Microbial pollution of water from agriculture

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ABSTRACT

Microbial contamination of small streams in agricultural areas was monitored for two years. Microbiological indicators of faecal pollution (faecal coliforms, *Escherichia coli* and intestinal enterococci were detected by standard methods based on the cultivation of bacteria on selective media). The obtained results showed that running contamination of streams from agricultural areas was not extremely high, but it showed marked seasonal fluctuations (the average values and maximal values revealed great differences). Microbial contamination also increased several times in relation to high precipitation. The water quality in three (and/or four) localities exceeded the acceptable counts of faecal coliforms and enterococci given by the Czech legislation (40 CFU/ml for faecal coliforms and 20 CFU/ ml for enterococci). In agriculturally polluted streams, there were detected more enterococci than faecal coliforms, and also some less frequent species related to farm animals (*Streptococcus equinus* and *S. bovis*) or plant rests (*E. mundtii*, *E. gallinarum*, *E. casseliflavus*) were present. *E. faecalis* and *E. faecium* strains (these are the most common species related to human faecal pollution) were less frequent there.

Keywords: *Enterococcus*; faecal coliforms; pollution source identification; antibiotic resistance; surface water quality

Generally, the quality of surface waters in the Czech Republic has improved recently, but microbial pollution of streams remains high in many areas and it frequently puts constraints on water usage.

The subject of this study is to assess the level of microbial pollution resulting from agricultural activities in our country. The studied issues concern non-point sources of pollution from agriculture – both from plant (fields, runoff water from fields, fertilisation), and animal production (pasture, farms). The study does not cover point sources of pollution, including sources from agriculture (such as agrarian waste water and waste water from the processing industry), which are easier to identify, monitor and assess – and were, therefore, investigated more frequently. However, in many cases, pollution from non-point and point sources is hard to distinguish. Crop and animal production in the Czech Republic does not make such a big part of economy as in other countries (England, some areas in the USA, Australia), and the method of animal husbandry results in point, rather than non-point pollution (free pastures are used much less).

Within the evaluation of water quality, it is important to detect the source of microbial pollution (to distinguish between human and animal sources). The ratio of counts of faecal coliforms and enterococci (faecal streptococci) – 'FC:FS ratio' in water sample taken from one sampling point was used to distinguish between faecal pollution of human and animal origin by some authors (e.g. Young and Thackston 1999). It was shown that faecal coliforms are relatively more abundant in stool of human origin, whereas enterococci prevail in faeces of warm-blooded animals. The FC:FS ratio for faecal pollution of human origin should be > 4, of farm animals origin between 0.1 and 0.6 and of wild animals origin < 0.1, respectively. Chou et al. (2004) distinguished between faecal pollution of human and animal origin (from pigs) quite clearly. Moreover, they introduced the ratio of the enterococci strains, isolated from waters of different type of pollution '(*E. durans* + *E. hirae*)/(*E. faecalis* + *E. faecium)*', which was

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0.9 with a human source of faecal pollution and up to 5.5 with a pollution source from pigs in waste water. Some works challenged these ratios, and so variability can be expected (e.g. Edwards et al. 1997, Brion and Lingireddy 1999). Brion and Lingireddy (1999) unsuccessfully attempted to develop a model based on the FC:FS ratio and to distinguish the faecal pollution origin. Therefore, this ratio can be used only as 'subsidiary' index.

Many authors attempted to distinguish between faecal pollution of animal and human origin based on different resistance of isolated bacteria to antibiotics; it comes from the fact that people and animals were subjected to different spectrum of antibiotics. However, the use and consequently also the exposure of various animal groups and humans to antibiotics have changed markedly over time, and so the studied and suggested profiles of antibiotic resistance of isolates of different origin will be applicable just for short time. Kelsey et al. (2003) investigated multiple antibiotic resistances on isolates of *E. coli* in South Carolina. On the basis of their results, they found that most faecal pollution in Murrells Inlet is of non-human origin. Wiggins et al. (1999) and Choi et al. (2003), among others, studied enterococci isolates and their antibiotic resistance. The former authors studied reliability and reproducibility of enterococci isolates classification based on resistance to 9 antibiotics and their separation according to their origin into 4 types of faecal pollution (human, livestock, poultry and wild animals). When the isolates were assessed individually, the correct classification was reached in 64–78%, when all isolates of the individual types were averaged, the correct classification was obtained in 96–100%. Choi et al. (2003) performed discriminat analysis on occurrence of resistance to 7 chosen antibiotics on 2991 enterococci strains and found that the main source of enterococci in the surfing zone of the Californian Huntington Beach is from bird excrements.

Formerly, *Myxobacteria*, which belong among sliding bacteria, were considered to be suitable indicators of the water trophy level and of contamination with humus from agricultural production in the Czech Republic. They are gram-negative,

Table 1. Characteristics of studied localities

Locality number	No. of samples	Soil type	Region/Locality	Catchment area (ha)	Q (l*/s) (2007 and 2008)
1	12	Haplic Chernozem	Central Bohemia, the area of Polabi/Jordan (Dolnostrizinsky stream)	253.5	2.85
2	17	Haplic Chernozem	East part of central Bohemia/left tributary to Zehunsky stream	354.3	3.6
3	11	Dystric Cambisol	West Bohemia, near of the Touzim city/left tributary to Utvinsky stream	251.3	6.95
4	14	Eutric Cambisol	Vysocina area/right tributary to Vintirovsky stream	158	3.4
5	12	Eutric Cambisol	North west Bohemia, Lounsko/Lhotecky stream	128	1.8
6	9	Eutric Cambisol	Moravia, the area of Hana/Hrebecovsky stream	956	6.8
7	14	Dystric Cambisol	Central Bohemia, near the city of Benesov/Lhotsky stream	249.1	6.85
8	13	Dystric Cambisol	South Bohemia, near the city of Tabor/Brusnik stream	67.8	1.75
9	10	Dystric Cambisol	South west Bohemia, near Horažďovice/west tributary to the pond of Velky Smrkovec	189.7	3.45
10	13	Dystric Cambisol	East Bohemia, near Trutnov/right tributary to Vlcicky stream	321.1	19
11	10	Albic Luvisol	East Bohemia near Broumov/left tributary to Bozanovsky stream	105.2	1.25

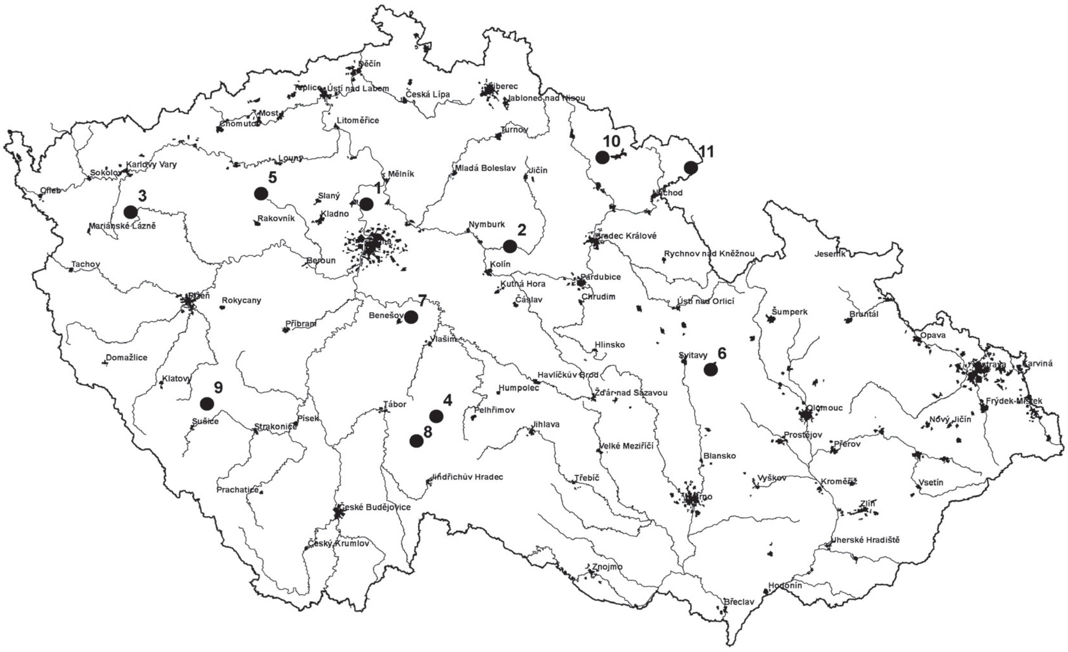

Figure 1. The map of studied localities (the specific data are given in Table 1)

aerobic, heterotrophic, and able to create small fruit bodies. They are cellulolytic, and therefore they decompose cellulose in aerobic way, and cellulose gets into water environment as plant residues, runoffs and waste. Using enzymes, the *Myxobacteria* are able to decompose in aerobic way some other hard-to-decompose polysaccharides (hemicellulose, lignin, pectins); they appear, besides the cellulose, in the herbivores digestive tract, and also in manured soil, silage, etc. Their limits to assess the level of water pollution were suggested by Lecianova (1987).

METHODOLOGY

The presented study consists of three parts:

(1) Assessment of results of microbial pollution of streams in agricultural areas (11 localities), monitored every two months during two years (the total number of samples studied at each locality are given at Table 1).

(2) Detection of microbial pollution of streams in pasture areas (Borova near Chvalsiny, South Bohemia Region) – sampling was performed four times during the years of 2007 and 2008, the concrete data on sampling are given at Table 4.

(3) Identification of strains of enterococci isolated from waters of different type of pollution (both agricultural and anthropogenic). A total of 237 strains were identified.

The list of studied localities and their characteristics are given in Table 1; the map of the localities is shown in Figure 1. The studied profiles are situated at the small streams in agriculture areas, the size of catchments areas and average flows are also given in Table 1. The streams were of quite low organic pollution (COD up to 25 mg O_2/l), and the total phosphorus was up to 50 µg/l (median values).

Sampling for microbiological analysis: Grab samples of water were taken into sterile glass bottles (300 ml) and microbiological analyses were performed up to 18 h after sampling. Samples were transported at the temperature between 4–8°C.

Microbiological methods: Faecal (thermotolerant) coliform bacteria were determined by the membrane filtration method (membrane filter size of 0.45 µm), under cultivation on mFC agar, for 24 h at 44°C. *Escherichia coli* was determined among faecal coliform bacteria according to the activity of β-ᴅ-glucuronidase enzyme using fluorogenic substrate (4-methyl-umbelliferyl-β-ᴅ-glucuronide) under cultivation for 4 h at 37°C; blue fluorescence was detected under UV radiation (460 nm). Intestinal enterococci were determined by membrane filtration (membrane filter size of 0.45 µm) of samples and cultivation on the Slanetz-Bartley agar for 48 h at 37°C and confirmation on bile esculin agar with sodium azide. All these methods correspond to the Czech and European standards

Table 2. Results of faecal coliforms (FC), *E. coli* (ECOLI), enterococci (ENT) and *Myxobacteria* (MYX) – all in CFU/ml. Arithmetic mean (mean), maximal value (max) and percentil 95 (*P*95) are given

Locality	FC			ECOLI			ENT			MYX
	mean	max	*P*95	mean	max	*P*95	mean	max	*P*95	CFU/ml
1	27.39	110	99.55	25.33	110	94	25.12	200	107	7
2	14.9	207	55	1.58	9	7.5	4.32	21	18.6	2
3	2.13	6.4	6.2	1.79	5.4	5.2	4.37	16	12.7	3
4	2.88	8.6	8.21	1.92	7	7	8.08	36	26.4	20
5	1.61	9	7.35	1.83	9	6.5	2.74	16	11.6	2
6	1.75	8	6.775	0.82	3.4	2.91	4.67	26	19.1	0
7	6.95	80	33.2	6.32	70	31.6	3.22	15	13.1	7
8	0.23	1.3	0.76	0.1	0.36	0.264	1.57	9	6.48	7
9	1.5	5	4.96	1	3.6	2.96	3.71	13	10.9	4
10	8.12	60	42.75	1.91	9.2	9.06	9.07	43	34.6	1
11	4.21	40	22.63	1	9	5.2	7.03	67	37.3	2

(CSN 75 7835, CSN EN ISO 7899-2). *Myxobacteria* were cultivated on nutrient agar with mushroom extract and sterile filter paper, for 28 days at 30°C.

Microtests STREPTOtest 16, EN-COCCUStest, HIPPURATEtest and PYRAtest by the PLIVA-Lachema Diagnostika s.r.o. company were used for identification of enterococci.

The disc diffusion method with antibiotic discs (penicilline 10 units (P), vancomycine 30 μg (VA), erythromycine 15 μg (E), tetracycline 30 μg (T), ciprofloxacine 5 μg (Cf) by Himedia, high gentamycine 120 μg (hG) by Oxoid and high streptomycine 300 μg (hS) by Biorad) were used to determine sensitivity of enterococci to antibiotics.

RESULTS AND DISCUSSION

Part 1. Microbial pollution of streams in agricultural areas. The results of determination of microbiological indicators in studied localities are given in Table 2. Besides the arithmetic mean, maximum value and percentile p95 are presented, because the results of microbial pollution show unambiguous seasonal fluctuations (e.g. Tillet et al. 2001). Moreover, a positive effect of increased precipitation was regularly observed (in agreement with Buck et al. 2004), and therefore the arithmetic mean (and possibly also median) are quantities with poor value for prediction.

Table 3. Profiles in the pasture area Borova near Chvalsiny – description and characteristic of expected pollution

Profile 1	the stream Borova, at 2/3 NW pasture	agricultural pollution – livestock
Profile 2	the stream Borova above the village Borova	agricultural pollution – livestock
Profile 3	the stream Borova under the village Borova	mixed pollution (village and pasture)
Profile 4	the stream Zrcadlovy	agricultural pollution – livestock
Profile 5	the end of the area studied; the stream Borova, about 800 m under the Zrcadlovy stream inflow and 100 m under the village Borova	mixed pollution, the stream is revitalised, lower contamination than in the profile 3 can be expected

The detection of *Myxobacteria* was performed in one time in the autumn 2007. *Myxobacteria* were observed in a large majority of profiles, but just in the order of units. The differences in the presence of *Myxobacteria* in all studied localities were not significant.

The results where the values of percentile 95 exceeded the limits for allowed pollution of streams set by the Government Decree No. 61/2003 Coll., later amended by the Government Decree 229/2007 Coll., are in bold. The strongest pollution of agricultural sources was detected at localities of Jordan (Dolnostrizinsky stream), Vintirovsky stream and Bozanovsky stream. More enterococci than faecal coliforms appeared in most profiles (except localities No. 2 and 7), which may indicate agricultural pollution as was suggested in the results of other authors (Young et al. 1999, Chou et al. 2004, etc.). Ratios of faecal coliforms and enterococci 'FC:FS' ranged from 0.1 to 9.9, but most frequently they were around 0.6, which indicates the influence of farm animals (according to Young et al. 1999). However, at localities No. 2 and 7 (Zehunsky and Vlcicky streams), although the counts of enterococci were lower than counts of faecal coliforms, the communal (anthropogenic) pollution was not detected. This supports the argument that the ratio of FC:FS is only subsidiary index.

No differences in microbial pollution in water coming from different soil types were observed.

Myxobacteria appeared in the studied areas only in the order of units (up to ten) CFU/ml. According to Lecianova (1987), it indicates unpolluted (most of studied localities) or slightly polluted water (locality No. 4). In the latter locality (Vintirovsky stream) the higher counts of enterococci were also detected.

Part 2. Microbial pollution of streams in the pasture area (Borova near Chvalsiny, South Bohemia Region). Pastures in the area of the municipality of Borova near Chvalsiny were monitored. Profiles in streams 'Borova' and 'Zrcadlovy' were studied. The list of profiles and their characteristics are given in Table 3; results of microbiological indicators are given in Table 4.

Four samplings took place all of which were quite different. On May 17, 2007, livestock was not on pasture yet, but it was stalled in the Borova village. On September 6, 2007, an extreme rainfall situation occurred (whole-day intensive rain); at that time livestock was on pasture by the Zrcadlovy stream. On June 19, 2008 and August 28, 2008, livestock was on the north-west pasture and no important rainfall occurred.

The results were not processed using statistics, as only four samplings took place, and each of them was done under different conditions. The general results have well completed the results for microbial contamination in various agricultural areas (season changes and runoffs in time of high precipitation). More faecal bacteria were detected when the livestock was present at pastures (since June to September) with the exception of profile 3 (the continual influence of sewage from the Borova village). The highest counts of bacteria were detected on September 6, 2007, during the strong precipitation event.

Part 3. Identification of enterococci, isolated from streams affected with agricultural pollution. Three sets of results of enterococci strains identification were processed.

Set 1 represented 45 strains from streams from agricultural areas given – localities No. 10, 11, 2 and 5. In this case, these were integrated sets

Table 4. Results of faecal coliforms and enterococci in Borova near Chvalsiny. The description of profiles is given in Table 3

Profile	Faecal coliforms (CFU/ml)				Enterococci (CFU/ml)			
	17.5.2007	6.9.2007	19.6.2008	28.8.2008	17.5.2007	6.9.2007	19.6.2008.	28.8.2008
1	0.08	21	0.7	17	0.66	82	4.6	5
2	0.07	44	1.1	1.4	0.7	250	5	2.4
3	58	390	18	6	7.8	1200	4	2.3
4	1.5	220	0.52	0.4	0.9	210	0.6	0.9
5	1.6	350	4	0.6	1.2	400	1.8	1.3

Table 5. Results of antibiotic resistance (percentage of resistant strains are given) of enterococci strains isolated from water with anthropogenic or agricultural source of pollution

	P	VA	E	T	Cf	hS	hG
Strains from anthropogenic source of pollution	96.2	3.8	18.9	7.6	1.9	1.9	9.4
Strains from agricultural source of pollution	98.1	9.4	1.9	3.8	0	0	1.9

P – penicillin; VA – vancomycine; E – erythromycin; T – tetracycline; Cf – ciprofloxacine, hG – high gentamycine; hS – high streptomycine

of all grown enterococci on a membrane filter (about 10 CFU from a sample). All these samples contained more enterococci than faecal coliforms. Strain *Enterococcus faecalis* did not appear and *E. faecium* appeared just in the minimum amount, but streptococci and enterococci strains related to farm animals (*Streptococcus equinus* and *S. bovis*) or plant rests (*E. mundtii*, *E. gallinarum*, *E. casseliflavus*) appeared. The identified number – 10 enterococci strains from the sample – should be high enough to distinguish between the pollution of human and agricultural origin, which is important for possible practical usage in laboratories.

Set 2 represented 86 strains from the area Borova near Chvalsiny, 44 from profile 2 (NW pastures, above village Borova, possible agricultural pollution) and 42 from profile 3 (below the village Borova, mixed pollution). Relation between hippurate negative strains and agricultural pollution was not confirmed. It was found that there is more or less mixed pollution (particularly below the village). *E. faecalis* was not detected in the pasture locality, however over 9.5% was found below the village. The strain of *E. faecium* was balanced in both localities (about 30%). The *S. equinus* strain was markedly more isolated from enterococci from pastures (23%) compared to the locality below the village (3%). It is in agreement with our expectations based on the knowledge of *Enterococcus* species and their characteristics (Holt et al. 1994).

Set 3 represented 106 enterococci strains (53 strains were of anthropogenic origin – these strains were isolated from the Luzicka Nisa stream in the agglomeration of the Liberec city, and 53 strains were of agricultural origin – mixture from pastures in the Orlicke mountain area. Results of the study of antibiotic resistance are given in Table 5.

Some differences in antibiotic resistance in strains of anthropogenic and agricultural sources appeared, but much more strains (at least by order) would be necessary to process to confirm them. For instance, Wiggins et al. (1999) and Choi et al. (2003) examined more than 2000 strains per set. This is quite labour-consuming and unsuitable for practical usage. Moreover, the enterococci strains from the agricultural sources grow poorly and antibiotic resistance is hard to count on them.

In was found that the micro EN-COCCUStest is more suitable for identification of enterococci than STREPTOtest 16, with addition of the strip HIPPURATEtest. There are just 8 reactions in EN- COCCUStest, which is sufficient for classification into main groups for purposes of this work, and selection of reaction is more suitable (STREPTOtest is oriented on clinically important streptococci). It was found that in the case of anthropogenic (municipal) pollution, most isolated enterococci belonged to the group of *E. faecalis/hirae* (41%), whereas in the case of agricultural pollution, more hippurate negative strains appear among enterococci (75% compared to less than 20% for strains of anthropogenic origin), and strains *Streptococcus bovis* and *S. equinus* (connected with farm animals) appeared. Furthermore, a new STREPTOtest 24 is now available and it will be used and tested in our next study.

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