Alphaproteobacteria* were more highly represented by their relative proportions in those profiles with some form of natural self-purificaton (profiles Prosec and Machnin); for instance, the genus *Caulobacter* belonging to the *Alphaproteobacteria*. The proportions of all phylogenetic groups of *Alphaproteobacteria* detected was 28-48 % of total counts, data which corresponded to similar results in the literature (Araya et al. 2003; Bouvier & del Giorgio 2003).

Certain characteristics of the FISH method were chosen for estimation for the purposes of quality assurance and control (QA/QC). Repeatability of counting (number of cells being evaluated more than once by one worker) for the FISH method was between 0 and 42%, 14% on average (more specifically it was 2-40% for *Alphaproteobacteria*, 0-37% for *Betaproteobacteria*, 0-38% for *Gammaproteobacteria*, 0-29% for *Cytophaga-Flavobacterium* and 5-42% for *Archaea*). The repeatability of direct counts (DAPI) was 6 to 55%, 25% on average. The repeatability of counting for FISH was quantified from two measured values and for DAPI from two to four measured values. In addition, we should mention that the repeatability of counting results for *Gammaproteobacteria* from seven

values obtained from one filter was 35%, and for DAPI from 8 results was 27%. The repeatability of counting the number of cells on one microscopic image was 1 to 2 %.

It was also found that samples for FISH detection were stable for up to 1 month; decreases in the numbers of counts of from 1 to 2 orders of magnitude after 4 month of storage were also detected. Our studies confirmed that it was better to store samples on the filter and not in the formaldehyde solution; numbers of cells on the filter were stable for longer (data not shown).

Conclusions

Microbiological indicators for the detection of municipal pollution were more sensitive than that of chemical indicators, mainly because these microbiological indicators showed increases above the limits given by Czech legislation very soon after the point of pollution. From the chemical indicators, one of the worst results was the detection of ammonia nitrogen; values exceeding the permissible limit were detected in the profile downstream of the city of Jablonec nad Nisou. On the other hand, amounts of organic compounds (dichromate value results; COD) were of a suitable level even in the profile at Machnin downstream of the city of Liberec. Of the phylogenetic groups studied, the phylum of *Proteobacteria* was more abundant in comparison to the *Cytophaga-Flavobacterium* group or the *Archaea* domain. The profile at Lucany (before urban areas begin) was very different from the rest of the profiles, having a very low content of faecal bacteria, low counts of *Gammaproteobacteria,* and a clear dominance of the *Cytophaga-Flavobacterium* group together with *Betaproteobacteria.* The profiles further along the stream did not exhibit such large differences among themselves. The *Gammaproteobacteri*a group was mainly common in those profiles with significant quantities of untreated faecal pollution. The repeatability of counting bacteria using the FISH method was 14 % on average, which is similar to the "uncertainty" commonly found in cultivation methods.

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Appendix

Tab 1: List of sampling points

Sampling point	GPS characteristics	Characteristics of profile and number of samples (n)
Lucany	50°44´ N 15°13´ E	unpolluted profile above the urban area of Jablonec (2006-2007, n=8)
Jablonec nad Nisou – Paseky	50°44´ N 15°11´E	polluted profile, in the middle of urban area of Jablonec nad Nisou (2006-2007, n=8)
Prosec nad Nisou	50°43´N 15°08´ E	below the urban area of Jablonec nad Nisou, the last polluted profile with some self-purification processes* (2006-2009, n=11)
Liberec – Postovni square	50°45´N 15°04´E	in the middle of the city urban area of Liberec, strongly anthropogenically- affected profile (2008-2009, n=8)
Liberec above the WWTP	50°46´ N 15°02´E	below the city urban area of Liberec, but above the effluent of the WWTP Liberec, a highly anthropogenically-affected profile (2006-2009, n=12)
Liberec under the WWTP	50°47´N 15°02´E	below the city urban area of Liberec, and below the effluent of the WWTP Liberec, > 100 000 PE, profile strongly affected by wastewaters (2008-2009, n=8)
Machnin	50°47´N 14°59´E	4 km below the effluent of the WWTP Liberec, some signs of natural self-purification processes observed (2006-2009, n=12)

Note: self-purification processes - biomass of microorganisms can increase due to utilization of final products of chemical and biological decomposition of polluting compounds.

Tab 2: Probes used for the detection of phylogenetic groups

Target group	Probe	Sequence	Reference
Alphaproteobacteria	ALF968	5'- GGT AAG GTT CTG CGC GTT -3'	Neef 1997
Betaproteobacteria	Probe labelled: BET42a	5'- GCC TTC CCA CTT CGT TT -3'	Manz et al. 1992
	Competitor: c BET42a	5'- GCC TTC CCA CAT CGT TT -3'	
Gammaproteobacteria	Probe labelled: GAM42a	5'- GCC TTC CCA CAT CGT TT -3'	Manz et al. 1992
	Competitor: cGAM42a	5'- GCC TTC CCA CTT CGT TT -3'	
Cytophaga-Flavobacterium	CF319a	5'-TGG TCC GTG TCT CAG TAC-3'	Manz et al. 1996
Archaea *	ARC344	5'- TCG CGC CTG CTG CIC CCC GT -3'	Raskin et al. 1994
	ARC915	5'-GTG CTC CCC CGC CAA TTC CT-3'	Stahl & Amann 1991

* Probes ARC344 and ARC915 for detection of *Archaea* were applied together in the ratio 1:1.

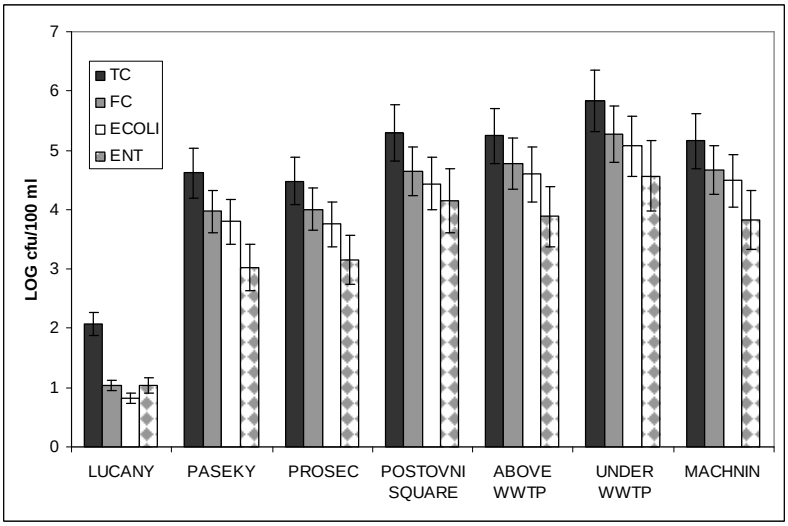

Fig 2: Microbiological indicators measured in the stream of Luzicka Nisa: TC – total coliforms; FC- faecal coliforms; ECOLI- *Escherichia coli*; ENT- intestinal enterococci; (geometric mean and relative standard dev.)

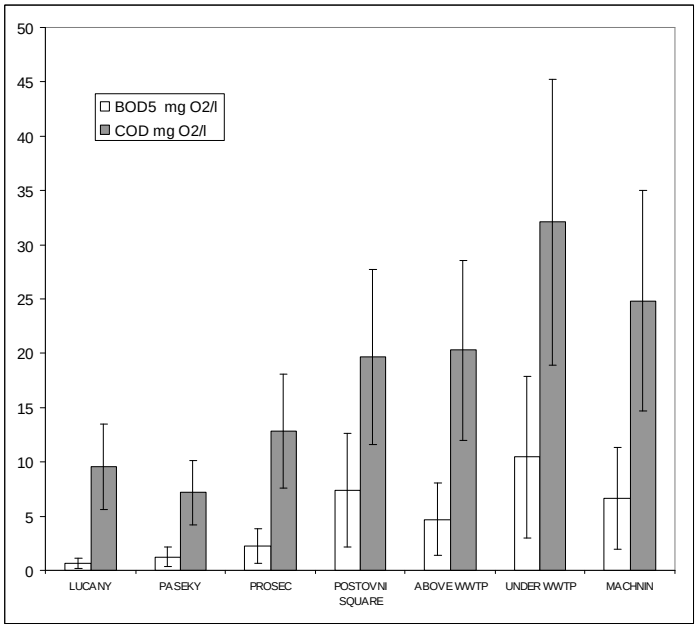

Fig 3: Chemical indicators BOD_5 and COD (arithmetic mean and relative standard deviation) in the stream of Luzicka Nisa – Part I

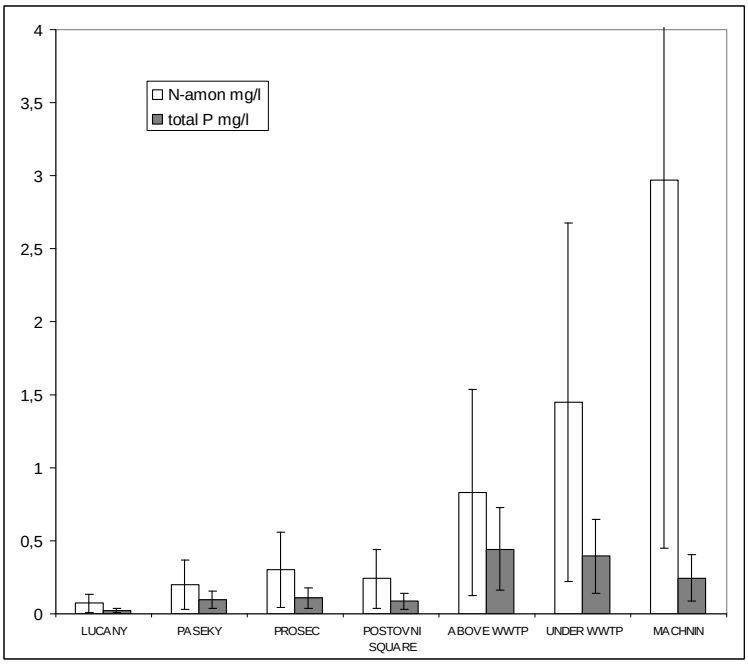

Fig 4: Chemical indicators N_{Amon} and total phosphorus (arithmetic mean and relative standard deviation) in the stream of Luzicka Nisa – Part II

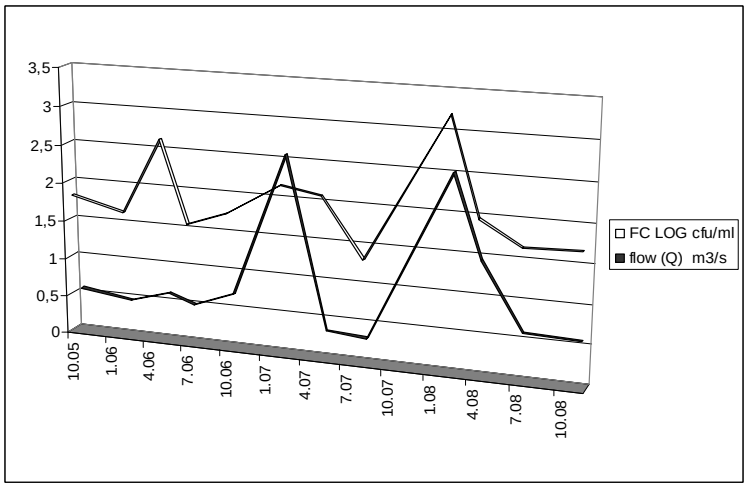

Fig 5: Changes of counts of faecal coliforms and actual stream flow in profile at Prosec

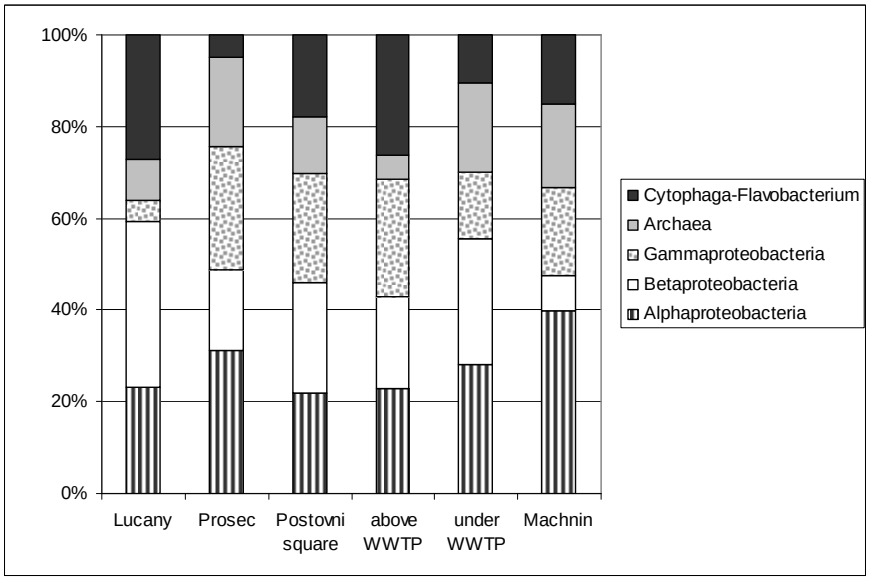

Fig 6: Relative proportions of groups of detected bacteria in studied profiles