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Changes in prokaryotic community composition in the small wastewater treatment plant of Zbytiny during treatment processes

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Abstract
Changes in bacterial community composition during treatment processes were detected in the Zbytiny wastewater treatment plant (Czech Republic, South Bohemia). Samples were taken from the wastewater inflow, outflow from biological lines, the filter, and both stabilization ponds. Indicators of faecal pollution (faecal coliforms, *Escherichia coli*, and intestinal enterococci) were detected by standard cultivation methods, and fluorescence microscopy was used for direct prokaryotic counts. The phylogenetic groups (the domain Archaea, from domain Bacteria classes Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, and *Cytophaga-Flavobacterium*) were detected by the method of fluorescence *in situ* hybridization (FISH). Screening was performed for pathogenic bacteria (thermotolerant campylobacters, salmonellae, and coagulase-positive staphylococci). The counts of indicators of faecal pollution in wastewater entering the treatment plant were of 10^3–10^4 cfu.ml^{-1}, whereas in the outflow from biological lines it averaged 10^1–10^2 cfu.ml^{-1}. Counts of microbiological indicators in the final outflow (after advanced treatment) were less than 1 cfu.ml^{-1}. In effluents the numbers of pathogenic bacteria were very low (in total effluents even less than 10^{-2} cfu.ml^{-1}). The total counts of Prokaryotes ranged from 10^7 (inflow) to 10^6 cell.ml^{-1}. The most abundant phylogenetic groups were the classes of Betaproteobacteria and Gammaproteobacteria across all sampling points.

Key words: wastewater treatment plant, faecal indicators, fluorescence *in situ* hybridization, waterborne pathogens, removal efficiency

Introduction

In the Czech Republic, treatment processes in wastewater treatment plants (WWTPs) are most often based on mechanical pre-treatment and subsequent biological treatment; the application of any additional/advanced treatment processes (filters, wetlands, stabilisation ponds, etc.) is, however, rather rare. Mechanical-biological WWTPs are capable of eliminating microbial pollution (based on the detection of faecal indicators) mostly in the range of 2–3 orders of magnitude, i.e. more than 95% (Fleischer et al. 2000, Koivunen et al. 2003). Advanced treatment (e.g., biological ponds) can improve the reduction of hygienically-important microorganisms up to 5 orders of magnitude (Tagliareni & Ecker 1997). A study on a comparison of the efficiency of microbiological pollution removal in six wastewater treatment plants with different treatment systems (larger plants with tertiary treatment, smaller plants with enhanced secondary treatment, and very small compact facilities) has been presented by Kistemann et al. (2008); the average microbial reduction of each WWTP depended on its capacity and treatment processes, ranging between 1.9 and 3.5 $\log_{10}$.

Bacterial communities can be studied by different approaches, the most common being

indirect 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 a standard cultivation method as prescribed in European or national standards.

Pathogenic microorganisms, namely bacteria, those that can cause human illnesses, can be detected directly. Koivunen et al. (2003) presented salmonellae elimination results of 94–99.9% (salmonellae were undetectable in effluents in a volume of 100 ml). *Campylobacter* spp. were detected in sewage in amounts of 10^0–10^3 cfu.ml^{-1} (cfu = colony forming units); the primary sedimentation was able to remove more than 78% of incoming campylobacters. Campylobacters are reduced relatively efficiently during the course of the activated sludge process, possibly because of its sensitivity to aeration (Stelzer et al. 1991). Moreno et al. (2003) studied the specific detection of campylobacters in water and sewage by polymerase chain reaction (PCR) and fluorescence *in situ* hybridisation (FISH).

Another approach is based on culture independent methods that can assign prokaryotic cells to appropriate groups without any cultivation: an obvious advantage, as it is estimated that only about 0.1–10% of all prokaryotic species are cultivable. For the classification of Prokaryota 16S and 23S rRNA molecules 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 (Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, and Epsilonproteobacteria). The latest edition of Bergey's Manual (Garrity 2005) is based on this phylogenetic system. For the study of complex microbial communities, rRNA-targeted oligonucleotide probes are ideal. Mlejnková & Sovová (2010) studied phylogenetic groups in wastewater (the influent and effluent of two constructed wetlands in Moravia, the Czech Republic). In municipal wastewater they found the highest proportions of microbes belonged to Betaproteobacteria, Alphaproteobacteria, and Gammaproteobacteria, followed by the *Cytophaga-Flavobacterium* group; the lowest counts were found in the domain Archaea.

There have been many studies on the phylogenetic groups of bacteria in activated sludge. The bacterial groups most predominantly found were from Betaproteobacteria (Manz et al. 1994, Wagner et al. 1994, Amann et al. 1996, Kämpfer et al. 1996, Bond et al. 1999, Jiang et al. 2008). Lee et al. (2002) showed the predominance of Proteobacteria in a WWTP with enhanced biological phosphorus removal, with and without nitrogen removal; counts of *Cytophaga-Flavobacterium* and Archaea (detected with an Arch915 probe) were low (1–5% of the total bacterial area for *Cytophaga-Flavobacterium* and less than 1% of total prokaryotic cell numbers for Archaea). Manz et al. (1994) compared the presence of phylogenetic groups in municipal and industrial WWTPs, Proteobacteria predominating in municipal WWTPs: the class Betaproteobacteria were the most abundant (60% of bacteria detected with EUB338 probe), followed by Gammaproteobacteria (24% of bacteria), and *Cytophaga-Flavobacterium* represented 23%. Wagner et al. (1994) also showed a predominance of Betaproteobacteria in municipal WWTPs: they detected 42% of cells of the bacterial population as Betaproteobacteria, 32% as Gammaproteobacteria and 10% as Alphaproteobacteria.

Jiang et al. (2008) have constructed a 16S rDNA gene clone library for activated sludge: the most retrieved clones belonged to Proteobacteria, more specifically Betaproteobacteria, followed by Gammaproteobacteria and Alphaproteobacteria. Snaidr et al (1997) sequenced 65 clones isolated from activated sludge, 35 of which belonged to the class Betaproteobacteria (i.e. 52.2%), while Gammaproteobacteria represented 13 clones (20%) and Alphaproteobacteria 4 clones (6%).

This paper describes the changes in composition of the prokaryotic community in the WWTP of Zbytiny (Czech Republic, South Bohemia) during treatment and aims to confirm

the excellent quality of its treated water, which is most advisable in this area.

The following microbial communities were monitored: (i) indicators of faecal pollution (faecal coliforms, *E. coli*, and intestinal enterococci); (ii) pathogenic microorganisms (salmonellae, thermotolerant campylobacters, and coagulase-positive staphylococci); and (iii) total counts of Prokaryotes and phylogenetic groups (classes Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria, group of *Cytophaga-Flavobacterium*, and the domain Archaea).

The Zbytiny WWTP was chosen for this study because, in this special case, the environmental requirements for wastewater treatment efficiency are much higher than the regular legal demands. The municipality of Zbytiny, with its approximately 200 inhabitants, is located in South Bohemia, the Czech Republic, the village being situated inside the Šumava Protected Landscape Area.

Material and methods

Wastewater treatment plant

The Zbytiny WWTP was planned for 450 PE with a design discharge Q_{24} of 67.5 m^3. The WWTP was put into operation in November 2008. Zbytiny WWTP is equipped with mechanical pre-treatment, consisting of a fine screen and a sand catcher. The biological part consists of two parallel lines: each line consists of an anoxic and oxygenic part, divided by a barrier. The anoxic part of the aeration tank is mixed by two coarse-bubble elements, while the oxygenic part, separated by a barrier, is aerated by 15 fine-bubble elements. Sludge separation is ensured by two inbuilt clarifiers in the oxygenic zones. A microscreen drum filter for sludge leak elimination has been placed behind the outflows from both clarifiers.

For the advanced treatment, the treated wastewater is led through two serial stabilization ponds (SP1, SP2) placed behind the wastewater treatment plant. These stabilization ponds were built together with the WWTP. Initially they used to be filled by water from the Blanice River, but nowadays their only influent is the discharged wastewater from the WWTP. Their task is to improve the final quality of treated water. In the case of any accident at the WWTP, which would normally result in an operational shut-down, wastewater can be led directly into the stabilization ponds.

A scheme of Zbytiny WWTP and the sampling points are shown in Fig. 1. The retention period in the WWTP during the design discharge was 1.3 days, while in the pond SP1 it was 42 days and in pond SP2 108 days. Average concentrations (arithmetic mean) of chemical parameters in the WWTP's effluent measured behind the drum filter screen during the period from October 2008 to December 2009 are given in Table 1.

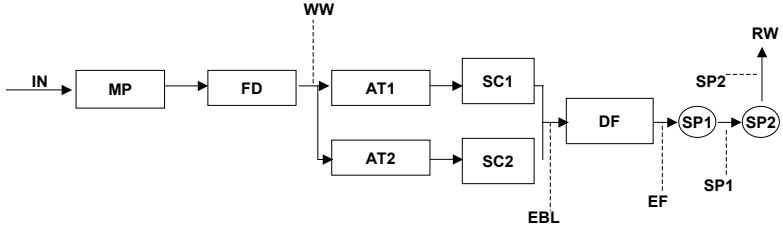

Fig. 1. A scheme of the Zbytiny wastewater treatment plant (WWTP): IN – inflow; MP – mechanical pre-treatment; FD – flow dividing; AT1, AT2 – aeration tanks; SC1, SC2 – secondary clarifiers; DF – micro-screen drum filter; SP1, SP2 – stabilization ponds; sampling points: WW – WWTP inflow; EBL – effluent from biological line; EF – effluent from filter; SP1, SP2 – effluents from SP1 and SP2.

Table 1. The statistical characteristics of chemical data in effluent from the WWTP measured behind the drum filter screen and in the final effluent, period from October 2008 to December 2009. COD – chemical oxygen demand, BOD_5 – five-day biological oxygen demand, SS – suspend solids, N_{TOT} – total nitrogen, P_{TOT} – total phosphorus, RSD – relative standard deviation, min – minimum, max – maximum.

Parameter	Effluent from the WWTP					Final effluent				
	mean	median	Min	max	RSD	mean	median	min	max	RSD
	$mg.l^{-1}$	$mg.l^{-1}$	$mg.l^{-1}$	$mg.l^{-1}$	%	$mg.l^{-1}$	$mg.l^{-1}$	$mg.l^{-1}$	$mg.l^{-1}$	%
COD	46	41	29	178	80	31	29	25	103	64
BOD_5	5	4	3	51	242	4.6	4	3	19	89
SS	9	5	2	58	168	11	7	2	43	113
N_{TOT}	27	26	16	40	25	19	18	5	35	38
P_{TOT}	3	3	0.1	7	53	0.3	0.3	0.1	0.8	58

Sampling

Sampling was performed monthly for one year (2009), grab samples (a total volume of 2 l) being taken. Microbiological analyses were finished up to 18 hrs after sampling; samples for microscopic examination (total counts of bacteria and FISH) were fixed with sterile 38% formaldehyde (50 $\mu l.ml^{-1}$ of sample; final concentration 2% according to Mlejnková & Sovová (2010)) immediately after sampling and stored at 4°C until further processing.

Samples were taken from five sampling points – influent of wastewater (WW), effluent from biological lines (EBL), effluent from filter (EF), and effluent from both stabilization ponds (SP1, SP2). In addition, single samples from the Blanice River and the Zbytinský Potok stream below the municipality Zbytiny (without and with the WWTP) were taken.

Microbiological methods

The following indicators of faecal pollution were determined: faecal (thermotolerant) coliform bacteria (according to Czech Standard CSN 75 7835); *Escherichia coli* (determined among faecal coliform bacteria according to the activity of enzyme β-D-glucuronidase using a fluorogenic substrate; according to Czech Standard CSN 75 7835); and intestinal enterococci (according to Standard EN ISO 7899-2).

The detected pathogenic microorganisms included: salmonellae; thermotolerant campylobacters; and coagulase-positive staphylococci. Salmonellae were detected in a volume of 1 000 ml according to ISO 19250. Thermotolerant campylobacters were detected by membrane filtration and cultivation on CCDA medium in a microaerophillic environment (24 hrs at 42°C); confirmation was performed biochemically (oxidase and catalase) and microscopically (phase contrast – monitoring of typical movement in broth, and FISH). Staphylococci were detected by cultivation on Baird Parker medium with tellurite and egg yolk (24 hrs at 36°C).

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

Microorganisms from the domain Bacteria – classes Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria, the group of *Cytophaga-Flavobacterium,* and microorganisms from the domain Archaea were detected by the method of fluorescence *in situ* hybridization. A microscopic examination was performed under an Olympus BX41 epifluorescence microscope equipped with a camera DP70 with phase contrast. A filter set for DAPI and for Cy3 was used. For the probes used see Table 2. We used competitor probes where appropriate. Probe sequences were downloaded from the ProbeBase (Vienna Ecology Centre, Facul-

Table 2. Probes used for the detection of phylogenetic groups.

Target group	Probe	Sequence	Reference
Alphaproteobacteria	ALF968	5'-GGTAAGGTTCTGCGCGTT-3'	Neef 1997
Betaproteobacteria	Probe labelled: BET42a	5'-GCCTTCCCACTTCGTTT-3'	Manz et al. 1992
	Competitor: c BET42a	5'-GCCTTCCCACATCGTTT-3'	
Gammaproteobacteria	Probe labelled: GAM42a	5'-GCCTTCCCACATCGTTT-3'	Manz et al. 1992
	Competitor: cGAM42a	5'-GCCTTCCCACTTCGTTT-3'	
Cytophaga-Flavobacterium	CF319a	5'-TGGTCCGTGTCTCAGTAC-3'	Manz et al. 1996
Archaea*)	ARC344	5'-TCGCGCCTGCTGCICCCCGT-3'	Raskin et al. 1994
	ARC915	5'-GTGCTCCCCCGCCAATTCCT-3'	Stahl & Amann 1991
Thermotolerant *Campylobacter* spp.	Catherm	5'-GCCCTAAGCGTCCTTCCA-3'	Poppert et al. 2008

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

ty of Life Sciences at University of Vienna, Department of Microbial Ecology; Loy et al. 2007). The sequence for the thermotolerant *Campylobacter* specific probe was taken from Poppert et al. (2008). Probes for the detection of Archaea were applied in the ratio of 1:1. The FISH procedure was performed according to Amann (1995), Poppert et al. (2008), and Nielsen et al. (2009).

Hybridization was performed on black epoxy-resin-coated glass with 6 or 8 wells (Marienfeld, Germany). Filter sections were dehydrated in an ascending ethanol series (3 min each: 50%, 80%, and 100% ethanol) and placed on single wells. Hybridization was carried out in a humid chamber for 2 h at 46°C (2.5 h for *Campylobacter spp.*) in an 18 µl hybridisation buffer according to the protocol of Amman (1995). After air drying in the dark, filter sections were stained with DAPI (Sigma-Aldrich, 15 ml, conc. 1 mg.ml-1), 15 ml of solution being applied on the filter. Filters were incubated for 5 min at room temperature, in the dark and washed with deionised water. After drying in the dark and embedding in Citifluor oil AF1 (Citifluor Ltd., UK), the filters were inspected by microscope.

Results and discussion

Indicators of faecal pollution

An evaluation of the results of faecal pollution indicators is presented in Table 3, while the results of faecal bacteria in stabilization pond SP2 are shown in Fig. 2.

Table 4 shows the elimination of faecal pollution indicators: the efficiency of removal is very high, and this system can almost completely eliminate faecal bacteria. The elimination of faecal bacteria by biological treatment at Zbytiny WWTP – more than 95% on average (log reduction 2.7) – is comparable to results found by other authors (Fleischer et al. 2000, Kistemann et al. 2008, Koivunen et al. 2003). The counts of faecal bacteria (*E. coli* and intestinal enterococci) at sampling points EBL and EF are comparable to those of Kistemann et al. (2008) for smaller plants with enhanced secondary treatment, i.e., 10^2 cfu.ml $^{-1}$ on average. The relative standard deviation (fluctuation of counts during the year) is also similar (96–157%, vs. 50–210%). The higher relative standard deviation in the sampling points EBL and EF detected here were caused by some technological problems (a leak of sludge) in the biological line (May 2009). This situation also increased the average counts of faecal indi-

Table 3. Indicators of faecal pollution – statistical analysis (arithmetic mean, median, and relative standard deviation – RSD) in all sampling points at different points in the treatment processes from all dates received during the year (n = 12).

	Faecal coliforms	*E. coli*	Enterococci
Inflow			
Mean (cfu.ml^{-1})	117 000	865 000	41 000
Median (cfu.ml^{-1})	85 000	55 500	19 500
RSD	88%	100%	150%
Outflow from biological line			
Mean (cfu.ml^{-1})	667	430	108
Median (cfu.ml^{-1})	195	155	42
RSD	217%	182%	150%
Outflow from filter			
Mean (cfu.ml^{-1})	501	364	88
Median (cfu.ml^{-1})	130	120	33
RSD	213%	186%	143%
Stabilisation pond 1			
Mean (cfu.ml^{-1})	2.37	1.67	0.98
Median (cfu.ml^{-1})	1.75	1.05	0.4
RSD	114%	142%	149%
Stabilisation pond 2 (total effluent)			
Mean (cfu.ml^{-1})	0.39	0.34	0.27
Median (cfu.ml^{-1})	0.15	0.15	0.1
RSD	139%	120%	141%

cators at these sampling points.

The advanced treatment (the two biological ponds) improved the reduction of hygienically-important microorganisms down to counts less than 1 cfu.ml^{-1}, which complies with values given by European Directive 2006/7/EC for bathing waters. In some cases (mostly in summer), the elimination of faecal bacteria in the whole system was up to 5 $\log_{10}$ units (in agreement with Tagliareni & Ecker 1997). The role of the stabilization ponds is also very important in the case of any technological problems during biological treatment (as, for example, happened in May 2009). No deterioration in the bacteriological quality of SP2 was observed (according to our results) during this period.

The microbiological water quality in the Blanice River and the Zbytinský Potok stream

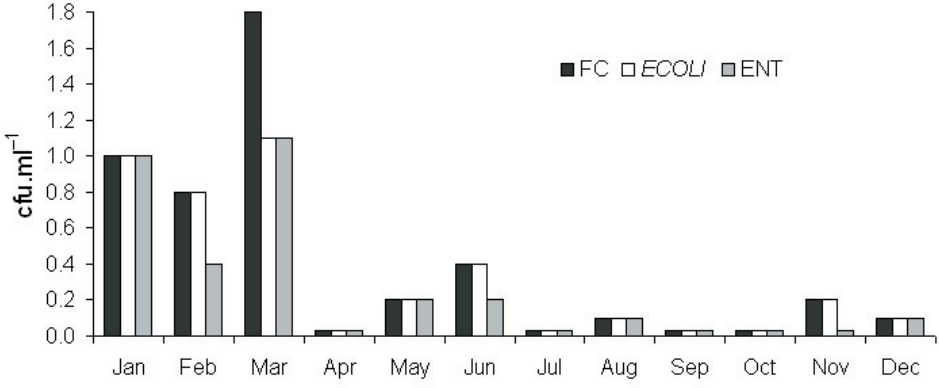

Fig. 2. Counts of faecal bacteria (FC – Faecal coliforms; *E. coli*; and ENT – intestinal enterococci) in total effluent (effluent from stabilization pond 2) during the whole year of 2009.

Table 4. Elimination (log reduction) of faecal bacteria at WWTP Zbytiny – mean (range).

	Faecal coliforms	*E. coli*	Enterococci
Biological line	2.58 (1.43–3.48)	2.57 (1.03–3.48)	2.79 (1.52–3.62)
Biological line and filter	2.72 (1.57–3.79)	2.65 (1.10–3.67)	2.90 (1.58–4.18)
Biological line and filter, and stabilization pond 1	4.97 (3.87–6.17)	5.02 (4.09–6.48)	4.95 (3.63–6.26)
Biological line and filter, and stabilisation ponds 1 and 2	5.74 (4.70–6.70)	5.61 (4.70–6.48)	5.33 (4.18–6.88)

was tested in the years of 2005 and 2011. No noticeable changes in the Blanice River were detected (faecal coliform 0.06 cfu.ml^{-1} (2005) and 0.14 cfu.ml^{-1} (2011); *E. coli* 0.05 cfu.ml^{-1} both years; enterococci 0.24 cfu.ml^{-1} (2005) and 0.1 cfu.ml^{-1} (2011). However, the microbiological quality in the Zbytinský Potok stream was much better in 2011 compared to 2005: faecal coliform 9 cfu.ml^{-1} (2005) and 0.5 cfu.ml^{-1} (2011); *E. coli* 1 cfu.ml^{-1} (2005) and 0.1 cfu.ml^{-1} (2011); enterococci 4.4 cfu.ml^{-1} (2005) and 0.05 cfu.ml^{-1} (2011).

Pathogenic bacteria

Salmonellae were detected in EBL, EF and SP1 in 50% of cases (in a volume of 1 l), and in SP2 in 25% of cases. Thermotolerant campylobacters and coagulase-positive staphylococci were not detected in SP2 in 100 ml of sample, and they were detected on average less than 0.1 cfu.ml^{-1} in EBL, EF and SP1. The counts of thermotolerant campylobacters detected in WW were similar to the results given in Stelzer et al. (1991), i.e., 10^2 cfu.ml^{-1} on average. Counts of coagulase-positive staphylococci in WW were similar to the counts of campylobacters, i.e., 10^1–10^2 cfu.ml^{-1}.

Total counts of Prokaryotes

The total counts of Prokaryotes (on a logarithmic scale) are shown in Fig. 3. The highest counts of Prokaryotes were detected in WW, with a decrease being observed in EBL and EF; however, an increase in comparison to the effluents from the biological lines was most frequently detected in SP1 (though total counts of bacteria were lower than in WW).

The changes of total prokaryotic counts are much less pronounced (increasing or decreasing by 1 order of magnitude on average) than in the case of faecal indicators. A significant increase of total counts was observed in the sampling point of SP1 compared to the preceding sampling points because of the presence of natural (self-purification) processes.

Prokaryotic phylogenetic groups

A statistical evaluation of the results of prokaryotic phylogenetic groups is presented in Table 6. Minimal values in most cases correspond to negative results (i.e., no cells detected), which is less than 1.6×10^3 cell.ml^{-1}. The size of the microscopic field and filtration area of the filter were chosen for the determination of the detection limit 1.6×10^3 cell.ml^{-1}.

Counts of Alphaproteobacteria were under the detection limit from January to May 2009 (except January results for EBL – 2.8×10^5 cell.ml^{-1}). A similar trend was observed for Archaea from January to April 2009. We assume that changes of bacteria counts during treatment process are not affected by the dilution in activated sludge because we demonstrated the predominance of Gammaproteobacteria in the effluent, whereas in activated sludge Betaproteobacteria are the most abundant according to the literature (Manz et al. 1994, Wagner et al. 1994, Amann et al. 1996, Kämpfer et al. 1996, Bond et al. 1999, Jiang et al. 2008). The percentages of phylogenetic groups in the total counts (DAPI) at the various sampling points are given in Table 5. Bond et al. (1999) investigated bacteria in both efficient and inefficient

Table 5. Statistical analysis of microorganisms total counts and phylogenetic groups at all sampling points during the sampling period (Alpha – Alphaproteobacteria, Beta – Betaproteobacteria, Gamma – Gammaproteobacteria, CF – *Cytophaga-Flavobacterium*, and Archaea; n = 12).

	DAPI	Alpha	Beta	Gamma	CF	Archaea
Inflow						
Mean (10^3 cell.ml^{-1})	35 500	67.1	1020	1270	310	99.4
Median (10^3 cell.ml^{-1})	21 500	<1.60	755	1200	105	<1.60
RSD	133%	293%	93%	80%	126%	288%
Outflow from biological line						
Mean (10^3 cell.ml^{-1})	4162	39.5	360	791	102	194
Median (10^3 cell.ml^{-1})	4150	<1.60	67.0	159	25	1. 55
RSD	76%	211%	242%	168%	176%	310%
Outflow from filter						
Mean (10^3 cell.ml^{-1})	4350	173	509	950	106	17.4
Median (10^3 cell.ml^{-1})	3100	3.85	67.5	330	5.05	2.15
RSD	117%	333%	164%	156%	297%	215%
Stabilisation pond 1						
Mean (10^3 cell.ml^{-1})	5230	42 .3	90 .8	61. 5	315	86.3
Median (10^3 cell.ml^{-1})	3400	5.12	22.0	15. 5	69.0	3.10
RSD	78%	159%	178%	176%	170%	215%
Stabilisation pond 2 (total effluent)						
Mean (10^3 cell.ml^{-1})	4020	10.3	203	93.3	106	15.3
Median (10^3 cell.ml^{-1})	2900	<1.60	49.0	23.5	19.5	2 .79
RSD	96%	183%	153%	196%	169%	192%

biological phosphorus-removal activated-sludge systems; in both of them Betaproteobacteria were the most abundant (50% of bacteria detected by DAPI). Alphaproteobacteria represented 1% of Prokaryotes detected by DAPI in the inefficient system and 4% in the efficient system, while Gammaproteobacteria represented less than 1% of the DAPI population in both systems. KÄMPFER et al. (1996) detected the phylogenetic groups in a large municipal wastewater treatment plant: The distribution of Proteobacteria was similar for both the aerobic and anaerobic zones and represented 60–75% of DAPI-detected prokaryotic counts. The predominant proteobacterial community was the class Betaproteobacteria (33% DAPI population), followed by 13% of DAPI-detected bacteria being members of the class Alphaproteobacteria, and 10% were members of the class Gammaproteobacteria. Members of the

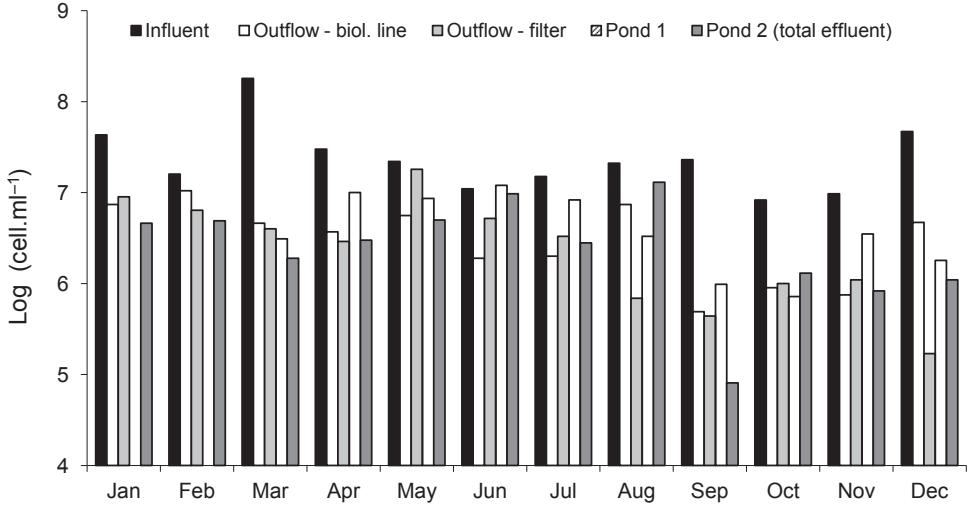

Fig. 3. Total counts of Prokaryotes at all sampling points during the whole year of 2009.

Table 6. Percentage of phylogenetic groups in total counts (DAPI) at all sampling points during the sampling period (Alpha = Alphaproteobacteria, Beta = Betaproteobacteria, Gamma = Gammaproteobacteria, CF = *Cytophaga-Flavobacterium*, and Archaea; n = 12).

	Alpha	Beta	Gamma	CF	Archaea
Inflow	0–1%	0–18%	1–13%	0–6%	0–2%
Outflow from biological line	0–6%	0–30%	0–68%	0–53%	0–38%
Outflow from filter	0–38%	0–42%	0–57%	0–28%	0–3%
Stabilization pond 1	0–4%	0–17%	0–4%	0–30%	0–7%
Stabilization pond 2 (total effluent)	0–3%	0–56%	0–33%	0–21%	0–7%

group *Cytophaga-Flavobacterium* (1% of DAPI population) were of minor importance.

Our results showed a domination of Prokaryota in the following descending order: Gammaproteobacteria, Betaproteobacteria, Alphaproteobacteria, *Cytophaga-Flavobacterium*, and Archaea. Of these Alphaproteobacteria and Betaproteobacteria showed the most stable results. The counts of Prokaryota in particular phylogenetic groups are shown in Fig. 4 (100% represents the count of Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, *Cytophaga-Flavobacterium*, and Archaea). It is clear from Fig. 4 that Gammaproteobacteria and Betaproteobacteria predominated in the influent, the effluent from the biological line, and the effluent from the filter. Also, about 50% of the *Cytophaga-Flavobacterium* group was detected in SP1. Betaproteobacteria were abundant in the total effluent (about 50%). Archaea constituted maximally 15% of all detected phylogenetic groups (Fig. 4); the low occurrence of Archaea agrees with the findings of other authors (e.g., Mlejnková & Sovová 2010).

In contrast to faecal bacteria, counts of the representatives of phylogenetic groups were more variable in the grab samples. On the basis of RSD we can declare that Gammaproteobacteria were the most stable (according to counts at individual sampling points; Table 5) – as was detected at WW, EBL and EF. Results from individual sampling points showed that the most stable were *Cytophaga-Flavobacterium* (variation among average values was 61%).

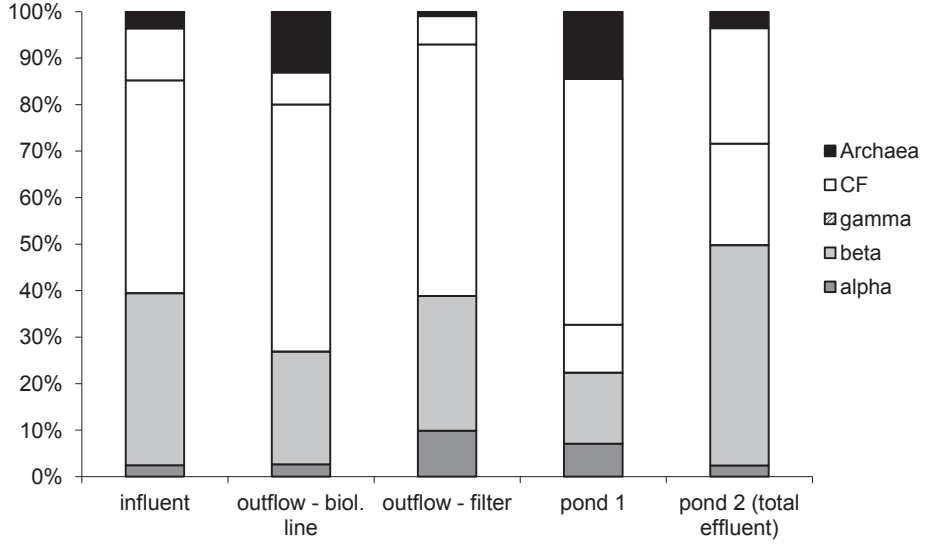

Fig. 4. Distribution of Prokaryotes in particular phylogenetic groups (average: Alpha – Alphaproteobacteria, Beta – Betaproteobacteria, Gamma – Gammaproteobacteria, CF – *Cytophaga-Flavobacterium* and Archaea; 100% represents the sum) in every sampling point.

Among the Proteobacteria, Betaproteobacteria showed the highest stability (RSD 83%), whereas the same statistical characteristic for Alphaproteobacteria, Gammaproteobacteria, and Archaea was 95%, 85%, and 89%, respectively. Counts of detected Prokaryota in SP2 were lower compared to the influent in 90% of cases. A decrease of Prokaryota under the detection limit during the treatment process (comparing WW and SP2) was detected twice for Betaproteobacteria and Gammaproteobacteria, and once for *Cytophaga-Flavobacterium*. In four cases, no cells were detected in WW but there was a positive detection of these bacteria in SP2 – the total effluent; this means an increase of prokaryotic counts by hundreds of units per ml. Biomass could increase because of its growth on the substratum existing in the system (*Cytophaga-Flavobacterium*), or it concerned biomass being released from the system.

The prevalence of phylogenetic groups from the classes Betaproteobacteria and Gammaproteobacteria in the wastewater and biologically-treated wastewater corresponds to results from other authors (Mlejnková & Sovová 2010); similar results have also been observed in the active sludge (Jiang et al. 2008, Snaidr et al. 1997). Bacteria from the families *Enterobacteriaceae* (including, for example, *E. coli*) and *Pseudomonadaceae* are important parts of the cultivable bacteria from the class Gammaproteobacteria. Changes in the microbial communities were detected in both stabilization ponds (see Figs. 2–4); most importantly, a significant portion of *Cytophaga-Flavobacterium* bacteria was detected in SP1 (these bacteria are responsible for the decomposition of polymer carbohydrate).

Conclusions

The elimination of faecal bacteria by biological treatment at the Zbytiny WWTP was better than 95% on average. The additional treatment (the two biological ponds SP1 and SP2) reduced hygienically-important microorganisms to less than 1 cfu.ml^{-1}. The stabilization ponds SP1 and SP2 also played a very important role should any technological problems occur during the biological treatment. The counts of the studied pathogenic bacteria (thermotolerant campylobacters and coagulase-positive staphylococci) were very low in the effluents (less than 1 cfu.ml^{-1}). The changes in the total prokaryotic counts were much lower (increasing or decreasing by 1 order of magnitude on average) than was the case with the faecal indicators. Furthermore, a prevalence of Betaproteobacteria and Gammaproteobacteria in the wastewater and biologically-treated wastewater was revealed. Changes in the composition of the microbial community were detected in both stabilization ponds; notably a significant proportion of bacteria affiliated with *Cytophaga-Flavobacterium* was detected in SP1.

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Notes