UDK 636.083.1 Izvorni znanstveni članak
Original scientific paper

HYGIENIC AND ECOLOGICAL RISKS CONNECTED WITH SURVIVAL OF *Salmonella Typhimurium* IN THE SOLID FRACTION OF PIG SLURRY FROM AGRICULTURAL WASTEWATER TREATMENT PLANT

I. Plachá, J. Venglovský, P. Novák, Alenka Tofant, Marija Vučemilo

Summary

We studied the survival of *Salmonella typhimurium* and dynamics of indicator microorganisms (psychrophilic, mesophilic, fecal coliforms, coliforms, fecal streptococci) at different temperatures (4°, 20 °C) in solid fraction of pig slurry.

Under laboratory conditions the investigated strain of *S. typhimurium* survived on a leather carrier at 20 °C until day 97 of investigation. The decimation time value (T_{90}) was 3.59 days.

Decimation time of indicator microorganisms ranged within 30.69 days (fecal streptococci) to 189.19 days (coliforms). A significant dependance of the survival time of psychrophilic and mesophilic microorganisms on the dry matter as well as the influence of the dry matter upon the survival of fecal streptococci could be proved. In coliform microorganisms and fecal streptococci the effects of ammonia nitrogen were also observed. In indicator microorganisms, storage time of the solid fraction notably influenced survival.

The investigated strain of *Salmonella typhimurium* survived on a carrier stored at 4 °C 135 days which corresponds with a T_{90} value of 41. 12 days. Decimation time values in indicator microorganisms ranged from 82.54 days (fecal streptococci) to 157.66 days(psychrophilic microorganisms). Statistic significance could be proved for the dependance of survival time of mesophilic microorganisms and fecal streptococci on ammonia nitrogen and of the coliforms, fecal coliforms and feacal streptococci upon total nitrogen. In fecal streptococci and feacal coliforms a profound influence of pH was

I. Plachá, J. Venglovský, Research Institute of Veterinary Medicine, Hlinkova I/A, 04001 Košice, Slovak Republic, P. Novák; University of Veterinary Medicine and Pharmaceutical Science, Palackeho 1-3, 61242 Brno, Czech Republic; Alenka Tofant, Marija Vučemilo, Department of Animal Hygiene, Environment and Ethology, Faculty of Veterinary Medicine of Zagreb, Croatia.

also observed. With the exception of psychrophilic and mesophilic microorganisms, statistically significant effects of storage time of the solid fraction upon the survival of indicator microorganisms could be stated.

Key words: *Salmonella typhimurium*, indicator microorganisms, survival, pig farm, wastewater treatment plant

Introduction

Recently great attention has been paid to the hygienic and ecological risks connected with the production, disposal and utilization of secondary raw materials from animal production.

One of the possibilities how to eliminate the negative effects of secondary raw material production (represented mainly by the excreta of farm animals) upon the quality of the environment and to decrease the hygienic risks is to build wastewater treatment plants near animal farms. Attention has been paid to the non-hygienized solid fraction from WTP that occurs after separation of the liquid part from slurry. Although utilization of this fraction for dunging purposes or the production of compost is often declared, practice has proven that no proper attention is paid to manipulation with this fraction.

We have literature data on the survival of pathogenic bacteria in animal excreta, manure, slurry and sludge. But we did not find data on the survival of pathogens in solid fraction of slurry. From this point of view we investigated the survival pathogenic microorganisms in solid fraction of slurry.

All over the world, *Salmonella* is one of the most widely spread enteropathogens that causes serious infections in animals and man. The knowledge of tenacity of *Salmonellae* in the environment is rather important with respect to epidemiology as well as effective disinfection. From this viewpoint we focused on investigating the survival of a model strain of *Salmonella typhimurium* and the dynamics of indicator microorganisms in the solid fraction from an agricultural wastewater treatment plant under laboratory conditions.

It is well known that temperature is one of the most important factors influencing the survival of microorganisms; the model strain was therefore investigated at two different temperatures.

Materials and methods

The solid fraction of slurry used in our experiments was obtained from an aerobic wastewater treatment plant of a pig farm after mechanical separation

on vibrating screens and stored in the laboratory at 20 °C and in a refrigerator at 4 °C. At the beginning of the experiment the method of Philipp et al. (1990) was used to determine *Salmonella* spp. in the solid fraction.

Inoculation and isolation of *Salmonella typhimurium* from test carriers.

Test bacteria

A freeze-dried strain of *Salmonella typhimurium* SK 14/39 (ŠZU, Prague, Czech Republic) was used as the test strain.

Test carriers

As test carriers sterile leather squares (4x4 cm) were used. As a control, sterile 20 cm long glass ampules of 1 cm in a diameter were used.

Experimental procedure

From 24 h broth cultures (Nutrient broth No. 2, Imuna, Šarišske Michal'any, Slovak Republic) of *S. typhimurium* 0.2 ml were inoculated to the leather squares and 1 ml was filled into the control glass ampules, which were sealed. The dried pieces of leather were placed into a thermostat for 24 h at 37°C. This was done in order to achieve good adhesion of *Salmonellae* to the carriers. After the removal from the thermostat the actual concentration of *Salmonella typhimurium* was determined on the carriers. Both the carriers and the ampules were transferred directly into the solid fraction placed in a vessel and investigated at intervals given in Tables 1 and 4. The carriers and control ampules were checked according to the method of Müller (1973).

For quantitative analysis of the carriers and the control ampules XLD and SS agar (Imuna) were used with 24 and 48 h incubation at 37 °C.

Simultaneously qualitative determination of the presence of *Salmonellae* was carried out on each of the carriers. For non-selective cultivation buffered peptone water (BBL, Becton Dickinson, USA) was used as pre-enrichment medium with 24h of incubation at 37 °C. For selective cultivation Selenite medium (Imuna) and the medium according to Rappaport and Vassiliadis (Merck, Darmstadt, Germany) as enrichment media were used. Both selective media were incubated 48 h, the Selenite medium at 37 °C and the Rappaport-Vassiliadis medium at 43°C. XLD and SS agar (Imuna) were used as selective-diagnostic media and incubated for 24 and 48h at 37 °C.

Suspect colonies were examined biochemically using the system for identification of Enterobacteriaceae (BBL Enterotube II, Becton Dickinson).

The serological examinations were carried out at the Department of Microbiology of the State Veterinary Institute in Košiće, Slovak Republic.

The results of the quantitative and qualitative examinations given in the tables are the arithmetic means of three parallel analyses. In qualitative examinations the given sample was considered to be negative when all three successive analyses for *Salmonella typhimurium* were negative.

Observation of the dynamics of the indicator microorganisms

The solid fraction samples intended for observation of the dynamics of the indicator microorganisms were taken at time intervals given in tables 2 and 5 as mean samples weighing about 50 g. The samples were processed according to the method of Philipp et al. (1990) and were analysed for the dynamics of indicator microorganisms (psychrophilic, mesophilic, fecal streptococcus, feacal and total coliform).

Quantitative analysis

Psychrophilic and mesophilic bacteria were determined using nutrient agar No.2 (Imuna), psychrophilic bacteria were incubated at 20 °C for 72h and mesophilic at 37 °C for 24h.

Feacal coliforms and coliform bacteria were analyzed on Endo agar (Imuna), incubated at 43 °C for 48 h (fecal coliform) and 37 °C for 24 h (coliform).

Fecal streptococci were determined using Selective agar for isolation of fecal streptococci (Imuna), incubated at 37 °C for 24h.

The values given in the tables are the arithmetic means of three parallel analyses.

Determination of the physical and chemical parameters

Determination of the physical and chemical parameters, pH value, dry matter, ammonia nitrogen, total nitrogen and total phosphorus was carried out according to -APHA et al. (1985).

Statistical evaluation of the experiments

Regression analysis of logarithmically transformed data was used to evaluate the influence of the physical and chemical parameters both upon

Salmonella typhimurium survival and the dynamics of the investigated indicator microorganisms.

Since the experiments were not carried out simultaneously but independently of each other, the starting concentrations of *Salmonella typhimurium* differed. For this reason devitalization of the investigated microorganisms was expressed in the decimation time value (T_{90}) as the main unifying indicator of devitalization rate.

Decimation time T_{90} was calculated as - 1α where α is the decimation constant of the straight line. Determination of the T_{90} values was based on a test for straight line fitness. This analysis has been described in detail elsewhere (Schlundt, 1982).

Results

Experiments at 20 °C

At the beginning of the experiments no *Salmonella* spp. microorganisms could be cultured from the solid fraction of slurry.

At 20°C, the tested *S. typhimurium* strain survived on the leather carrier 97 days (Table 1). The starting concentration of *Salmonellae* on the carrier was 1.39×10^{12} CFU (colony forming units) per ml of sample and on day 12 of the experiment the number of microorganisms still reached 4.38×10^{9} CFU per ml. As early as day 22 of the experiment a pronounced decrease to 3.29×10^{4} CFU per ml of sample could be observed. Between days 32 and 97 the tested strain was only subjected to qualitative analysis. The decimation time value (T_{90}) in the tested *Salmonella typhimurium* strain on a leather carrier was 3.59 days. At the end of the experiment (i.e., on day 150), *S. typhimurium* counts in the control ampules presented 7.54×10^{6} CFU per ml and the T_{90} value was 25.74 days.

At the beginning of the experiment, indicator microorganism counts in the solid fraction stored at 20 °C ranged within 10^{8} and 10^{9} CFU per ml of sample (Table 2). In the course of the observation period (150 days) the counts decreased by two - six orders. The most pronounced decrease in the counts of microorganisms was observed in the first third of the experiment (until day 43). After the end of the experiment decimation time values (T_{90}) ranged within 30.69 (fecal streptococci) and 189.19 days (coliform microorganisms; Table 2).

Table 1. - DETECTION TIME OF *Salmonella typhimurium* IN SOLID FRACTION - 20 °C

Time (Days)	Leather carrier CFU.ml^{-1}	Control sample CFU.ml^{-1}
0		1.39×10^{12}
1	4.70×10^{9}	5.87×10^{12}
12	4.38×10^{9}	2.79×10^{12}
22	3.29×10^{4}	3.67×10^{12}
32	+	4.78×10^{11}
36	+	5.67×10^{10}
43	+	4.87×10^{9}
57	+	7.65×10^{8}
73	+	4.65×10^{8}
85	+	9.87×10^{7}
97	+	7.98×10^{7}
107	-	4.65×10^{7}
117	-	8.65×10^{6}
127	-	3.87×10^{6}
137	-	9.87×10^{6}
150	-	7.54×10^{6}
slope	-0.27892	-0.03885
T_{90}	3.59	25.74

Table 2. - DYNAMICS OF INDICATOR MICROORGANISMS IN SOLID FRACTION - 20 °C

Time (Days)	Psychrophilic CFU.ml^{-1}	Mesophilic CFU.ml^{-1}	Fecal coliforms CFU.ml^{-1}	Coliforms CFU.ml^{-1}	Fecal streptococci CFU.ml^{-1}
0	1.35×10^{9}	1.27×10^{9}	1.16×10^{9}	1.16×10^{8}	1.51×10^{8}
43	1.04×10^{6}	3.90×10^{5}	1.20×10^{4}	8.76×10^{6}	3.60×10^{4}
83	2.54×10^{6}	1.99×10^{6}	7.85×10^{4}	2.45×10^{7}	2.00×10^{4}
117	7.27×10^{4}	3.37×10^{5}	1.50×10^{4}	7.86×10^{7}	100×10^{3}
127	6.23×10^{4}	1.78×10^{5}	6.76×10^{3}	6.76×10^{6}	7.86×10^{3}
137	2.12×10^{4}	2.98×10^{4}	4.23×10^{4}	5.43×10^{7}	2.34×10^{3}
150	1.99×10^{3}	2.34×10^{4}	3.45×10^{3}	3.21×10^{6}	1.89×10^{2}
slope	-0.03121	-0.02713	-0.0255	-0.00529	-0.03259
T_{90}	32.04	36.86	39.21	189.19	30.69

Of the physical and chemical parameters of the solid fraction (Table 3) the most pronounced changes could be recorded in pH, the starting value of which (6.91) increased to 8.14 after 43 days. It decreased to 7.22 on day 83 and after irregular decreases and increases it reached the starting value of 6.90 at the end of the experiment. Total nitrogen, ammonium nitrogen, total phosphorus and dry matter values slightly decreased.

Table 3. - PHYSICO-CHEMICAL PARAMETERS IN SOLID FRACTION - 20°C

Time (Days)	pH	dry mater (%)	N-NH_4 ($g.kg^{-1}$)	N-total ($g.kg^{-1}$)	P-total ($g.kg^{-1}$)
0	6.91	16.75	0.39	19.81	19.90
43	8.14	15.87	0.41	17.98	19.11
83	7.22	14.78	0.40	17.63	18.98
117	6.70	12.52	0.38	16.91	13.68
127	7.71	13.31	0.39	16.44	13.21
137	7.10	13.98	0.37	16.59	11.90
150	6.90	13.45	0.34	16.39	10.34

The dependance of survival time of psychrophilic and mesophilic microorganisms upon dry matter content was statistically significant at the level of $P<0.05$. A more pronounced effect of the dry matter content upon survival time was observed in fecal streptococci ($P<0.01$). In coliform microorganisms and fecal streptococci effects of ammonia nitrogen could be also opserved ($P<0.05$). Survival of indicator microorganisms was notably influenced by the storage time of the solid fraction (level of significance $P<0.01$, in psychrophilic microorganisms $P<0.001$).

Experiments at 4°C

The *Salmonella typhimurium* strain stored on a leather carrier at 4 °C was subjected to quantitative and qualitative analyses until day 92 and 135, respectively. From day 156 on neither qualitative analysis revealed *Salmonellae*. In the control ampule *Salmonella* counts at the end of the experiment decreased by two orders. Decimation time value (T_{90}) of *Salmonella typhimurium* on the leather carrier and in the control sample was 41.12 and 118.46 days, respectively (Table 4).

Table 4. - DETECTION TIME OF *Salmonella typhimurium* IN SOLID FRACTION - 4°C

Time (Days)	Leather carrier $CFU.ml^{-1}$	Control sample $CFU.ml^{-1}$
0	$8.71x10^7$	
1	$2.12x10^5$	$1.12x10^7$
13	$1.70x10^5$	$3.20x10^7$
37	$8.70x10^2$	$1.56x10^6$
63	$2.10x10^3$	$2.34x10^7$
92	$1.20x10^4$	$2.13x10^6$
111	+	$6.78x10^6$
135	+	$2.23x10^6$
156	-	$3.21x10^6$
190	-	$4.43x10^5$
220	-	$2.34x10^5$
slope	0.02432	-0.00844
T_{90}	41.12	118.46

Indicator microorganism counts in the solid fraction stored at 4 °C ranged between 10^4 and 10^7 CFU per ml of sample. The counts of mesohilic microorganisms, coliforms and fecal coliforms tended to increase until day 37 and to decrease from day 63 on. A decreasing tendency was also seen in the psychrophilic microorganisms. The counts of fecal streptococci tended to decrease as early as from day 13 on. Decimation time values (T_{90}) ranged between 82.54 days (fecal streptococci) and 157.66 days (psychrophilic microorganisms; Table 5).

Table 5. - DYNAMICS OF INDICATOR MICROORGANISMS IN SOLID FRACTION - 4 °C

Time (Days)	Psychrophilic ($CFU.ml^{-1}$)	Mesophilic ($CFU.ml^{-1}$)	Coliforms ($CFU.ml^{-1}$)	Fecal coliform ($CFU.ml^{-1}$)	Fecal streptococci ($CFU.ml^{-1}$)
0	$4.13x10^6$	$1.10x10^7$	$3.87x10^4$	$4.30x10^4$	$3.72x10^5$
13	$9.10x10^6$	$4.45x10^5$	$3.31x10^6$	$5.35x10^5$	$8.30x10^4$
37	$5.24x10^6$	$1.84x10^8$	$2.50x10^6$	$1.51x10^5$	$7.20x10^4$
63	$3.33x10^4$	$7.40x10^5$	$6.31x10^5$	$5.90x10^3$	$1.70x10^3$
92	$2.47x10^5$	$1.12x10^5$	$7.55x10^4$	$4.37x10^4$	$6.50x10^2$
111	$3.43x10^5$	$2.23x10^4$	$3.14x10^4$	$2.11x10^3$	$7.87x10^2$
135	$3.12x10^4$	$5.34x10^4$	$6.87x10^4$	$4.32x10^3$	$1.21x10^3$
156	$1.23x10^4$	$6.45x10^5$	$2.23x10^4$	$5.64x10^3$	$8.17x10^3$
190	$6.78x10^5$	$6.98x10^5$	$3.31x10^3$	$6.54x10^2$	$1.43x10^2$
220	$7.87x10^5$	$8.12x10^5$	$7.87x10^3$	$7.34x10^2$	$6.43x10^2$
slope	-0.00634	-0.01074	-0.01041	-0.01126	-0.01221
T_{90}	157.66	93.12	96.03	88.82	82.54

In the course of the experiment pH values decreased from the starting value of 8.80 to the final one of 6.20. pH values tended to decrease until day 63 of the experiment when a slight increase occurred, followed by a repeated decrease from day 111 on until the end of the experiment. Dry matter values of the solid fraction slightly decreased at the end of the experiment. Ammonia nitrogen values at the end of the experiment (0.19 $g.kg^{-1}$ dry matter) presented about the half of the starting value (0.42 $g.kg^{-1}$ dry matter). A rather pronounced decrease was observed on days 92 and 111. Total nitrogen values tended to decrease from the starting value of 19.78 $g.kg^{-1}$. The values of total phosphorus slightly decreased the extreme decrease was recorded on day 111 of the experiment (Table 6).

Table 6. - PHYSICO - CHEMICAL PARAMETERS IN SOLID FRACTION - 4 °C

Time (Days)	pH	dry matter (%)	$N\text{-}NH_4$ ($g.kg^{-1}$)	N-total ($g.kg^{-1}$)	P-total ($g.kg^{-1}$)
0	8.80	15.62	0.42	19.78	19.36
13	7.92	13.79	0.34	13.46	16.99
37	6.70	13.50	0.39	15.92	17.41
63	7.10	16.30	0.36	14.62	16.21
92	7.30	13.30	0.08	19.91	15.99
111	7.08	14.40	0.04	19.24	16.17
135	6.93	16.76	0.21	18.49	17.31
156	6.63	14.16	0.19	16.87	17.91
190	6.01	13.91	0.21	16.54	18.02
220	6.20	12.82	0.19	16.21	17.98

The dependance of the survival time of mesophilic microorganisms and fecal streptococci upon ammonia nitrogen as well as that of coliform microorganisms upon total nitrogen was statistically significant at the level of $P<0.05$. The effect of total nitrogen upon microorganism survival was more pronounced in fecal coliforms and fecal streptococci ($P<0.01$). In fecal streptococci and fecal coliforms pH levels were also found to significantly influence the survival of these microorganisms ($P<0.05$). Except in psychrophilic and mesophilic microorganisms, storage time of the solid fraction was determined to influence the survival of indicator microorganisms at the level of significance $P<0.01$.

Discussion

The results achieved in our experiments point to the fact that the investigated strain of *S. typhimurium* is able to survive for long periods in the solid fraction of slurry from a wastewater treatment plant. Although quantitative analysis revealed the presence of the microorganism only until day 92, qualitative analysis proved it to survive as long as until day 135. These results cannot be compared with those of other investigators since in the accessible literature we could not find data on the survival of *S. typhimurium* in the solid fraction of slurry.

Comparison of *S. typhimurium* survival at 4 °C and 20 °C reveals a longer survival time at the lower temperature (until day 135) than at the higher temperature (until day 97). The rate of *Salmonella typhimurium* concentration

decrease was more pronounced at 20 °C than at 4 °C and was expressed in the slope and T_{90} value (-0.27892 and 3.59 days; -0.02432 and 41.12 days).

The observations by Strauch (1987) according to whom 90% of *Salmonella* reduction is connected with pH decrease in the substrate could be confirmed. The decrease of the pH value during storage is influenced by the natural bacterial flora producing fatty acids which have toxic effects upon *Salmonellae*. The latter - in comparison to natural bacterial flora - are not able to secure nutrients; this probably causes their death.

It is evident from our results that pH is an important factor affecting the dynamics of indicator microorganisms, thus it can be presumed that, from the viewpoint of the devitalization of pathogenic microorganisms, the effective methods of secondary raw material processing are accompanied by extreme pH value changes combined with temperature effects which are the main factors of devitalization.

Hess and Breer (1975) studying the survival of *Salmonellae* in sludge, slurry and soil observed low temperatures to be a suitable condition. We can confirm these results since *S. typhimurium* survived longer at 4 °C than at 20 °C (Tables 1 and 4).

The percentage of dry matter content ranged within 12.5% and 16.8% which points a rather long survival time of indicator microorganisms as well as a pasty consistence of the solid fraction. Mitscherlich and Marth (1984) and Strauch (1987) claimed the survival time in slurry to increase with the decreasing ratio of the solid and luquid fraction. The prolonged survival period can also be explained by the fact that excreta with a lower dry matter content do not self-heat during storage, thus the survival of pathogenic microorganisms is promoted by insufficient temperatures.

It is evident from our results that indicator microorganisms survive long periods in the solid fraction of agricultural wastewater treatment plants. This is also indicated by the decimation time values (T_{90}) which exceed the T_{90} values recorded in aerated and non-aerated slurry (Larsen et al., 1988).

Pepper et al. (1991) observed the dynamics of indicator microorganisms in sewage sludges prior to and after anaerobic treatment and 5 weeks after spreading over soil. They recorded the starting values of fecal streptococci, coliforms and fecal coliforms ($6.1x10^6$; $1.3x10^8$; $3.7x10^7$; $CFU.kg^{-1}$ of dry matter) to decrease after treatment to $9.6x10^6$; $4.6x10^6$; $4.6x10^4$ $CFU.kg^{-1}$ of dry matter. However, after 5 weeks, when temperature in the soil over which the sludge was spread decreased, the values of indicator microorganisms again increased and reached $1.0x10^3$; $9.8x10^6$; $4.5x10^6$ $CFU.kg^{-1}$ of dry matter, respectively.

When comparing these results with the values of the indicator microorganisms recorded in our experiments in which a lower decrease was observed at 4 °C than at 20 °C it seems to be a matter of fact that low temperature promotes the viability of bacteria (Tables 2 and 5).

According to Larsen and Munch (1986) spreading of 10 t of liquid manure (with a concentration of 10^4 *S. typhimurium* per 1 ml which is a concentration often met with in practice) over 1 ha of soil would cause a mean starting concentration of 10^7 *S. typhimurium* per square meter of area. When comparing the *Salmonella* counts recorded in our experiment 1.20×10^4 $CFU.ml^{-1}$ after 92 day storage of the solid fraction at 4 °C and qualitative observation of the tested microorganisms on day 97 at 20 °C with the above facts, the danger of spreading non-treated (non-disinfected) solid fraction over soil is obvious.

In view of these data it is important that in agricultural wastewater treatment plants attention is paid not only to the effectiveness of liquid fraction treatment but also to handling, storage and processing of the solid fraction of slurry.

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HIGIJENSKI I EKOLOŠKI RIZIK U SVEZI S PREŽIVLJAVANJEM *Salmonella typhimurium* U KRUTOJ FAZI SVINJSKOG TEKUĆEG GNOJA IZ UREĐAJA ZA PROČIŠĆAVANJE

Sažetak

U laboratorijskim uvjetima istraživano je preživljavanje *Salmonellae typhimurium* kao i dinamika indikatorskih mikroorganizama (psihrofilnih, mezofilnih, fekalnih koliforma, koliforma, fekalnih streptokoka) pri različitim temperaturama (4°, 20°C) u krutoj fazi svinjskog tekućeg gnoja.

Na kožnim nosačima kod 20 °C vrijeme preživljavanja za *S. typhimurium* iznosilo je 97 dana. Decimalna redukcija (90%) mikrobne populacije (T_{90}) iznosila je 3,59 dana.

Decimalna redukcija T_{90} za indikatorske mikroorganizme kretala se od 30.69 dana za fekalne streptokoke do 189,19 dana za koliforme. Može se dokazati i značajna ovisnost vremena preživljavanja psihrofilnih, mezofilnih i fekalnih streptokoka o sadržaju suhe tvari u gnoju. Kod koliforminh mikoorganizama i fekalnih streptokoka ustanovljen je i značajan utjecaj amonijačnog dušika. Vrijeme skladištenja krutog gnoja također ima znatan utjecaj na preživljavanje indikatorskih mikroorganizama.

Kod 4 °C istraživani soj *S. typhimurium* preživio je na nosaču 135 dana, što odgovara T_{90} vrijednosti od 41,12 dana. Za indikatorske mikroorganizme T_{90} je varirao od 82.54 dana za fekalne streptokoke do 157,66 dana za psihrofilne mikroorganizme. Može se dokazati statistički značajna zavisnost vremena preživljavanja mezofilnih mikroorganizama i fekalnih streptokoka o sadržaju amonijačnog dušika, a koliforma, fekalnih koliforma i fekalnih streptokoka o sadržaju ukupnog dušika. Vrijednost pH je imala izražen utjecaj samo na fekalne streptokoke i fekalne koliforme. Uz izuzetak psihrofilnih i mezofilnih mikroorganizama vrijeme skladištenja krute faze gnoja ima statistički značajan utjecaj na preživljavanje indikatorskih mikroorganizama.

Primljeno: 15. 10. 2001.