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MICROBIOLOGICAL ANALYSIS OF LESSIVE SOIL FERTILISED WITH ANIMAL WASTE

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ABSTRACT

The present research aimed at defining the survival rate of faecal bacteria selected in the lessive soil fertilised with cattle slurry. The field trials over dry and moist summers involved slurry at the amount of 3 lm^{-2} with the suspended matter of *S. senftenberg*, *E. coli* and faecal streptococci. Soil was sampled from different depths prior to the application of slurry, a week after the application, and then four times with monthly intervals. On average over the total soil profile over the dry period, the survival time of *E. coli* amounted to 17.7 weeks, faecal streptococci – 18.7, while *Salmonella* bacilli - 14.1 weeks. Over the moist period, it was much longer and amounted to 26.7, 25.9 and 19.9 weeks, respectively. A prevailing number of faecal bacteria were retained in the upper layers of soil profiles. Over the dry period microorganisms migrated to the depth of 43 cm, while over the moist period - to 70 cm. The research showed that facing soil structural disturbances, one cannot eliminate the risk of groundwater infection with pathogenic microorganisms present in slurry.

Key words: slurry, lessive soil, survival rate, *S. senftenberg, E. coli*, faecal streptococci

INTRODUCTION

Animal waste management in the countries of intensive animal breeding remains an important environmental concern [11,12,17,18]. Depending on the species and the animal health status, slurry may contain numerous bacteria and pathogenic viruses as well as parasite eggs [3,5,14,16]. Slurry infection is of a great significance when we face latent infections, notably animals produce with their faeces and urine pathogenic microorganisms, yet no disease symptoms occur [22]. Slurry deposits are usually oxygen-free, which, in turn, prevents from an intrinsic increase in its temperature which would destroy slurry pathogens. Slurry, usually only after a few months of being stored, undergoes a self-purification process and can be employed as a fertiliser in agriculture [23]. In Poland, especially in the vicinity of large-size farms, too intensive slurry fertilisation can disturb the soil self-purification.

The research aimed at defining, under natural conditions, faecal bacteria survival and migration in soil fertilised with slurry.

MATERIALS AND METHODS

72 l of cattle slurry was applied on plots of 24 m^2, over dry summer (L1) and moist summer (L2). To increase the concentration of the microorganisms researched, the slurry was enriched, prior to application, with 1 l of broth suspended matter of *E. coli* and D-group streptococci (10^8-10^9 of bacteria per 1 ml) and *Salmonella senftenberg* bacilli (10^6-10^7 of bacteria per 1 ml). The final concentration of *E. coli* bacilli in the slurry applied over L1 amounted to 7.5 x 10^6 cfu/ml, faecal streptococci 9.5 x 10^6 cfu/ml and 2.0 x 10^4 cfu/ml of *Salmonella* bacilli. Over L2 the respective values were as follows: 1.7 x 10^8 cfu/ml, 9.5 x10^6 cfu/ml and 7.5 x 10^4 cfu/ml.

1. Soil sampling

Each time the soil was sampled at the respective depths of 12, 25, 43, 70 and 90 cm prior to slurry application, a week after its application, and later 4-times, with monthly intervals. The soil profile of the soil pits prepared was horizontally penetrated with the pilot sampling device and then with the proper sampling device of a smaller diameter.

2. Physical and chemical soil analysis

Soil reaction was defined with the pH meter in water and in the KCl solution. The soil samples were also analysed to determine the total carbon content with the Tiurin method, total nitrogen content with the Kiejdahl method, while the contents of calcium, magnesium, phosphorus and potassium oxides with the methods commonly applied in agricultural chemistry [13].

3. Soil microbiological examination

Each time from different soil profile depths 3 samples, of 10 g of soil each, were added to 90 ml of enriched broth and 3 samples of 1 g which were put in 9 ml of broth and carefully distributed in the ultrashaker. 3 rows of dilution of varied concentration values from 10^0 to 10^{-7} from the test-tubes with 1 g of soil sample were additionally prepared. Following the incubation, the material researched derived from each dilution was transferred onto solid substrates where three inoculations with the oeze were completed, which allowed to define the number of the microorganisms researched following the NPL method.

3.1. Quantitative analysis of *E. coli* in soil

The soil samples prepared were first added to the liquid substrate, Mac Conkey's, (43° C for 24 hours). Then the material was sieved through onto the tergitol-agar adding 1% of the 2,3,5-TTC solution - acid (24 hours at 43° C). Whenever *E. coli* was difficult to identify, the material was transferred onto agar to obtain pure colonies (37° C for 24 hours). To discriminate *E. coli* from other soil coli-group bacilli time-effectively, a test was applied to detect glutamic decarboxylase [21]. Finally the lactose decomposition capacity with gas evolution was defined (44° C for 48 hours).

3.2. Quantitative analysis of D-group streptococci in soil

To obtain a selective enteric cocci growth, a liquid broth applied contained glucose and azide (48 hours at 37° C). The turbid sample material was transferred onto solid medium, namely agar with esculin and azide (37° C for 48 hours). The final identification of the D-group streptococci employed serologic test of newly obtained pure bacterial cultures.

3.3. Quantitative analysis of *Salmonella* bacilli in soil

First the soil samples were placed into the 1% of peptone water (24 hours at 37° C), and then the 0.1 ml of the material from each test-tube was transferred to the test-tubes filled with 10 ml of selective-enrichment liquid medium following Rappaport (43° C for 24 and 48 hours). Then the material was sieved onto the BPLA selective medium following Kaufmann (24 hours at 37° C). The final identification included serologic test with polyvalent serum (HM).

4. Result statistical analysis

The results obtained were verified and analysed statistically; a primary analysis was made based on the changes in the bacteria quantity in soil with time. The formula describing the changes was as follows:

$$\ln (N) = ax + b$$

N - the number of bacteria at a given time in soil,
x - period, weeks,
a - slope coefficient corresponding with a mean change in the number of bacteria defined as ln per week,
b - intercept, in theory corresponding with the ln of bacteria number at the 0 time involved in a given process.

The bacteria soil survival time was defined as x_o determining the period until there was a single bacterium left. Only the coefficients of the equations where the linear correlation of $y = ax + b$ was significant at $p_o < 0.05$ were analysed.

RESULTS

The research were conducted over two summer periods; the first one was exceptionally dry with high sun exposure and a very high air temperature, while the other period showed a lower air temperature and high rainfall (Table 1).

The lessive soil chemical composition analysis is presented in Table 2. The contents of C and N in the soil researched were lower in the parent rock, as compared with the topsoil layers, while the C:N ratio ranged from 6.7 – 10.47. The highest contents of Ca and K were observed at the depths from 40 to 70 cm and from 70 to 100 cm, while the lowest at the depths from 0 to 25 cm and from 25 to 40 cm. The P_2O_5 content, depending on the soil genetic layer, ranged from 1.84 to 4.60 mg/100 g of the soil.

The activity of faecal microorganisms in lessive soil fertilised with cattle slurry is presented in Fig. 1-3. Over the two research periods, there was observed an occasional inconsiderable number of microorganisms in the topsoil; it did not exceed usually a few bacterial cells per 100 g of the soil. No sample contained *Salmonella* bacilli.

Table 1. Mean air and soil temperatures, vapour pressure deficit, sun exposure and rainfall

Month		Decade	Air temperature °C	Soil temperature, °C				Vapour pressure deficit hPa	Sun exposure hours	Rainfall mm
				Depth, cm						
				5	10	20	50			
L1	VI	1	20.4	22.3	21.0	20.8	18.8	15.8	11.2	22.0
		2	18.5	20.5	19.7	19.4	18.2	9.8	7.7	9.0
		3	20.3	23.0	22.1	21.9	20.3	15.9	10.4	0.0
		x	19.7	21.9	20.9	20.7	19.1	13.8	9.8	31.0
	VII	1	20.5	23.9	22.8	22.5	21.0	16.6	9.1	1.3
		2	20.3	22.4	21.1	21.6	20.5	11.0	7.8	21.1
		3	22.3	24.2	23.4	23.3	22.0	16.3	10.2	5.3
		x	21.1	23.5	22.8	22.5	21.2	14.6	9.1	27.7
	VIII	1	24.6	25.1	24.1	24.0	22.2	19.8	9.2	4.9
		2	18.6	20.5	20.2	20.6	20.6	10.1	5.8	4.8
		3	21.1	21.6	20.8	20.9	19.9	11.7	6.8	12.0
		x	21.4	22.4	21.7	21.8	20.9	13.8	7.3	21.7
	IX	1	13.7	15.1	25.2	15.5	15.3	5.1	3.5	17.6
		2	14.0	14.8	14.6	14.8	14.8	5.3	4.1	1.9
		3	13.8	14.1	13.8	14.2	14.4	7.0	5.5	0.0
		x	13.8	14.7	14.5	14.8	14.8	5.8	4.4	19.5
L2	VI	1	18.3	21.2	20.4	20.4	18.5	13.7	10.4	0.0
		2	15.9	18.6	18.5	18.6	18.1	6.0	4.5	30.0
		3	14.0	16.6	16.3	16.4	16.2	4.8	4.6	33.3
		x	16.1	18.8	18.4	18.4	17.6	8.2	6.5	63.3
	VII	1	17.6	19.0	18.4	18.2	17.5	9.1	7.8	34.5
		2	16.6	18.7	18.1	17.9	17.5	6.6	5.6	27.9
		3	17.5	19.4	18.7	18.5	17.9	6.7	7.3	28.8
		x	17.2	19.0	18.4	18.2	17.6	7.4	6.9	91.2
	VIII	1	18.8	20.4	19.9	19.8	19.3	7.3	5.4	21.7
		2	18.2	19.8	19.1	18.6	18.6	10.8	8.4	9.8
		3	13.6	16.0	15.8	16.6	16.6	4.8	8.1	15.3
		x	16.9	18.6	18.2	18.1	18.1	7.5	8.6	46.8
	IX	1	12.4	14.7	14.5	14.9	14.9	3.1	2.5	46.8
		2	11.3	13.0	13.1	13.6	13.6	2.4	2.5	24.2
		3	13.0	13.3	13.2	13.7	13.7	4.4	5.7	15.4
		x	12.2	13.7	13.6	14.0	14.0	3.3	3.6	86.4

Table 2. Chemical composition of soil investigated

C mg/100g	N mg/100g	Depth cm	pH		C:N	Total contents			
			H_2O	KCl		CaO mg/100 g	K_2O mg/100 g	P_2O_5 mg/100 g	MgO mg/100 g
1026	98	0-25	6.9	6.6	10.47	16.40	6.60	4.60	4.40
142	21	25-40	6.9	6.4	6.76	25.12	2.50	2.80	4.90
226	30	40-70	6.9	5.8	7.53	30.20	8.30	2.80	*
210	23	70-100	6.9	6.2	9.13	37.00	9.11	1.84	*

*** non-determined**

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Fig.1. Number of E. coli bacteria (ln) per 100g of lessive soil at different depths over dry (L1) and moist (L2) summers

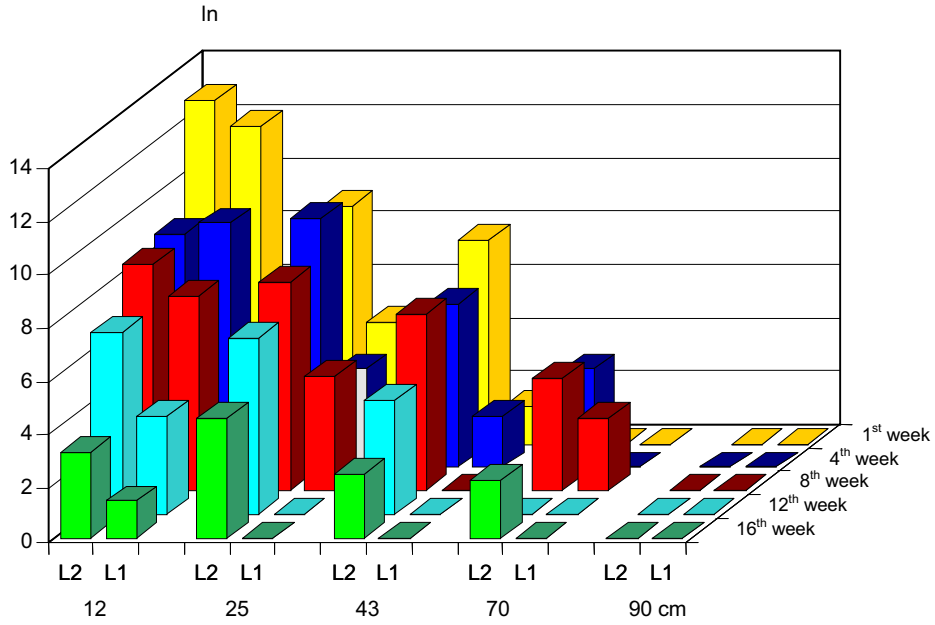

Fig. 2. Number of D-group streptococci (ln) per 100g of lessive soil at different depths over dry (L1) and moist (L2) summers

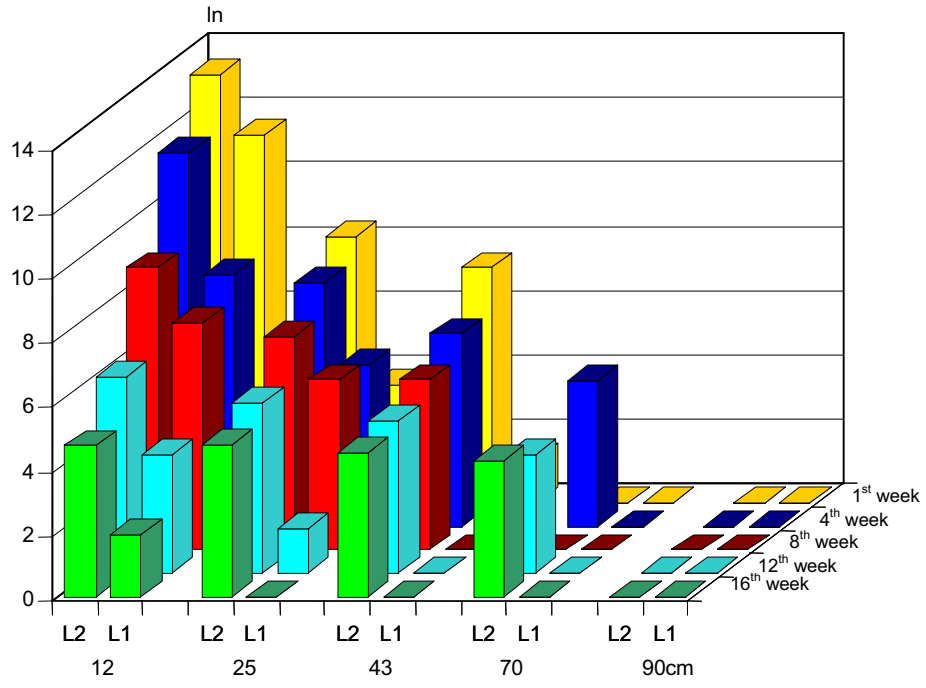

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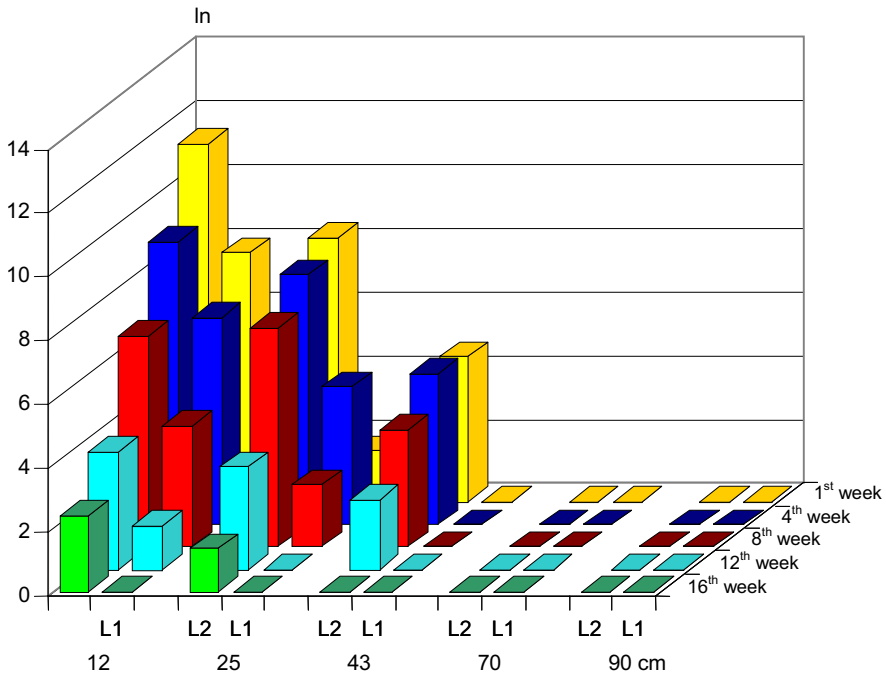
Fig. 3. Number of Salmonella spp. bacilli (ln) per 100g of lessive soil at different depths over dry (L1) and moist (L2) summers



7 days since the liquid slurry had been applied, the topsoil was observed to contain, over the first (L1) and the second (L2) research periods, from 1.5 x 10^5 to 4.0 x 10^5 *E. coli* bacteria and from 9.5 x 10^4 to 6.0 x 10^5 of D-group streptococcus colonies per 100 g of the soil. In the deeper soil profile layers the number of bacteria was considerably lower. Over dry summer at the depth of 27 cm, the number of D-group streptococci did not exceed 4.0 x 10^1, while the number of *E. coli* bacteria did not exceed 9.5 x 10^1 per 100 g of soil. They were also observed sporadically at the depth of 43 cm and at greater depths. Over the moist period the microorganism penetration after one week was much easier; at the depth of 27 cm there were noted 7.5 x 10^3 *E. coli* bacilli and 4.0 x 10^3 D-group streptococci per 100 g of soil. Also some microorganisms penetrated to the depth of 43 cm; no more than 2.0 x 10^3 *E. coli* bacteria and 1.5 x 10^3 entericcoccus colonies per 100 g of soil. A similar phenomenon was observed while analysing the activity of *Salmonella* bacilli in soil. However after one week their migration deep down the soil profile was limited over the dry period (L1). The bacilli were isolated in small numbers (75 bacteria per 100 g of soil) from the soil sampled up to 27 cm down, yet they were not detected at greater depths. Over the moist period, at three upper soil profile layers Salmonella bacilli were isolated in much greater numbers. At the depth of 12 cm - 7.5 x 10^4 and at 43 cm - 9.5 x 10^2 bacteria per 100 g of soil were detected.

Microorganism distribution in lessive soil throughout further research weeks over both L1 and L2 periods was still different. Over the dry period throughout the research cycle the bacteria were mostly observed at 12 cm, their inconsiderable number was detected at 25 cm (7.5 x 10^1 of *E. coli* bacilli and 2.0 x 10^2 of D-group streptococcus colonies per 100 g) as well as incidental presence at the depth of 43 cm. Over the period of higher rainfall, microorganisms penetrated deeper. Neither the number of *E. coli* nor D-group streptococci exceeded 9.5 x 10^2 bacteria per 100 g of soil even as deep as 70 cm. However no faecal bacteria were detected in soil sampled deeper.
In the course of research there was observed a decrease in the number of faecal bacteria in all the lessive soil profiles, especially the upper ones; the rate of decrease differed in both research periods. Over the dry summer the decrease incidence remains the same. At 12 cm the number of *E. coli* bacteria dropped from 1.5 x 10^5 to 4.0 x 10^0, while the number of D-group streptococcus from 9.5 x 10^4 to 7.0 x 10^0 colonies per 100 g of soil. Over the moist summer the decrease was observed in two stages; the first 8 weeks showed a rapid slump and then the number of bacteria in soil remained at the same level. At the final stage of the experiment (L2) the number of bacteria in soil was much higher than the number detected in L1. On average all over the soil profile and over the dry period, the highest rate of decrease (0.70 ln per week) in the number of *E. coli* bacteria was quite unexpected (Table 3).

Table 3. Regression curves for bacteria mean survival rate in the total lessive soil profile over dry (L_1) and moist (L_2) summers

Period	E. coli	D-group streptococci	Salmonella
L_1	Y = - 0.47x +12.75 r = - 0.975	Y = - 0.51x + 13.2 r = - 0.944	Y = - 0.61x + 19.6 r = - 0.812
L_2	Y = - 0.70x + 12.41 r = - 0.842	Y = - 0.61x + 11.40 r = - 0.893	Y = - 0.60x + 8.69 r = - 0.894

The microorganism survival period calculated with the course of the regression curve amounted to 17.7 weeks. Similar regression coefficient values calculated for the weekly decrease in the number of bacteria in soil researched were observed for the other microorganisms and amounted to - 0.61 for D-group streptococci and –0.60 for *Salmonella* bacilli. The survival period for D-group streptococci amounted to 18.7 weeks, while the one for *Salmonella* bacilli to 14.1 weeks, only.

Over L2 the soil profile showed the greatest rate of decrease in the number of Salmonella (by 0.60 ln per week), a lower rate for *E. coli* (by 0.47 ln) and for D-group streptococci (by 0.51 ln). The survival period was longer for *E. coli* and increased from 17.7 to 26.7 weeks, for D-group streptococci from 18.7 to 25.9, while for *Salmonella* from 14.1 to 19.9 weeks.

The analysis showed a relationship between the depth where the bacteria survival rate and the regression coefficient describing their decrease over time was being determined. Under the experimental conditions for *E. coli* and D-group streptococci, the curve took the form of

$$a = - 0.018\ h\ (\pm 0.002) + 1.2\ (\pm 0.06)$$

$$r = 0.99\ P < 0.065$$

DISCUSSION

The soil environment, due to its low content of nutrients, its specific bacterial flora, low temperature and other numerous factors, is not a favourable habitat for intestinal bacteria. Consequently, the number of microorganisms present in the liquid slurry being applied decreases constantly [15]. One can expect that the process of topsoil bacteria dying out in the topsoil at a considerable rate could have been due to bacteria bioactivity. Especially autochthonous soil microflora, protozoa and nematodes, present mostly in the humic layer, play an important role here [1,9,10]. However, deeper down the soil, there are much fewer autochthonous microorganisms, which could have created more favourable conditions for intestinal microorganisms.

Undoubtedly, the soil intestinal microorganisms were influenced by the weather conditions; much less favourable for faecal bacteria over the cycles researched were found over dry hot summer. A slump was observed in the number of microorganisms in the soil researched throughout the period of microbiological analysis; their number decreased almost completely, especially in the upper parts of the soil profiles, in the 3rd or 4th month of the cycle or they were present there in minimal quantities.

A high temperature, irrespective of the soil moisture level, is not favourable for faecal bacteria maintaining their viability in soil [20]. Over moist summer, the process of faecal bacteria dying out took place in two stages; over the first 8 weeks of research it was very intensive to be followed by a moderate decrease in the number of microorganisms in soil. By the end of the observation period, (4 months) their number was relatively similar and in the top layer of the soil profile it ranged from several to a few hundred of microorganisms per each 100 g of soil. The number of the D-group streptococci and *E. coli* bacteria isolated over the 2nd, 3rd and 4th month of research ranged slightly and in fact remained unchanged, which means a capacity of long-time survival of an inconsiderable number of microorganisms if there is a balance between auto- and allochthonous macroorganisms. It seems that moist soils are more favourable habitats for intestinal microorganisms [7,9,10].
Out of all the faecal microorganisms, *Salmonella* bacilli appear especially hazardous; causing and influencing widespread outbreaks of diseases, also in animals [2]. In the present research the microorganisms, depending on the weather conditions, survived from 7.2 to 14.9 weeks. The results obtained differ from the data reported by other authors. Thunegard [25] defines the survival of the *Salmonella* bacilli from 6 to 64 weeks, while Hess et al. [8] detected them in soil as late as after 500 days.

One shall stress that the activity of the other two intestinal bacterium species investigated was similar. Throughout the research period, almost all the D-group streptococci adapted to the soil conditions, which was observed in their slightly longer survival period. Depending on the temperature and soil moisture level, *E. coli* bacteria survived from 15.2 to 28.1 weeks, while D-group streptococci from 13.0 to 33.8 weeks. The results of the present research partially confirm the observations made by Crane and Moore [4] who observed a longer survival time of D-group streptococci in soil, as compared with the respective time measured for *E. coli* bacteria. Hirte [9], depending of the initial concentration, defined their survival time from 20 to 56 days. Edmonds [6], however, having applied liquid animal waste in woodlands, observed *E. coli* bacilli after 447 days. With time, faecal microorganisms applied with liquid slurry penetrated deep down the soil profile to a different extent, which depended considerably on the weather conditions, rainfall especially. One shall stress a high mobility of microorganisms as in the initial stage of the experiment, 7 days since the liquid slurry had been applied. Over the dry period they were usually detected in the soil sampled from the depth of 25 cm, occasionally from deeper layers. It seems that an increased bacteria absorption by soil particles was one of the main factors limiting the faecal bacteria migration deep into the soil profiles over the dry period. The presence of bacteria, after such a short period, in deeper soil layers could have been due to their movement through soil mega- and macro- pores, e.g. earthworm and plant root channels, often as deep as 1 m. Bacteria mobility through megapores deep down to the plant root zone can be expected after a few hours, sometimes as early as after a few minutes [24].

The activity of bacteria researched in soil throughout an increased rainfall was different; between the 4^{th} and 8^{th} weeks since the slurry had been applied, the bacteria were isolated from lessive soil sampled from the depth of 75 cm (4.0 - 9.5 x 10^2 D-group streptococcus colonies and 9.0 x 10^0 - 7.0 x 10^2 *E.coli* colonies per 100 g of soil). A similar activity was observed in *Salmonella* microorganisms, isolated up to the depth of 43 cm. As affected by rainfall, microorganisms, previously absorbed by soil particles could have been remobilised and diffusion-driven down the soil profile. A hazard of faecal bacteria reaching the groundwater calls for defining the maximum faecal bacteria penetration depth. The reports on the maximum faecal bacteria penetration depth in soils fertilised with slurry define it from several cm [6] to 3 m [19].

Although a majority of faecal bacterium populations applied with liquid slurry is retained in the top layers of the soil profile, some of them can migrate over a short period to a considerable depth, mostly via macro- and mega- pores, especially over moist seasons, which calls for adequate measures which would minimise the potential source of infection, creating detailed guidelines for animal waste safe application in agriculture.

CONCLUSIONS

1. The survival rate of the faecal microorganisms in lessive soil varied and depended on the bacterium species as well as the weather conditions following the slurry application.
2. A clear majority of faecal bacteria present in slurry was retained in the upper layers of soil profiles.
3. The process of bacteria dying out was most intensive in the topsoil over the dry period.
4. Under extreme weather conditions (heavy thunderstorm rainfall), at disturbed soil structure, one cannot eliminate the risk of groundwater contamination with pathogenic microorganisms present in slurry.

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