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Effect of temperature on the number of selected microorganism groups and enzymatic activityof sewage sludge composted with different additions in cybernetic bioreactors

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Abstract. Microbiological characteristics of sewage sludge from a mechanical-and-biological sewage treatment plant composted in controlled conditions with straw and sawdust are presented. Prepared composts were placed in four bioreactors with differentiated oxygen flow (2L and 4L air·min^{-1}). In bioreactor K3 and K4, the composted mass consisted of 70% sewage sludge + 25% sawdust + 5% straw, while in bioreactor K1 and K2, the proportion was 50% sludge + 45% sawdust + 5% straw. Compost samples were taken from all chambers at the same time depending on the actual temperature.
Microbiological analyses consisted in the determination by plate method on selective medium the numbers of mesophilic and thermophilic bacteria, actinomycetes, molds and pathogenic bacteria from *Salmonella* genus *Clostridium perfringens* and from *Enterobacteriaceae* family. Furthermore, in the experiment, the activity levels of dehydrogenases were determined using 1% triphenyltetrazole chloride as substratum.
Studies have shown that the composting process had caused a total elimination of *Enterobacteriaceae* in all composted masses and a reduction of the remaining microorganism groups. During the experiment, a drop in the level of dehydrogenases activity was observed which was negatively correlated with temperature increase in the studied composts.

Key words: microorganisms, dehydrogenases activity, biowaste, compost

INTRODUCTION

Important ecological problems connected with the development of civilization include the necessity to utilize enormous amounts of wastes which not only create a burdensome ballast, but also may have an unfavourable effect on the environment (Szejniuk & Kluczek ,1996; Lasocka, 2000). One possibility for utilizing sewage sludge is to compost it and then use it in agricultural production (Czyżyk et al., 2002; Wolna-Maruwka et al., 2005).

The composting of sewage sludge is gaining increasing interest in Poland and abroad, because the process permits, among others, decreasing the amounts of sludge stored on storage yards and reintroducing many plant nutrition components into biological circulation; primarily, however, it allows minimization of the sanitary endangerment. According to Butler et al. (2001), Sidhu et al. (2001) and Cambardella et al. (2003), the introduction into the soil of uncomposted or nonstabilized sewage sludge can lead to immobilization of nutritive components and contribute to the agglomeration of substances toxic for plants and creates a bacteriological threat for microorganisms.

During the composting process, besides the final product in the form of humus, the following are released: heat, compounds of nitrogen, oxygen, phosphorus, CO_2, H_2O; in addition, a significant amount of microorganism biomass is created (Tiquia et al., 2002). The succession of mesophilic and thermophilic microorganisms in composted material is connected with temperature changes and indicates the correct course of the composting process (Finstein et al., 1986; Ishii et al., 2000). High temperature (60–75°C) reached in the thermophilic phase of composting is a factor which decreases or completely reduces the number of pathogens (Macgregor et al., 1981).

According to Strom (1985), the diversity and activity of microorganisms in composted material are affected by temperature but also by the degree of oxygenation and the availability of nutritive components.

Thus microorganisms play the key role in the composting of organic matter and in the creation of humus (full-value fertilizer). However, it is important that the final product obtained is not only useful but also safe from the hygienic perspective.

MATERIALS AND METHODS

The experiment was established in laboratory conditions in 2006. Material used in the studies consisted of sewage sludge originating from the left-side-bank of the Sewage Treatment Plant in Poznań and consisted of wheat and rye straw and sawdust. The microbiological and chemical analyses are presented in Tables 1 and 2. Studies were carried out in four bioreactors of 125dm^3 capacity and equipped with electronic sensors for constant recording of process parameters (temperature, carbon dioxide, methane, ammonia and oxygen) (Figure 1). Materials for the studies were thoroughly mixed in a container in weight proportion in relation to dry matter as follows: 70% of sewage sludge + 25% of sawdust + 5% of straw in bioreactor K3 and K 4; while in bioreactor K1 and K2, the proportion was: 50% of sewage sludge + 45% of sawdust + 5% of straw.

The experiment was conducted with a constant air flow amounting to 4L·min^{-1} in chambers 3 and 4 and 2L·min^{-1} in chambers 1 and 2.

Material in the bioreactors was composted for 552hrs, while compost samples were taken from all chambers at the same time depending on the actual temperature of the composted material.

Table 1. Chemical property values of components used in experiment

Characteristic		sewage sludge	straw	sawdust
Dry mass %		47.64	90.00	82.80
pH-H_2O		8.14	-	-
$C_{org.}$	$g \cdot kg^{-1}$ d.m.	222.20	426.30	493.10
$N_{tot.}$		20.58	3.37	4.210
C : N		10.80	126.50	117.10

Table 2. The number of microorganisms in sewage sludge, straw and sawdust (beginning of experiment)

Groups of microorganisms	sewage sludge	Straw	sawdust
$cfu \cdot g^{-1}$ d.m. of material			
Salmonella sp.	0	0	0
Mesophilic bacteria	$1687.66 \cdot 10^6$	$9.50 \cdot 10^6$	$7.00 \cdot 10^6$
Theromphilic bacteria	$354.13 \cdot 10^3$	$1.90 \cdot 10^3$	$46.20 \cdot 10^3$
Molds	$2586.66 \cdot 10^4$	$39.90 \cdot 10^4$	$9269.86 \cdot 10^4$
Actinomyces	$3251.19 \cdot 10^5$	$79.80 \cdot 10^5$	$429.33 \cdot 10^5$
Enterobacteriaceae	$316.92 \cdot 10^3$	$18.45 \cdot 10^3$	0
Clostridum perfringens	$110.60 \cdot 10^3$	0	0
$mg \cdot TPF \cdot kg^{-1}$ d.m. of compost$\cdot 5h^{-1}$			
Dehydrogenase activity	109.97	21.91	15.82

On microbiological selective medium, using the plate method, the number of colony forming units (cfu) of fungi, actinomycetes, mesophilic and thermophilic bacteria, as well as pathogenic bacteria of *Salmonella* genus, *Clostridium perfringens* and *Enterobacteriaceae* family were determined.

The number of mesophilic bacteria was determined on simple nutritive agar by incubation of plates at 26°C for 48 hrs (Kańska et al., 2001). Thermophilic bacteria were determined on 3% nutritive agar. Plates were incubated for 24hrs at 55°C (Kańska et al., 2001). Mould fungi were determined on Martin's nutrient at 24°C for 5 days (Martin, 1950). The number of actinomycetes was determined on Pachon's selective medium (Kańska et al., 2001) by incubation of plates for 7 days at 26°C.

Salmonella sp. was determined on XLT medium of Merck after 18-24 hours at 37°C (Miller & Tate, 1990). In order to make sure that the bacteria were of *Salmonella* sp. genus, the procedure was conducted according to the Polish Standard PN-Z-19000-1 with a confirming identification (Polish Normalization Commiittee, 2001).

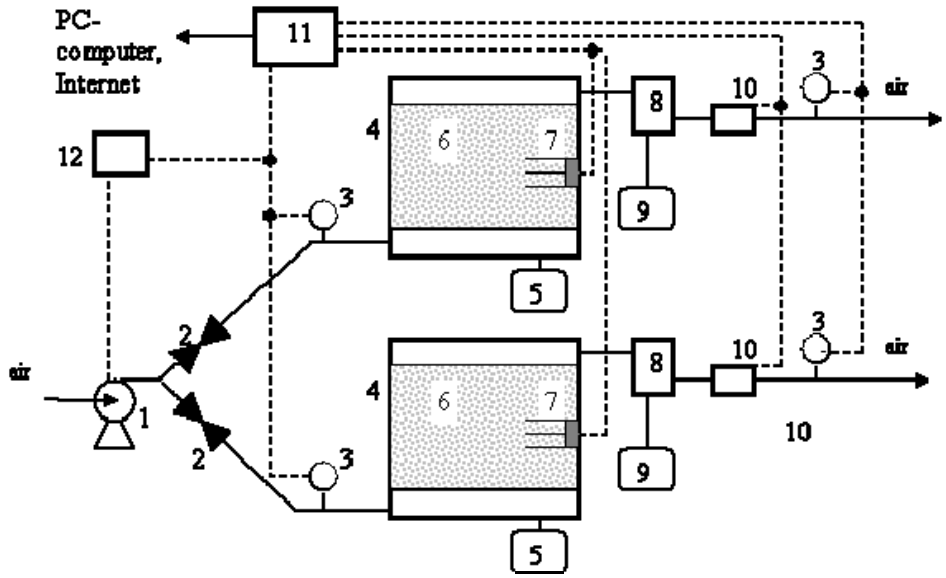

Fig. 1. Schematic diagram of the 2-chamber bioreactor: 1. pump, 2. flow regulator, 3. flow meter, 4. isolated chamber, 5. drained liquids container, 6. composted mass, 7. sensors set, 8. air cooling system, 9. condensates container, 10. column of gases content analysis (NH_3, O_2, CO_2, CH_4, SH_2), 11. 16-channel recorder, 12. air pump steering system

In order to determine the number of bacteria from *Enterobacteriaceae* family, the selective substratum of Merck was used. Plates were incubated at 37°C for 24 hours (Manafi & Kneifel, 1989). *Clostridium perfringens* was determined on TSC agar substratum with triptose, sulphate and cycloserine by incubation of plates in a thermostat with 22% CO_2 content, at 37°C for 24 hours (American Public Health Association, 1984). Furthermore, in the sampled composted material, the activity of dehydrogenases was identified by spectrophotometric method, using as substrate 1% TTC (triphenyl-tetrazole chloride), after 5-hour incubation in 30°C, at wave length 485nm. Enzyme activity was expressed in mg TPF·kg^{-1} d.m. of compost·$5h^{-1}$ (Thalman, 1968).

Statistical analyses applied in the experiment were used on the basis of Statistica 7.1 program (Ott, 1984).

RESULTS AND DISCUSSION

On the basis of microbiological analyses, it was found that an increase of temperature during the composting process (Figure 2) was the main factor causing changes in the number of the analysed microorganism groups. According to Taiwo & Oso (2004) the temperature of composted matter determines the rate of many biological processes and plays a basic role in microorganism succession understood as a change in the quantitative and qualitative composition of the microorganism population.

On the basis of the number of mesophilic bacteria (Table 3) in the analysed composts, it was found that their highest number the day of the experiment's establishment (term I) occurred in the compost K3 and amounted to 705.05 cfu·10^6·g^{-1}·d.m. of compost. A 24-hour composting process in controlled conditions caused an increase of temperature in bioreactors by 15-22°C, which contributed to the increase of mesophilic bacteria number in all chambers from 5 to 20.5%.

In the successive analysis term (term III–analysis after 48h) a sudden drop was recorded in the proliferation of mesophilic bacteria most probably connected with the successive 10-degree plus increase of temperature in the composted materials.

Starting with term III, the number of mesophilic bacteria was maintained on a very low level connected with a high temperature dominating in composts which reached 73°C. The decrease of proliferation of mesophilic bacteria with the increase of temperature in the composted communal wastes was observed also by Hassen et al. (2001).

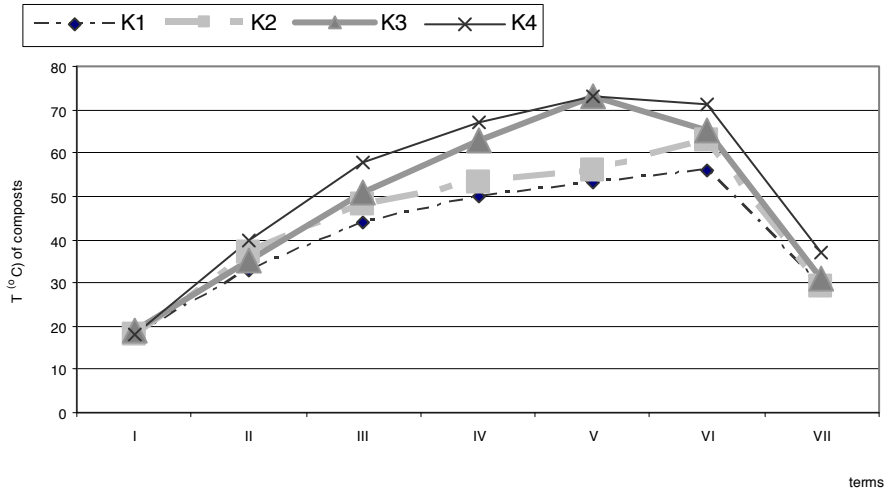

Figure 2 The changes of temperature in biowastes during composting process

Fig.2. The changes of temperature in biowastes during composting process

After 552h of composting, temperature decrease, which contributed to a slight increase of mesophilic bacteria proliferation, was recorded in all chambers.

From the results shown in Table 3, it follows that in the early phase of the composting process, when the temperature oscillated between 20 and 40°C, the mesophilic bacteria were the dominating group in the decomposited organic matter. However, the increase of temperature above 40°C (Figure 1) contributed to their destruction or inactivation and thereby to the domination of thermophilic bacteria in composts (Table 3).

According to Schlegel (2003), in the thermophilic phase, in the compost, bacteria from genus *Bacillus* dominate, while the temperature of 70°C is regarded as the critical temperature of thermophilic bacteria development. Strom (1985) reported that in the temperature range 49-55°C, the species *B. coagulans* and *B. circulan* dominated Temperature increase to the value of 55-65°C causes a change in the domination which is taken over by *B. brevis* and *B. stearothermophilus* species. In turn, in a higher temperature, exceeding 70°C, *Caldibacillus cellulvorans* decomposing cellulose is dominant (Berquist et al., 1999). According to Janda & Falkowski (2003), thermophilic microorganisms possess the ability for growth and development in conditions of high temperatures to the specific biochemical properties of their cells and to a lesser degree because of their morphological structure. Furthermore, the resistance of microorganisms to the negative effect of high temperature depends also on environmental factors (pH, chemical composition of substratum).

On the basis of data presented in Table l, it was found that starting with term III, when the temperature in the composted materials increased up to 44-58°C, the proliferation of thermophilic bacteria started increasing as well. The above status was maintained until term V, when the number of the discussed microorganisms reached the maximal values in the range of 12965.3–30524.0 $cfu \cdot 10^4 \cdot g^{-1}$ d.m.

A drop in the proliferation of themophilic bacteria in all analysed composts was recorded after 120h of composting (term VI).

The sudden decrease in the number of thermophilic bacteria in all chambers could have been caused by the appearance in the substratum of metabolism products inhibiting their development, or as a result of the depletion of organic matter subject to decomposition.

Table 3. The number of mesophilic, thermophilic bacteria, fungi and actinomycetes in composts

Kind of com-post	T° of com post (°C)	cfu·10^6·g^{-1} d.m. of compost	SD*	cfu·10^4· g^{-1} d.m. of compost	SD*	cfu·10^5· g^{-1} d.m. of compost	SD*	cfu·10^4· g^{-1} d.m. of compost	SD*
		Mesophilic bacteria		**Thermophilic bacteria**		**Actinomycetes**		**Fungi**	
I date– beginning of experiment									
K1	18	**634.43**	116.43	**27.50**	3.77	**1319.76**	795.21	**1106.36**	310.82
K2	18	**634.43**	116.43	**27.50**	3.77	**1319.76**	795.21	**891.33**	92.91
K3	19	**705.05**	13.72	**15.35**	21.70	**1321.89**	293.23	**656.11**	68.39
K4	18	**397.27**	28.22	**34.98**	174.4	**1227.39**	75.31	**485.47**	138.00
II date– after 24 h									
K1	33	**848.34**	132.18	**117.63**	10.78	**2232.60**	102.19	**53.36**	37.47
K2	37	**764.42**	123.60	**185.36**	27.98	**1958.53**	466.91	**37.47**	8.89
K3	35	**1040.07**	81.07	**77.53**	38.79	**2986.64**	730.94	**50.69**	14.75
K4	40	**616.33**	53.09	**70.30**	2.87	**2436.03**	1249.15	**34.64**	11.30
III date– after 48 h									
K1	44	**9.05**	1.13	**221.30**	42.83	**76.64**	1.45	**30.89**	1.61
K2	48	**3.61**	0.24	**1239.72**	895.79	**40.66**	11.61	**7.71**	2.63
K3	51	**0.97**	0.56	**1126.46**	133.87	**85.93**	32.14	**0.57**	0.22
K4	58	**3.62**	0.29	**979.11**	204.64	**145.75**	3.75	**4.78**	1.19
IV date– after 72 h									
K1	50	**0.03**	0.00	**464.02**	27.34	**28.44**	3.29	**1.37**	0.38
K2	53	**0.04**	0.00	**3526.04**	1789.72	**33.39**	4.71	**0.38**	0.04
K3	63	**0.03**	0.00	**1176.25**	862.69	**10.75**	1.61	**0.002**	0.00
K4	67	**0.02**	0.00	**19509.7**	685.50	**14.67**	4.65	**0.03**	0.007
V date– after 96 h									
K1	53	**0.38**	0.06	**12965.3**	1616.68	**3.04**	0.35	**0.77**	0.73
K2	56	**0.34**	0.07	**30524.0**	2134.42	**3.06**	0.29	**1.53**	0.00
K3	73	**0.38**	0.03	**15650.7**	814.25	**3.26**	1.10	**0.01**	0.00
K4	73	**0.07**	0.02	**16342.8**	840.58	**1.51**	0.16	**0.02**	0.01
VI date – after 120 h									
K1	56	**0.01**	0.01	**166.54**	162.72	**6.48**	2.17	**1.04**	0.06
K2	63	**0.34**	0.07	**112.87**	65.16	**5.13**	0.84	**0.009**	0.00
K3	65	**0.09**	0.03	**587.89**	55.02	**6.75**	0.16	**0.009**	0.002
K4	71	**0.002**	0.00	**23.07**	0.00	**3.55**	0.48	**0.007**	0.003
VII date– after 552 h									
K1	29	**0.01**	0.00	**1.15**	0.15	**11.27**	2.36	**0.09**	0.01
K2	29	**0.4**	0.20	**9.88**	3.26	**21.78**	1.56	**0.12**	0.05
K3	31	**0.04**	0.02	**0.47**	0.00	**14.71**	0.32	**0.06**	0.01
K4	37	**0.03**	0.01	**0.46**	0.27	**17.25**	1.38	**0.06**	0.04

On the basis of the analysis of the proliferation of actinomycetes in all chambers (Table 3) during composting of biowastes, it was found that their number increased by 50-120%, after 24 hours of material composting. The reason for the above phenomenon was the temperature increase in the composted masses by 13-22°C. In the successive terms of analyses, it was found that as the temperature in bioreactors increased, the number of actinomycetes decreased. A repeated increased proliferation of the discussed group of microorganisms was recorded in the last term (VII), when the temperature in the composted masses dropped to the value of 29-37°C. An increase in the number of actinomycetes in composted materials connected with the drop of temperature below 40°C was also reported by Wieland & Sawicka (2000).

The role of actinomycetes in the composting process is very significant because these organisms participate in the degradation of ligninocelluloses being the precursors in the creation of humus. Furthermore, it is one of the microorganism groups taking part in the synthesis of vitamin B_{12} in the composted material (Wieland & Sawicka, 2000).

On the basis of statistical analysis, it was found that the term of material sampling had a highly significant effect (at α>0.01) on the development of actinomycetes which had not been shown in the case of the application of compost combinations.

Analogically as in the case of actinomycetes, the quantitative changes of fungi showed similar changes during the experiment (Table 3). The greatest number of fungi in all chambers was recorded in term I, i.e. on the day of experiment establishment and it oscillated within the limits: 485.47–1106.36 cfu$\cdot 10^4 \cdot g^{-1}$ d.m. In the successive terms of analyses, with the increase of temperature in the composted materials, a drop in the proliferation of fungi was recorded. Also Weyman-Kaczmarkowa & Głuchowska (2000) recorded a decrease in fungi proliferation with temperature increase in sewage sludges composted with different additions in laboratory conditions. A successive factor resulting in the decrease of fungi number during the experiment could be the increase of pH in the composted materials (Figure 3).

Analyses of results shown in Table 3 indicated that the decrease of the number of fungi continued until term VI and then, in term VII, a small increase in their proliferation was observed in the majority of chambers, while the composting process had lasted 552h.

Wieland & Sawicka (2000) also stated that with the temperature drop below 40°C, the number of fungi in the composted mass increased. These microorganisms, besides further organic matter decomposition, produce in that time antibiotic substances serving for natural disinfection of the composts.

Among factors limiting agricultural utilization of sewage sludges, a significant role is attributed to their presence in human and animal pathogenic microorganisms. In spite of many factors inhibiting the development of pathogens in the soil (pH, drought, insolation etc.), it is necessary to expose sewage sludges to processes aiming at their full hygienization (Watanabe et al., 1997).

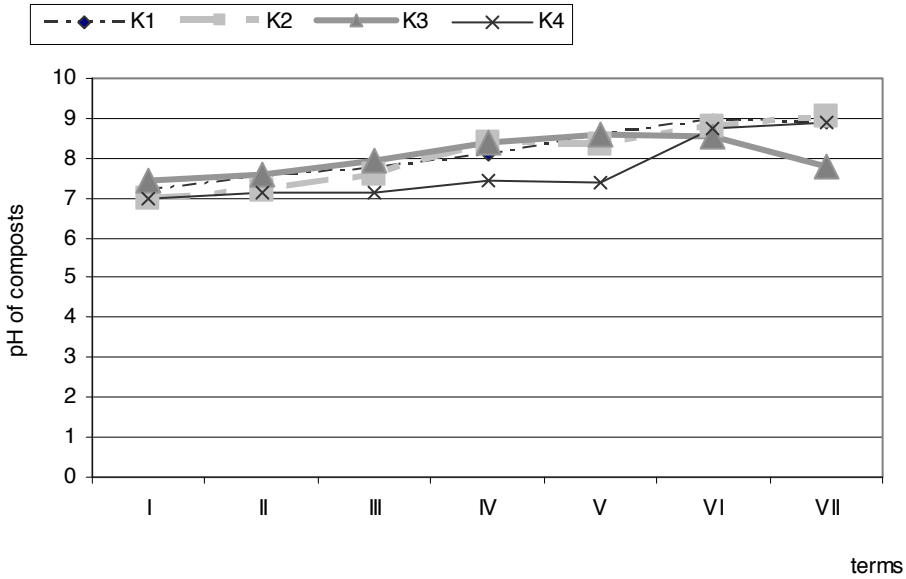

Figure 3 The changes of pH in biowastes during composting process

The effectiveness of sewage sludge composting depends on the value of temperature obtained during composting (Paluszak et al., 2004). Because of the absence of *Salmonella* spp. bacteria in the analysed microbiological materials, they have not been discussed in this paper.

On the basis of results presented in Table 4, it was found that the composting process was characterized by a high effectiveness in the elimination of bacteria from *Enterobacteriaceae* family regarded as the sanitary indicator of the purity of sewage sludge, soil and water (Hassen et al. 2001; Directive of the Minister of Agriculture and Rural Development, 2004).

Analysis of the above mentioned bacteria starting from the day of the experiment's establishment (term I) has indicated that after 24 hours of composting, there followed a repeated drop in the bacteria number in all chambers. Further decrease of the proliferation of the discussed bacteria was recorded in the successive terms of analyses, while a total destruction of bacteria was observed after 72h of composting (term IV) in bioreactors K3 and K4. A complete reduction of *Enterobacteriaceae* in the above term was connected with the increase of temperature in the chambers above 60°C. Analogical conclusions were drawn in the case of the mass composted in chambers K1 and K2 after 120h of composting (term VI), when the temperature reached 56°C in K1, and 63°C in K2.

An inhibiting effect of high temperature obtained in the thermophilic phase in relation to bacteria belonging to *Enterobacteriaceae* family was also reported by other authors (Hassen et al. 2001; Deportes et al. 1995; Shaban 1999).

Similarly as in the case of *Enterobacteriaceae,* both the composition of the composted material and the term of analyses had a significant statistical effect on the proliferation of *Clostridium perfringens* bacteria (Table 4). On the day of the experiment's establishment, the highest number of bacteria was recorded in composts K1 and K2, amounting to 2542.67 cfu·10^{2}·g^{-1} d.m. However, a 24-hour composting process had already caused a repeated drop of *Clostridium perfringens* bacteria number. In the successive terms of analyses, the proliferation of the discussed bacteria continued decreasing, however, in none of the composted materials was a complete elimination of those pathogens found. Studies of Kaźmierczuk & Kalisz (1997) indicated the presence of *Clastridium perfringens* in sewage sludges even after a 3-year period of storage. The ability of the discussed bacteria to survive in high temperatures and in other unfavourable environmental conditions was most probably connected with their production of surviving forms.

Composting of sewage sludges favours not only changes in microorganism number, but also supports changes of enzyme activity levels in composts being regarded as an indicator of a correct course of the composting process (Pelåez et al., 2004). According to Macgregor et al. (1981), a strong decomposition of organic matter follows in the thermophilic phase of the composting process. In turn, McKinley & Vestal (1984) reported that the greatest metabolical activity occurred in temperatures ranging from 25–45°C. The same was confirmed by the studies of Tiquia et al. (2002), who stated that the activity of dehydrogenase reached the highest level at the beginning of the composting process and decreased as the process progressed. A similar tendency was observed in our own studies (Figure 4). In chambers K1 and K4, the activity of dehydrogenases originally increased, however, after surpassing 50°C, a temperature drop was observed. In the remaining two composted masses, a drop in the activity of dehydrogenases was recorded after 24 hours (term II) of the process, The decreasing tendency in the enzymatic activity level was maintained thereafter in all composted materials until term VI, and then, in the last term (VII), together with temperature drop to the value of 29–37°C, an increase of dehydrogenases activity was recorded.

Furthermore, on the basis of statistical analysis (Figures 5–8), it was found that the activity of hydrogenases in all chambers was negatively correlated with the level of temperature values in the composted biowastes.

The composting process had a more violent course in composts with the higher 70% share of sewage sludges (compost K3 and K4). In spite of that, changes in the number of the analysed microorganism groups in the composted biowastes, in the majority of cases (except *Enterobacteriaceae)* showed a similar course.

Table 4. The number of *Enterobacteriaceae* and *Clostridium perfringens* in composts

Kind of compost	Temperature of compost (°C)	cfu·g^{-1} d.m. of compost	SD	cfu·g^{-2} d.m. of compost	SD
I date– beginning of experiment					
K1	18	**3154.95**	1061.89	**2542.67**	1009.11
K2	18	**3154.95**	1061.89	**25.42.67**	1009.11
K3	19	**3789.27**	1321.87	**2344.73**	269.89
K4	18	**3726.65**	758.62	**1832.93**	389.57
II date– after 24 h					
K1	33	**219.59**	34.03	**938.40**	363.23
K2	37	**179.24**	72.87	**854.98**	439.60
K3	35	**353.65**	38.58	**796.75**	221.48
K4	40	**75.95**	14.51	**550.37**	31.12
III date– after 48 h					
K1	44	**31.62**	3.87	**181.32**	84.08
K2	48	**15.13**	5.10	**69.77**	46.03
K3	51	**43.68**	6.46	**90.24**	81.21
K4	58	**106.76**	3.30	**240.78**	137.34
IV date– after 72 h					
K1	50	**17.70**	0.00	**198.45**	47.81
K2	53	**54.38**	42.22	**66.03**	15.28
K3	63	**0.00**	0.00	**227.78**	188.28
K4	67	**0.00**	0.00	**308.96**	8.37
V date– after 96 h					
K1	53	**111.95**	9.01	**48.81**	12.28
K2	56	**8.26**	1.36	**93.83**	32.27
K3	73	**0.00**	0.00	**48.69**	46.26
K4	73	**0.00**	0.00	**11.97**	7.98
VI date– after 120 h					
K1	56	**0.00**	0.00	**120.42**	81.18
K2	63	**0.00**	0.00	**438.77**	117.14
K3	65	**0.00**	0.00	**41.90**	40.32
K4	71	**0.00**	0.00	**12.69**	3.99
VII date– after 552 h					
K1	29	**0.00**	0.00	**12.17**	6.73
K2	29	**0.00**	0.00	**50.79**	10.80
K3	31	**0.00**	0.00	**5,82**	4.03
K4	37	**0.00**	0.00	**2.30**	0.00

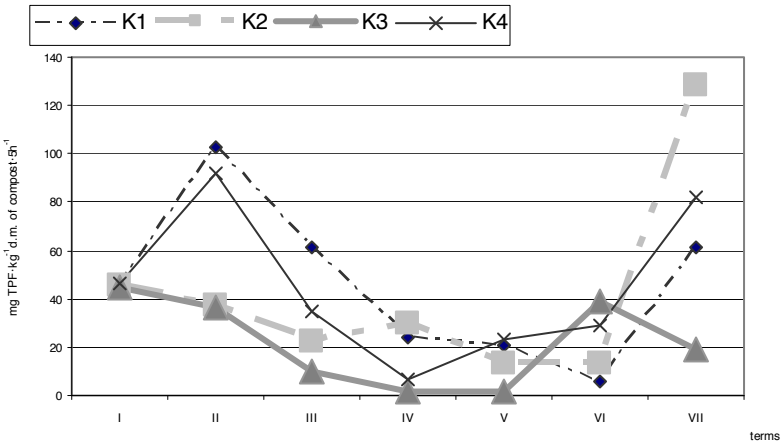

Fig. 4. The changes of dehydrogenases activity in biowastes during composting process.

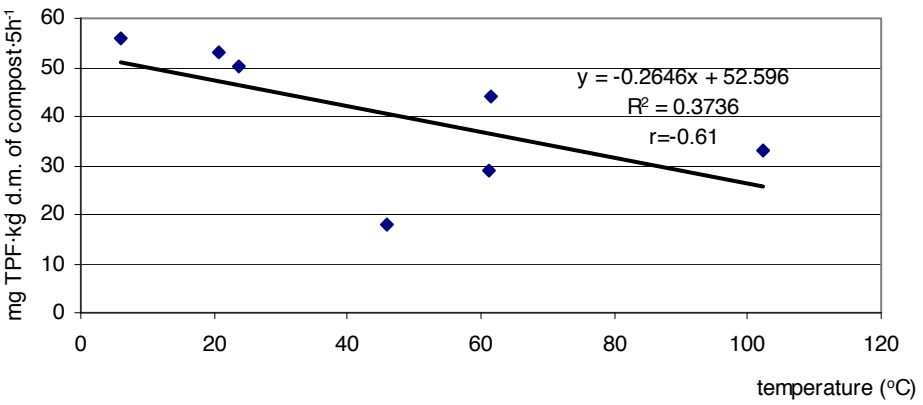

Fig. 5. Relationship between dehydrogenases activity and temperature in K1 compost.

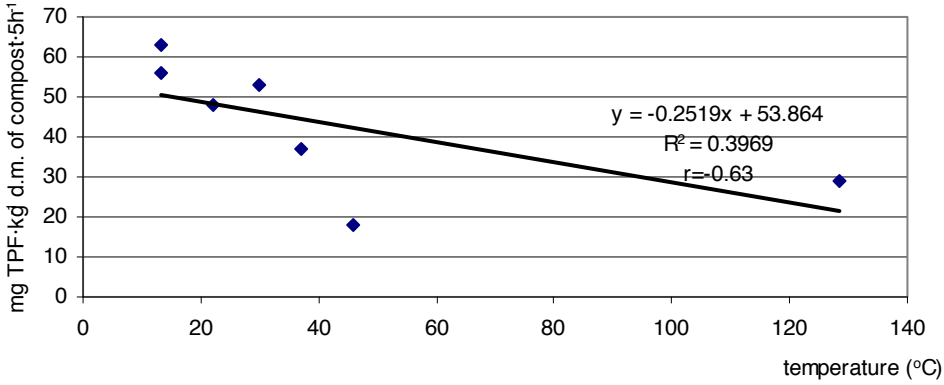

Figure 6 Relationship between dehydrogenases activity and temperature in K2 compost

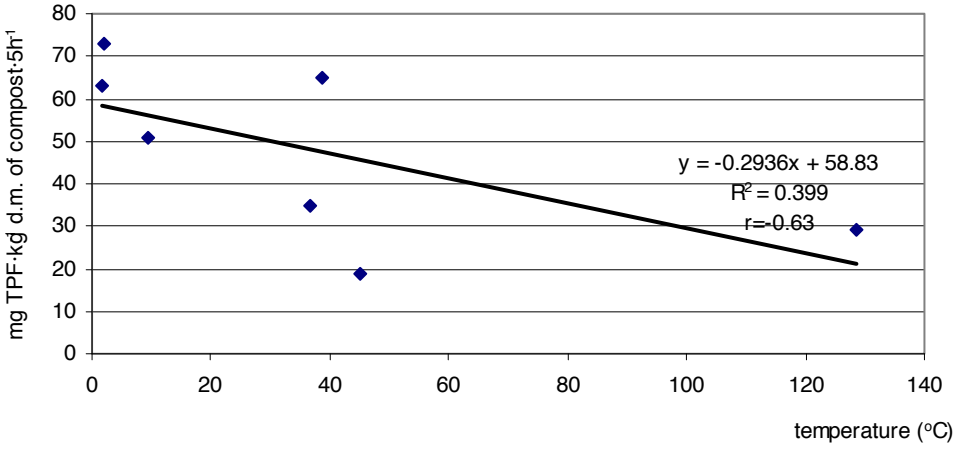

Figure 7 Relationship between dehydrogenases activity and temperature in K3 compost

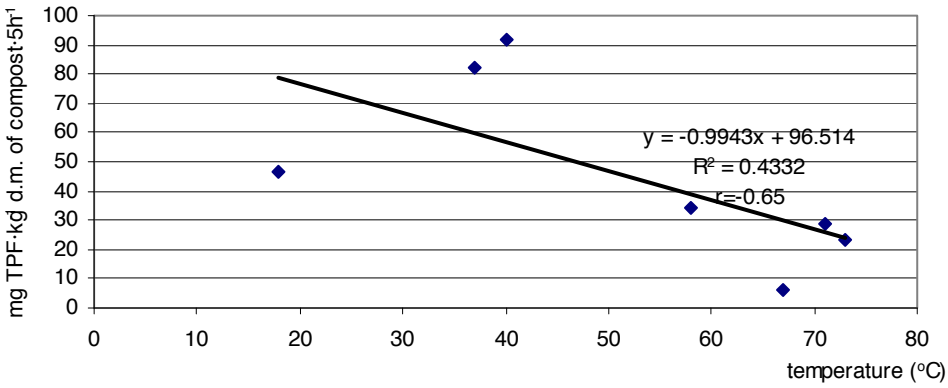

Figure 8 Relationship between dehydrogenases activity and temperature in K4 compost

CONCLUSIONS

1. The reduction dynamics of the studied groups of microorganisms (with the exception of thermophilic microorganisms) in composts was shifted in time, in the majority of cases, according to the temperature increase.
2. Both the term of analyses and the type of compost had a high statistically significant effect on the changes in the number of the analysed microorganism groups (except in actinomycetes) and on the level of dehydrogenases activity.
3. The studied sewage sludges applied in the experiment did not meet the sanitary standards required by the Directive of the Minister of Agriculture and Rural Development (2004), since in spite of the absence of bacteria from *Salmonella* genus, the number of bacteria from *Enterobacteriaceae* family was higher than 1000 cfu.
4. Differentiated proportional composition of the particular components in the applied composts caused different rates of the elimination of bacteria from *Enterobacteriaceae* family (in composts K1 and K2, the rate was shifted in time in relation to composts K3 and K4).
5. The total inactivation of bacteria from *Enterobacteriaceae* family and reduction of the number of *Clostridium perfringens* bacteria testify to a high hygienical effectiveness of composting processes guaranteeing their safe use for agricultural purposes, according to the Directive of the Minister of Agriculture and Rural Development (2004) and the Instruction of WE Commission No. 185/2007 of February 2, 2007.

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