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THE EFFECT OF WATER STRESS ON WHEAT KERNEL SIZE, COLOR AND PROTEIN COMPOSITION*

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Key words: image analysis, proteins, wheat, water stress.

A b s t r a c t

The present study determines the influence of water deficit during the vegetation period of three wheat cultivars on the accumulation of proteins in kernels. Additionally, it examines which trait of the kernel image (dimensions, shape or color) is the best marker for the occurrence of such type of stress. It was found, that as the result of water stress wheat kernels were smaller, mostly due to the reduction in their thickness and width, while their surface was lighter and redder. The color of their cross-section did not differ from the image of control kernels. Water deficit also resulted in an increase in mass density of kernels and in the level of protein density in endosperm. Kernels obtained under stress conditions were less abundant in albumins and globulins, γ gliadins and low and high molecular weight glutenins. Nawra spring wheat proved to be the most sensitive to water stress.

WPŁYW STRESU WODNEGO NA WIELKOŚĆ, BARWĘ ORAZ SKŁAD BIAŁEK ZIARNIAKA PSZENICY

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S ł o w a k l u c z o w e: analiza obrazu, białka, pszenica, stres wodny.

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Abstrakt

W pracy określono wpływ deficytu wody podczas wegetacji trzech odmian pszenicy na akumulację białek w ziarniakach oraz analizowano, która z cech obrazu ziarniaka (wymiary, kształt, barwa) jest najlepszym markerem wystąpienia tego rodzaju stresu. Stwierdzono, że w wyniku stresu wodnego ziarniaki pszenicy stały się drobniejsze, głównie na skutek zmniejszenia grubości i szerokości, miały jaśniejszą i bardziej czerwoną powierzchnię. Barwa ich przekroju nie różniła się od obrazu ziarniaków kontrolnych. Efektem niedoboru wody był również wzrost masy właściwej ziarniaków oraz gęstości białek w bielmie. Ziarniaki otrzymane w warunkach stresowych zawierały mniej albumin i globulin, γ gliadyn oraz glutenin wysoko- i niskocząsteczkowych. Najbardziej wrażliwa na stres wodny była pszenica jara odmiany Nawra.

Introduction

Water stress is one of the most important factors reducing the yield of cultivated plants (GRZESIUK et al. 1999, STARCK et al. 1995). The reason for water deficit in plants is a lack of available water in the ground, atmospheric drought, frequently occurring with high temperatures, as well as the transpiration process overbalancing water absorption (BOCZEK, SZLENDAK 1992, FORDOŃSKI et al. 1994). Water deficit is regarded as „the most important limiting factor in crop productivity in semi-arid agricultural areas" (WILHITE 1993). Water deficit conditions bring about the changes in hormone content and activity; first of all, the content of abscisinic acid (ABA) increases (CHAVES 1991, RISTIC et al. 1992), while the content of cytokines and gibberellins decreases (AHARONI et al. 1977).

Food made of processed wheat kernels are one of the main elements of the human diet. Its composition is determined both by the wheat genotype and the conditions under which it is cultivated. It is generally known that the amount and the quality of storage material in wheat kernel mainly depends on the accessibility of nutritional components in soil; however, its translocation to endosperm is determined by climatic conditions during the seed-filling stage. It has been shown that the accumulation of nutrients in kernel ends at the moment when the percentage of dry mass reaches about 0.55 g per g of fresh mass (SCHNYDER, BAUM 1992). Considering the effects on the baking value of flour, a question arises as to the influence of water stress on changes in proteins quantity and quality, particularly of storage prolamines. According to ZHAO et al. (2005), water stress in the kernel-filling phase increases the protein content. Similar results were obtained by OZTURK & AYDIN (2004), who claimed that continuous water stress increased its content by 18%, sedimentation value by 16% and wet gluten content by 22% compared with fully irrigated samples. Thus, the data indicate that the wheat grain obtained under water deficit conditions is definitely of better baking quality and should be

demanded by flour producers. However, this favorable effect is accompanied by unfavorable changes, such as: a decrease in yield and in mass of 1000 seeds, an increase in the ash level of kernels (NAGARAJAN et al. 1999, DANIEL, TRIBOI 2002, OZTURK, AYDIN 2004), and particularly of starch (NAGARAJAN et al. 1999, AHMADI, BAKER 2001). According to ALTENBACH et al. (2003), a water deficit during a wheat vegetation period at a 37/17°C (day/night) regimen has a stronger effect on the reduction of the starch than on the protein content in the mature kernel. The authors explain this by the fact that the starch accumulation period is shortened in comparison to the duration of protein deposition. In drought conditions, the end of accumulation of the soluble proteins and the onset of the rapid insolubilisation occurred earlier (DANIEL, TRIBOI 2002).

The above-mentioned data show that stress during plant vegetation and kernel filling affects deposition of storage material. The level of unfavorable effects of water stress on the partitioning of carbon and nitrogen to the kernels depends on wheat genotype (NICOLAS et al. 1985, NAGARAJAN et al. 1999). It was stated that some cultivars are tolerant, while others are sensitive; however, the mechanism of this phenomenon has not yet been discovered. Perhaps it is related to the accumulation of specific proteins, as it was established, e.g., in wheat cultivars tolerant to thermal stress (SKYLAS et al. 2002). A visible symptom of stress is a decrease in the kernel size and mass, whose features provide the first indication for the assessment of the quality of grain in a commodity market.

The aim of the presented study is to determine the sensitivity of three wheat cultivars to water stress. The authors wanted to establish how water deficit changes protein accumulation and which features of kernel images (dimensions, shape, color) could be the best marker of the occurrence of a stress factor.

Material and Methods

Wheat samples

The experimental material were three cultivars of wheat, including two winter ones: Sukces and Tonacja of glutenins alleles N-7+9-2+12 and 2*-7+9-2+12, respectively and one spring wheat – Nawra, of alleles N-7+8-5+10 for glutenins (*Descriptive List of Cultivars* 2006). Winter and spring wheats were cultivated in pots, which were filled with proper brown soil (experiment was executed in the greenhouse of the University of Warmia and Masuria in Olsztyn). The grain used in the study was dressed with Funaben T fungicide.

After germination, eight plants were left in each pot. Plants were watered with distilled water. Mineral fertilization was used twice: before the sowing and in the 4-5 leaf growth stage. The following crop protection chemicals against disease and pests were used twice: Amistar 250 SC, Talstar 100 EC and Nurelle D 550 EC.

Plants were cultivated in two variants: 60-70% of capillary water capacity, treated as control conditions, and 30-35% of capillary water capacity, differentiated from the plant flowering stage, treated as stress conditions.

Dimensions and color of kernel

The digital image analyses were performed for the surface of kernels and prepared cross-sections as done previously (KONOPKA et al. 2005). The images were acquired by a high resolution, low-noise CCD Nikon DXM-1200 color camera and analyzed by LUCIA G ver. 4.8 software. The frame grabber was at 1280x1024 pixels resolution. The kernels were examined from a distance (lens to object) of 13 cm. The light source was a KAISER RB 5004 HF with 4x36 W fluorescence (color temperature 5400°K) lamps. The results are presented in HSI (H-hue, S-saturation, I-intensity) color space. H is expressed in degrees, and S and I in percents. Geometrical features (length, width, thickness, area and elongation) were calculated automatically by the LUCIA G ver. 4.8 software and the accuracy of measurements was $d = 0.05$ mm. While calculating the kernels volume, it was assumed that their shape corresponds to a rotational ellipsoid. Measurements were conducted on 50 kernels and their cross-sections.

Protein extraction and analysis

Before analyses grain was milled in a laboratory mill type IKA A10 (Labortechnik, Germany). Proteins were extracted using the solvent system developed by WIESER et al. (1998). Albumins plus globulins were twice extracted with 1 mL of 0.4 mol L^{-1} of NaCl with 0.067 of mol L^{-1} $HKNaPO_4$ (pH 7.6); gliadins were extracted with 1 mL of 60% ethanol (three-fold extraction), and glutenins were twice extracted with 1 mL of 50% 1-propanol, 2 mol L^{-1} of urea, 0.05 mol L^{-1} of Tris-HCl (pH 7.5), 1% DTT, under nitrogen. The chromatographic separation was carried out on a Hewlett-Packard apparatus series 1050: RP-18 Vydac 218TP54 column with 5 μm bead size and 300 Å pore size, 250x4.6 mm; a Zorbax 300SB-C18 pre-column, 4.6x12.5 mm; a column temperature of 45°C, a mobile phase flow rate of 1 ml min^{-1}, and an injection

volume of 20 μl. A two-component gradient was used. A component: 0 min 75%, 5 min 65%, 10 min 50%, 17 min 25%, 18 min 15% and 19 min 75%. The first component (A) was water with 0.1% of TFA and the second (B) was ACN with 0.1% of TFA. The spectra were determined by a diode-array detector (HP 1050). Quantification of proteins was done by UV absorbance at 210 nm The identification of gliadins was based on the second derivative of their UV spectra according to the method developed by DZIUBA et al. (2007).

Other analyses

The grain moisture and protein were analyzed according to Polish Standard PN-91/A-74010 and PN-75/A-04018, respectively. 1000 kernels were counted by Seed Counter and their mass was calculated on 15% of grain moisture. Kernel and protein density was calculated as a ratio of single kernel mass/or HPLC units and volume (volume was calculated as described in previous part: *Dimensions and color of kernel*).

Statistics

The experimental results were analyzed using Statistica 6.0 software (StatSoft, Tulsa, USA). Analysis of variance with Duncan tests was performed at a significance level of $p < 0.05$.

Results and Discussion

Kernel mass, dimensions and density

Mass of 1000 kernels of wheat cultivated at optimum level of moisture was steady, and it amounted to about 38 g (Table 1). The values are much lower than typical for the cultivars under examination (about 47 g), listed in the *Descriptive List of Cultivars* (2006). It seems to be an unfavorable effect of pot cultivation of plants. Water stress lowered the mass of 1000 kernels by 18, 20 and 24% for Sukces, Nawra and Tonacja cultivars, respectively. Decrease of 1000 kernels mass in stressed kernels was also stated by OZTURK & AYDIN (2004).

Table 1

Wheat kernel dimensions

Cultivar	Variant		1000 kernels mass (g)	Specific density** (mg mm^{-3})	Area (mm^2)	Length (mm)	Width (mm)	Elongation	Thickness* (mm)	Volume (mm^3)
Nawra	control	$\bar{x}$	38.6^a	1.322	15.91^a	7.25^a	2.88^a	2.54^a	2.68^a	29.2^a
		$\hat{s}$	2.6	–	2.39	0.32	0.32	0.29	0.31	3.87
		c.v.	6.74	–	15.00	4.47	11.05	11.42	11.63	13.25
	stress	$\bar{x}$	30.9^b	1.545	12.6^b	7.03*b*	2.41^b	2.94^b	2.25^b	20.0^b
		$\hat{s}$	2.2	–	2.01	0.39	0.26	0.27	0.27	2.84
		c.v.	7.12	–	15.90	5.48	10.91	9.05	11.88	14.20
Sukces	control	$\bar{x}$	38.8^a	1.442	14.74^a	6.64^a	2.94^a	2.28^a	2.63^a	26.9^a
		$\hat{s}$	3.1	–	2.25	0.39	0.32	0.24	0.30	3.92
		c.v.	7.99	–	15.24	5.89	10.86	10.61	11.60	14.57
	stress	$\bar{x}$	31.9^b	1.519	13.28^b	6.55^a	2.66^b	2.50^b	2.31^b	21.0^b
		$\hat{s}$	7.5	–	2.11	0.35	0.34	0.30	0.27	3.31
		c.v.	23.51	–	15.92	5.35	12.76	11.97	11.80	15.76
Tonacja	control	$\bar{x}$	37.6^a	1.279	15.75^a	6.86^a	3.04^a	2.29^a	2.69^a	29.4^a
		$\hat{s}$	2.1	–	2.88	0.46	0.40	0.27	0.36	5.23
		c.v.	5.58	–	18.30	6.73	13.17	11.75	13.26	17.79
	stress	$\bar{x}$	28.4^b	1.352	12.94^b	6.59^b	2.61^b	2.56^b	2.33^b	21.0^b
		$\hat{s}$	3.4	–	2.27	0.44	0.35	0.32	0.35	3.47
		c.v.	11.97	–	17.54	6.61	13.45	12.47	15.14	16.52
Control		$\bar{x}$	38.3^A	1.373	15.18^A	6.81^A	2.94^A	2.34^A	2.67^A	27.9^A
		$\hat{s}$	0.7	–	2.46	0.46	0.28	0.28	0.32	4.34
		c.v.	1.83	–	16.18	6.78	11.92	11.92	12.03	15.56
Stress		$\bar{x}$	30.4^B	1.469	12.93^B	6.72^B	2.56^B	2.67^B	2.42^B	20.7^B
		$\hat{s}$	0.1	–	2.15	0.45	0.34	0.35	0.35	3.26
		c.v.	0.33	–	16.60	6.68	13.16	13.25	14.47	15.75

* detected on cross–section of kernel, ** kernel specific density was calculated only for average data of each sample and was not statistically analyzed $\bar{x}$ – mean value, $\hat{s}$ – standard deviation, c.v. – variability coefficient (%). Means with the same letter in the same column, separately for each cultivar, are not significantly different ($p = 0.05$).

The size of kernels obtained under control conditions were related to wheat cultivar. In the case of Nawra and Tonacja, they were of the same volume but about 8% larger than the kernels of Sukces cultivar (Table 1). Water stress significantly reduced the kernels volume (the average effect was 26%), and the highest decrease was observed in kernels of Nawra spring wheat. This effect resulted mainly from a reduction in width and thickness – which reached 14%. The length of kernels decreased only slightly, by about 3%. This resulted in an increase in the coefficient of kernel elongation (length/width) from 2.37 to 2.67, which proves that kernels after water stress had more elongated, slender shapes (Figure 1). The results confirm previous observations (KONOPKA et al. 2007) that among three basic kernel dimensions, its length is the most conserved. Other dimensions, and thus the shape of the kernel, depend on filling the endosperm with nutrients, particularly with starch and proteins. Under optimal climatic conditions and proper fertilization, the duration of protein and starch accumulation is almost the same (DUPONT, ALTENBACH 2003). Strong stress can cause premature apoptosis of endosperm cells, which ends the accumulation of storage materials earlier. It has been shown that under water stress, the time of starch accumulation is shorter than the duration of protein accumulation by up to 10 days (ALTENBACH et al. 2003). Research conducted by AHMADI & BAKER (2001) confirmed that water stress caused a marked reduction in the starch content of the grains. It was due to a decline in soluble starch synthase activity and inactivation of adenosine diphosphate glucose pyrophosphorylase which affected the rate of grain growth and growth cessation, respectively.

Changes of single kernel mass and volume affected on it density. It was stated that stress increased values of this feature. The highest, up to 17%, increase in density, was observed for Nawra kernels (in comparison for kernels of both winter wheat cultivars this trait was higher only by about 5%). It is known that an increase in density can result either from a change of the content of individual nutrients or from the degree of their packaging in endosperm or from a combination of these effects. Under typical cultivation conditions, an increase in the kernels density results mainly from an increase in the starch content, which has the highest specific gravity among the main nutrients. However, the observed effect seems to result mostly from the increase in the degree of endosperm packaging. If proteins deposition influences on this will be analyzed in latest part of this chapter.

Color of kernel surface and endosperm

All wheat kernels, regardless of cultivation conditions, were of a similar surface hue (H), ranging from 26.4 to 27.6° (Table 2). These are values by a few

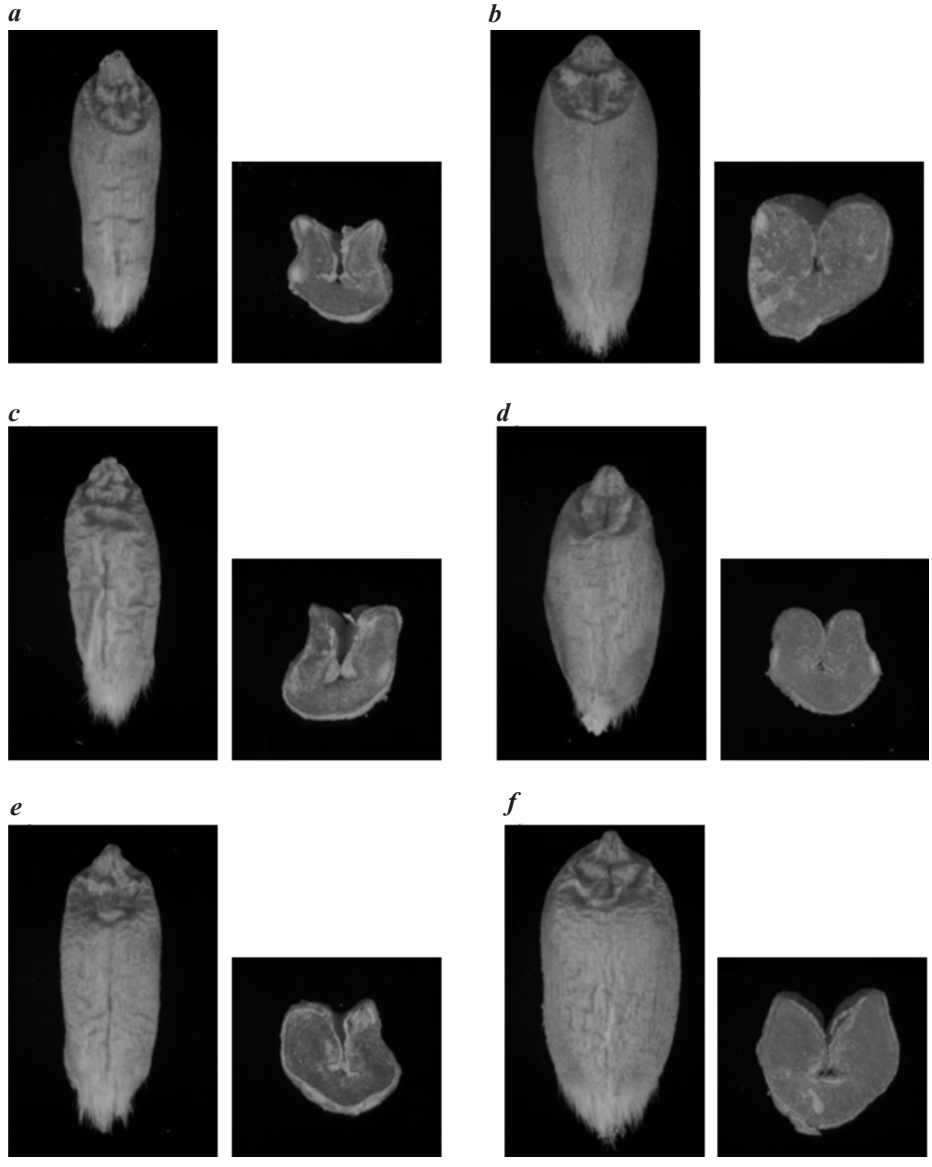

Fig. 1. Image of wheat kernels: *a* – Nawra – stress, *b* – Nawra – control, *c* – Sukces – stress, *d* – Sukces – control, *e* – Tonacja – stress, *f* – Tonacja – control

units lower than those presented in an earlier study (KONOPKA et al. 2004), which proves the shift of hue towards red. However, a precise comparison is not possible, as different cultivars were analyzed in both studies and other type of sample illumination was used. Decrease of saturation (S) and increase of

intensity (I) of surface color, particularly in the case of Nawra wheat kernels, let to detect of kernels cultivated under stress conditions. This shows that kernels of sensitive cultivar subjected to water stress had lighter and less colored surfaces.

Table 2

Color of wheat kernel surface and cross-sections

Cultivar	Variant		Surface			Cross-section		
			H (°)	S (%)	I (%)	H (°)	S (%)	I (%)
Nawra	control	$\bar{x}$	26.4^{a}	33.7^{a}	36.8^{a}	34.0^{a}	35.9^{a}	35.7^{a}
		$\hat{s}$	1.31	2.57	1.95	1.27	3.15	2.01
		c.v.	4.97	7.63	5.28	3.74	8.78	5.63
	stress	$\bar{x}$	27.1^{b}	30.8^{b}	40.3^{b}	33.4^{b}	36.4^{a}	35.0^{a}
		$\hat{s}$	1.07	2.04	1.56	0.91	4.36	3.28
		c.v.	3.97	6.63	3.87	2.73	11.96	9.38
Sukces	control	$\bar{x}$	27.6^{a}	30.0^{a}	40.1^{a}	33.6^{a}	37.4^{a}	34.5^{a}
		$\hat{s}$	0.87	1.99	1.83	1.87	3.66	2.09
		c.v.	3.15	6.64	4.55	5.58	9.79	6.06
	stress	$\bar{x}$	27.6^{a}	28.7^{b}	40.8^{b}	33.5^{a}	35.1^{b}	33.3^{b}
		$\hat{s}$	0.86	2.28	1.82	1.21	3.86	2.59
		c.v.	3.12	7.94	4.45	3.60	11.00	7.78
Tonacja	control	$\bar{x}$	26.9^{a}	28.6^{a}	41.6^{a}	33.3^{a}	38.7^{a}	33.4^{a}
		$\hat{s}$	1.10	2.09	1.99	1.67	3.12	2.35
		c.v.	4.09	7.31	4.80	5.01	8.05	7.03
	stress	$\bar{x}$	27.6^{b}	27.9^{a}	42.2^{b}	32.2^{b}	37.1^{a}	33.2^{a}
		$\bar{x}$	0.79	2.14	1.63	1.96	4.94	3.32
		c.v.	2.85	7.68	3.86	6.09	13.29	10.02
Control		$\bar{x}$	27.2^{A}	30.5^{A}	39.7^{A}	33.6^{A}	37.3^{A}	34.5^{A}
		$\hat{s}$	1.14	2.77	2.47	1.66	3.52	2.35
		c.v.	4.19	9.07	6.21	4.95	9.43	6.73
Stress		$\bar{x}$	27.5^{B}	29.1^{B}	41.2^{B}	33.1^{B}	37.0^{A}	34.2^{A}
		$\hat{s}$	0.95	2.48	1.86	1.77	4.28	2.96
		c.v.	3.45	8.53	4.51	5.35	11.57	8.65

$\bar{x}$ – mean value. $\hat{s}$ – standard deviation. c.v. – variability coefficient (%). Means with the same letter in the same column, separately for each cultivar, are not significantly different ($p = 0.05$).

Wheat cultivars differed in endosperm color only to a limited degree. The hue of kernel cross-sections ranged from 32.2 to 34.0°, so it was shifted towards yellow in comparison with the color of the surface (Table 2). A water deficit caused slight, statistically insignificant changes in the color of kernel cross-sections. Observed tendencies were, in most cases, unidirectional and consisted in reducing all analyzed components of a color. This indicates the shift of hue of kernel endosperm towards red (the opposite tendency was observed in

Nawra wheat kernels), a smaller saturation of this hue and a smaller percentage of whiteness in their image.

Previous studies (KONOPKA et al. 2004) have demonstrated a linear relationship between H and S values of kernel endosperm and the protein content (%) in grain. The current results seem to contradict this thesis (see the results of H, S and grain protein content of control samples, Tables 2 and 3). However, in previous studies experimental material originated from wider spectrum of inter-cultivar differentiation in technological quality. Additionally, analyzed kernels were from plots of wheat cultivated under classical conditions. The most important visual feature differentiating them from kernels of pot cultivation was the presence of the areas of mealy endosperm. In the research presented, none of the cross sections contained this type of endosperm and all kernels, including control kernels, demonstrated the vitreous structure. This may indicate that kernels from pot cultivation (considered to be control samples) are also subjected to a specific type of stress; however, only this type of cultivation makes it possible to control the water content in soil. An additional explanation of the differences observed could result from the fact that the endosperm color, besides its vitreousness/mealiness (FORNAL et al. 2003), could also be influenced by its carotenoid content, giving it a yellow hue. It is known that their biosynthesis increases under conditions of thermal and light stress (RABBANI et al. 1998, KONOPKA et al. 2006). Plants cultivated in greenhouse were exposed to natural sun illumination, what could modify the effect of main factor. It points that the phenomenon of the contribution of protein, carotenoids and endosperm structure in wheat kernel color is still yet not fully explained.

Protein content and composition

Control winter wheat grain samples were poorer in total protein, but the same was not observed in Nawra spring wheat (Table 3). Protein deposition in single kernel from control conditions was in all cases higher, and detrimental effect of water stress was especially visible in spring cultivar (ca. 30% decrease of protein content). It only partly confirmed results of ZHAO et al. (2005) and OZTURK & AYDIN (2004) that water stress increases protein content in grain.

An analysis of chromatograms showed a typical arrangement of protein fractions and a lack of qualitative differences between control and stressed kernels (Figure 2). Proteins soluble in 0.4 M NaCl (albumins and globulins) constituted from 16 to 19% of the total proteins in the control kernels (Table 3). Nawra spring wheat kernels were the richest. The gliadin content was differentiated in individual cultivars. The cultivar with the highest amount of

Table 3

Protein content of grain samples (mAU per 1 kernel)

Cultivar	Variant		Protein			A+B*	Gliadins			Glutenins	
			of grain N·5.7 (%)	of kernel (mAU)	density** (mAU mm^{-3})		ϖ	α/β	γ	HMW	LMW
Nawra	control	$\bar{x}$	12.3^{a}	43119^{a}	1477	8076^{a}	1112^{a}	8875^{a}	5167^{a}	5646^{a}	14243^{a}
		$\hat{s}$	0.3	345	-	9.6	22.1	6.0	20.5	0.3	66.3
	stress	$\bar{x}$	11.8^{a}	33232^{b}	1662	6324^{b}	793^{b}	6544^{b}	3901^{b}	4260^{b}	11409^{b}
		$\hat{s}$	0.3	602	-	35.4	24.2	2.8	7.0	21.0	63.7
Sukces	control	$\bar{x}$	10.5^{a}	36897^{a}	1372	6788^{a}	1132^{a}	7248^{a}	4207^{a}	4045^{a}	13477^{a}
		$\hat{s}$	0.3	456	-	49.9	18.1	10.4	132.4	201.2	270.0
	stress	$\bar{x}$	13.3^{b}	38682^{b}	1842	5531^{b}	1612^{b}	10109^{b}	4226^{a}	4380^{b}	12822^{b}
		$\hat{s}$	0.2	399	-	94.4	28.5	218.7	104.1	51.7	291.7
Tonacja	control	$\bar{x}$	11.7^{a}	39922^{a}	1358	6249^{a}	1201^{a}	11937^{a}	5353^{a}	3211^{a}	11969^{a}
		$\hat{s}$	0.2	664	-	81.3	22.9	65.1	4.0	55.6	151.0
	control	$\bar{x}$	13.8^{b}	35647^{b}	1697	5210^{b}	1157^{a}	11431^{b}	4587^{b}	2866^{b}	10395^{b}
		$\hat{s}$	0.4	522	-	5.6	27.9	144.8	180.6	13.3	202.1
Control		$\bar{x}$	11.5^{A}	39979^{A}	1433	7038^{A}	1148^{A}	9353^{A}	4909^{A}	4301^{A}	13230^{A}
		$\hat{s}$	1.0	2731	-	840.6	44.1	2129.5	553.7	1111.6	1045.8
Stress		$\bar{x}$	13.0^{A}	35854^{B}	1732	5688^{B}	1187^{A}	9361^{A}	4238^{A}	3835^{A}	11542^{B}
		$\hat{s}$	0.9	3112	-	514.8	367.9	2264.3	320.7	753.5	1102.0

* $A + B$ – albumin and globulin; ** calculated as mAU (HPLC units) per 1 mm^3 of kernel protein density was calculated only for average data of each sample and was not statistically analyzed

$\bar{x}$ – mean value, $\hat{s}$ – standard deviation. Means with the same letter in the same column, separately for each cultivar, are not significantly different ($p = 0.05$).

it was Tonacja, while the lowest was Sukces. In all examined cultivars, their smallest sub-fraction were ϖ gliadins (about 3% of total proteins), and the largest -α/β subunits (20% in proteins of Nawra and Sukces and 30% in Tonacja). Glutenins were the main group of storage proteins in Nawra and Sukces wheat kernels.

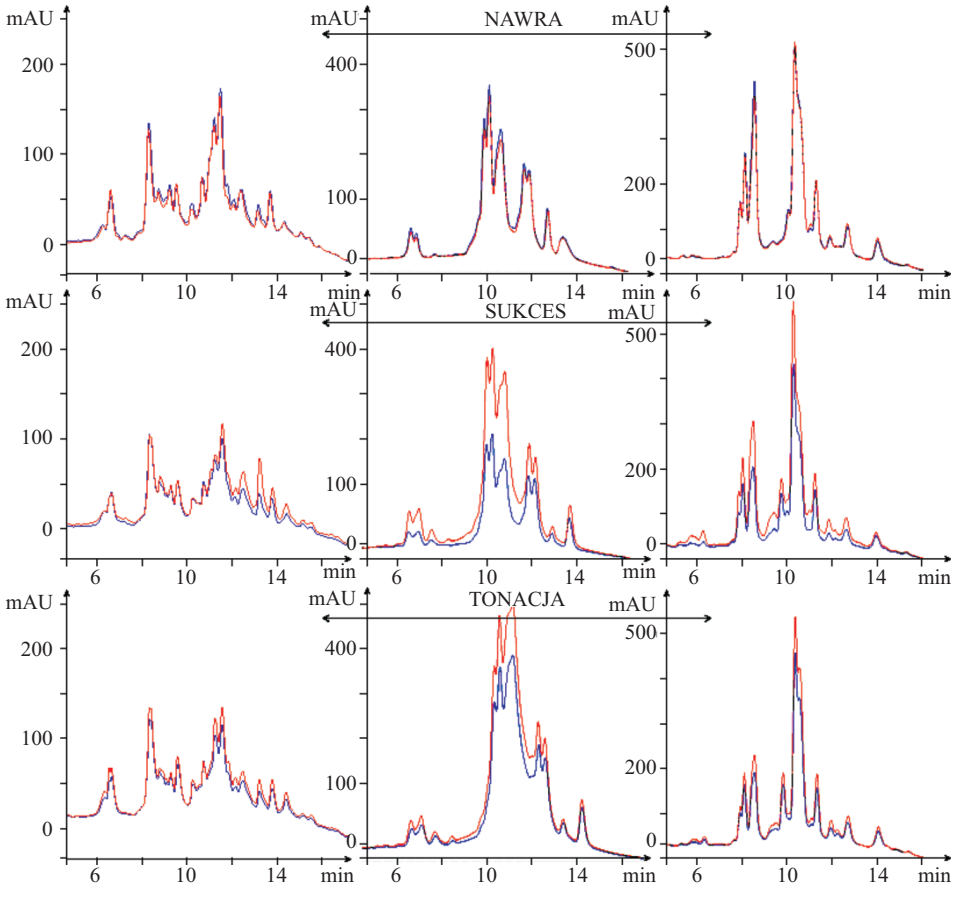

Fig. 2. RP-HPLC chromatograms of albumins and globulins (a), gliadins (b) and glutenins (c) from cvs Nawra, Sukces and Tonacja. Chromatograms show proteins extracted from 1 mg of grain (blue curve – control sample, red curve – stress sample)

In all cultivars, a water deficit brought about a decrease in the content of albumins and globulins, γ gliadins and both classes of glutenins (HMW and LMW). An average decrease was established as about 20% for albumins and globulins and between 11 and 14% for other proteins. The most intense

changes were observed in Nawra kernels. Changes in α/β gliadins accumulation under stress conditions were not unidirectional and depended on the cultivar. A water deficit caused their increase per 1 kernel in Sukces or decrease in Nawra and Tonacja cultivars. However, the differences in kernel size resulted in higher share of gliadins in the same grain mass after stress conditions in both winter cultivars (Figure 2). All stressed kernels were characterized by higher protein density. The amount of protein per a kernel volume unit was 1433 and 1732 mAU mm^{-3} in control and stressed kernel, respectively (Table 3).

The baking value of flour is favorably influenced, among others, by an increase in the number of HMW fractions, their proportions to LMW glutenins and content of polymers unextractable by SDS (MACRITCHIE 1999, DANIEL, TRIBOI 2002). Wheat cultivars analyzed in the study contained between 8 to 13% HMW fractions, and their proportion to low-molecular glutenins ranged from 0.27 to 0.40. The decrease in the amount of high-molecular glutenins is, therefore, unfavorable. This unfavorable effect was most visible in the case of Nawra wheat kernels, of a genotype containing alleles for HMW 1Dx5+1Dy10 glutenins. In previous research concerning the effect of thermal stress, BLUMENTHAL et al. (1995) found that wheat genotypes containing the same alleles were generally more tolerant to stress than 1Dx2+1Dy12 alleles (such was found in Sukces and Tonacja wheat kernels). This problem requires further explanation.

Conclusions

Water deficit during the wheat plant vegetation caused significant changes in the image and composition of proteins in kernels. The highest intensity of changes in all markers was found for kernels of Nawra spring wheat. It shows that it is the most sensitive to water deficit and should not be cultivated in the areas of Poland where droughts are very likely to occur. This is a one of the main cultivar in Poland – in 2005 it constituted 18% among 36 cultivars present in the register (*Descriptive List of Cultivars* 2006). It was generally stated that as a result of water stress, kernels were smaller, mostly due to the decrease in their thickness and width, with lighter, redder surface. The effect of water deficit was a simultaneous increase of kernel specific density and of the protein packaging. Kernels of all cultivars after stress conditions contained fewer albumins and globulins, γ gliadins and both classes of glutenins.

The results indicate the possibility of instant identification of grain cultivated under stress conditions, e.g. due to the use of digital image analysis and its segregation, using differences in geometric features of kernels, their density

and color. The observed effects of water stress on the changeability of dimensions and color of kernels can provide a partial explanation for the low effectiveness of algorithms developed by other researchers to identify and differentiate wheat cultivars. Discovering the sensitivity of individual cultivars to specified types of stress and effects of the environmental influence seems to constitute another challenge for science and practice, especially in view of the increasing occurrence of weather anomalies.

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References

AHARONI N., BLUMENFELD A., RICHMOND A.E. 1977. *Hormonal activity in detached lettuce leaves by leaf water content*. Plant Physiol., 59: 1169-1173.

AHMADI A., BAKER D.A. 2001. *The effect of water stress on the activities of key regulatory enzymes of the sucrose to starch pathway in wheat*. Plant Growth Regul., 35: 81-91.

ALTENBACH S.B., DUPONT F.M., KOTHARI K.M., CHAN R., JOHNSON E.L., LIEU D. 2003. *Temperature, water and fertilizer influence the timing of key events during grain development in a US spring wheat*. J. Cereal Sci., 37: 9-20.

BLUMENTHAL C., BEKES F., GRAS P.W., BARLOW E.W.R., WRIGLEY C.W. 1995. *Identification of wheat genotypes tolerant to the effects of heat stress on grain quality*. Cereal Chem., 72(6): 539-544.

BOCZEK J., SZLENDAK E. 1992. *Wpływ stresów roślinnych na porażenie roślin przez szkodniki*. Post. Nauk Rol. PAN, Wydz. Nauk Rolniczych i Leśnych, 2/237: 1-17.

CHAVES M.M. 1991. *Effects of water deficits on carbon assimilation*. J. Exp. Bot., 42: 1-16.

DANIEL C., TRIBOÏ E. 2002. *Changes in wheat protein aggregation during grain development: effects of temperatures and water stress*. Eur. J. Agron., 16: 1-12.

Descriptive List of Cultivars 2006. *Rośliny Rolnicze*. Cz. 1. Ed. by Centralny Ośrodek Badania Odmian Roślin Uprawnych Słupia Wielka (in Polish).

DUPONT F.M., ALTENBACH S.B. 2003. *Molecular and biochemical impacts of environmental factors on wheat grain development and protein synthesis*. J. Cereal Sci., 38: 133-146.

DZIUBA J., NAŁĘCZ D., HANASIEWICZ A., MINKIEWICZ P. 2007. *The application of spectroscopy to discriminate wheat* α/β – and γ-gliadins separated using high-performance liquid chromatography. Pol. J. Food Nutri. Sci. (in press).

FORDOŃSKI G., GÓRECKI R.J., BIENIASZEWSKI T., MAJCHRZAK B. 1994. *Wpływ tiuramu na kiełkowanie, wigor nasion i zdrowotność siewek roślin strączkowych w warunkach stresu chłodnowodnego*. Mat. konf. Uszlachetnianie Materiałów Nasiennych. PAN, ART Olsztyn, ss. 81-88.

FORNAL Ł., KOZIROK W., CHORĄŻEWICZ R. 2003. *New possibilities to characterizing wheat grain endosperm*. Pol. J. Natur. Sci., 13(1): 169-183.

GRZESIUK S., KOCZOWSKA I., GÓRECKI R.J. 1999. *Fizjologiczne podstawy odporności roślin na choroby*. Wyd. II. Wyd. ART Olsztyn.

KOBATA T., PALTA J.A., TURNER N.C. 1992. *Rate of development of postanthesis water deficits and grain filing of spring wheat*. Crop Sci., 32: 1238-1242.

KONOPKA I., CZAPLICKI S., ROTKIEWICZ D. 2006. *Differences in content and composition of free lipids and carotenoids in flour of spring and winter wheat cultivated in Poland*. Food Chem., 95: 290-300.

KONOPKA I., FORNAL Ł., DZIUBA M., CZAPLICKI S., NAŁĘCZ D. 2007. *Composition of proteins in wheat grain obtained by sieve classification*. J. Sci. Food Agric. (in press).

KONOPKA I., KOZIROK W., ROTKIEWICZ D. 2004. *Lipids and carotenoids of wheat grain and flour and attempt of correlating them with digital image analysis of kernel surface and cross-sections*. Food Res. Int., 37(5): 429-438.

KONOPKA I., KOZIROK W., TAŃSKA M. 2005. *Wheat endosperm hardness.* Part I. *Relationships to colour of kernel cross-section.* Eur. Food Res. Technol., 220(1): 11-19.

MACRITCHIE F. 1999. *Wheat proteins: characterization and role in flour functionality*. Cereal Foods World, 44: 188-193.

NAGARAJAN S., RANE J., MAHESWARI M., GAMBHIR P.N. 1999. *Effect of post-anthesis water stress on accumulation of dry matter, carbon and nitrogen and their partitioning in wheat varieties differing in drought tolerance*. J. Agron. Crop Sci., 183: 129-136.

NICOLAS M.E., LAMBERS H., SIMPSON R.J., DALLING M.J. 1985. *Effect of drought on metabolism and partitioning of carbon in two wheat varieties differing in drought-tolerance*. Ann. Bot., 55: 727-747.

OZTURK A., AYDIN F. 2004. *Effect of water stress at various growth stages on some quality characteristics of winter wheat*. J. Agron. Crop Sci., 190: 93-99.

PERKOWSKI J. 1999. *Badania zawartości toksyn fuzaryjnych w ziarnie zbóż*. Rocz. AR w Poznaniu. Rozpr. Nauk., 295: 1-136 (in Polish).

RABBANI S., BEYER P., v. LINTIG J., HUGUENEY P., KLEINIG H. 1998. *Induced* β-carotene synthesis driven by triacylglycerol deposition in the unicellular alga Dunaliella Bardawil. Plant Physiol., 116: 1239-1248.

RISTIC Z., GIFFORD D.J., CASS D.D. 1992. *Dehydratation, damage to the plasma membrane and thylakoids and heat – shock proteins in lines of maize differing in endogenous levels of abscisic acid and drought resistance*. J. Plant Physiol., 139: 467-473.

SCHNYDER H., BAUM U. 1992. *Growth of the grain of wheat (Triticum aestivum L.). The relationship between water content and dry matter accumulation*. Eur. J. Agron., 1: 51-57.

SHI Y.-C., SEIB P.A., BERNARDIN J.E. 1994. *Effects of temperature during grain-filling on starches from six wheat cultivars*. Cereal Chem., 71: 369-383.

SKYLAS D.J., CORDWELL S.J., HAINS P.G., LARSEN M.R., BASSEAL B.J., WALSH B.J., BLUMENTHAL C., RATHMELL W., COPELAND L., WRIGLEY C.W. 2002. *Heat shock of wheat during grain filling: proteins associated with heat-tolerance*. J. Cereal Sci., 35: 175-188.

STARCK Z., CHOŁUJ D., NIEMYSKA B. 1995. *Fizjologiczne reakcje roślin na niekorzystne czynniki środowiska*. SGGW, Warszawa.

WIESER H., ANTES S., SEILMEIER W. 1998. *Quantitative determination of gluten protein types in wheat flour by reversed-phase high-performance liquid chromatography*. Cereal Chem., 75(5): 644-650.

WILHITE D.A. 1993. *Drought Assessment, management and planning: Theory and case studies*. Dordrecht: Kluwer Acad. Publ.

ZHAO Ch., LIU L., WANG J., HUANG W., SONG X., LI C. 2005. *Predicting grain protein content of winter wheat using remote sensing data based on nitrogen status and water stress*. Int. J. Appl. Earth Observ. Geoinform., 7: 1-9.

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PROFTABILITY OF WILLOW PRODUCTION IN SHORT CYCLES IN THE LOW VISTULA VALLEY

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K e y w o r d s: willow (*Salix* spp.), biomass, yield, cost of production, profitability.

A b s t r a c t

The profit achieved from 1 ha of cultivation of seven clones of willow on alluvial soil loco plantation, calculated for one year, amounted to PLN 668.73 ha^{-1} in a one-year cycle, PLN 988.19 per hectare per year in a two-year cycle, and PLN 1,292.60 per hectare per year in a three-year cycle. In one-year production cycles, high profitability was achieved for the *Salix viminalis* 1023 and *Salix viminalis* x *Salix purpurea* clones: PLN 967.51 and PLN 833.22, respectively. It has been shown that the plants of the *Salix viminalis* x *Salix purpurea* clone generate reasonable profit only in cultivation in a one-year cycle; extending the production cycle reduces the profit. The highest profit of PLN 1,474.75 per hectare per year in a two-year cycle was achieved for the *Salix viminalis* 1023 clone. High profits in the three-year cycle were earned for the *Salix viminalis* var. *gigantea, Salix viminalis* JORR and *Salix viminalis* 1033 clones: PLN 1,779.97; 1,770.68 and 1,698.77 per hectare per year, respectively.

OPŁACALNOŚĆ PRODUKCJI WIERZBY W KRÓTKICH ROTACJACH W DOLINIE DOLNEJ WISŁY

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S ł o w a k l u c z o w e: wierzba (*Salix* spp.), biomasa, plon, koszty produkcji, opłacalność.

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A b s t r a k t

Zysk z 1 ha uprawy siedmiu klonów wierzby na glebie aluwialnej loco plantacja, w przeliczeniu na 1 rok użytkowania, wyniósł średnio w cyklu jednorocznym 668,73 zł · ha^{-1}, w dwuletnim 988,19 zł · · ha^{-1} · rok^{-1}, a w trzyletnim 1292,60 zł · ha^{-1} · rok^{-1}. W cyklach jednorocznych wysoką opłacalność produkcji uzyskano w przypadku klonów *Salix viminalis* 1023 oraz *Salix viminalis* x *Salix purpurea,* odpowiednio 967,51 zł i 833,22 zł. Wykazano, iż rośliny klonu *Salix viminalis* x *Salix purpurea* mogą być przydatne do zbioru tylko w cyklach jednorocznych, dalsze bowiem wydłużanie cyklu zbioru prowadzi do zmniejszenia zysku. W cyklu dwuletnim najwyższy dochód (1 474,75 zł · ha^{-1} · rok^{-1}) dał klon *Salix viminalis* 1023, w cyklu trzyletnim wysoką opłacalność produkcji stwierdzono w przypadku klonów *Salix viminalis* var. *gigantea, Salix viminalis* JORR oraz *Salix viminalis* 1033, odpowiednio 1 779,97, 1 770,68 i 1 698,77 zł · ha^{-1} · rok^{-1}.

Introduction

The current demand for forest wood and competition between power plants, wood processing, cellulose production plants, briquette and pellet producers as well as individuals may lead to a crisis due to the short supply of wood. This study analysed the feasibility of obtaining wood outside the forests in the Vistula river valley – part of which used to be a natural habitat of willow.

Stock-taking of all the soils in the Lower Vistula river valley suitable for the cultivation of fast growing willow, and establishing an energy plantation there would supply large amounts of raw material containing lignin and cellulose. For example, 100,000 ha of willow might yield about 3.0 M tonnes (Mg) of biomass each year (100,000 ha · 30 Mg · ha^{-1} · year^{-1} = 3 000 000 Mg · year^{-1} = 1.5 million Mg of dry substance) with the current market value of PLN 240 mln (3 mln Mg · 80PLN Mg^{-1} = PLN 240 million). Willow gives 6 times larger and 10 times quicker increments than forest trees. One does not need to wait 40 years for wood to grow, as is the case in forests: willow wood can be delivered after 2-4 years.

Success in willow cultivation is determined, among other factors, by the yield of biomass per unit area, the unit price of the wood, and, consequently, by the profit (KISIEL et al. 2004, 2006). Therefore, special attention should be devoted to the factors that determine the parameters. A potential plantation owner can primarily influence the yield from the unit area. The main elements which determine the yield of willow biomass are the choice of an appropriate site for cultivation, the species and variety to be planted and the somatic quality of the seedlings. Another important element is the proper treatment applied to the plantation – from the preparation of the site, planting, fertilisation, nurturing and protection treatment and the frequency of shoots harvesting.

According to forecasts, willow biomass will achieve a significant role as a source of energy in this decade (JEŻOWSKI 2003, SZCZUKOWSKI , TWORKOWSKI

2003). Current use of willow biomass includes solid fuel: wood chips, briquette, pellets (STOLARSKI et al. 2003, 2005). It can be supposed that implementation of the idea of cultivation of willow as fuel in rural areas will bring about agricultural, ecological and social benefits.

This study aimed at the determination of the cost and profitability of cultivation of 6 clones of willow (*Salix* spp.) as a source of energy, depending on whether the willow was cultivated in 1-, 2- or 3-year cycles.

Methods

The study was based on a field experiment conducted in 1999-2002 in the Kwidzyn Lowland. Detailed information about the method was presented in the paper by STOLARSKI et al. (2006).

The analysis of costs and profitability of Salix spp. cultivation was based on the mean values of all the clones under study and separately for each of them in the three harvesting cycles (1, 2 and 3-year long). The following stages of the process were isolated in a simulation of the costs of production of willow biomass: establishing the plantation, nurturing, harvest after the first year of vegetation, fertilisation, plant mowing and grinding, transport within the field and agricultural tax. The plants were fertilised with the use of a C-360-3P tractor with an N 031M sower. It was decided that after the first year of vegetation the willows would be mowed with a Stihl chain saw. In subsequent years, plants were harvested in the one-year cycles with a Z 364 forage harvester, and in two- and three-year cycles – with a Claas Jaguar 860 combine harvester. The appropriate number of Ursus C-360-3P tractors and T 604 trailers was planned for wood chip transport.

The cost of operation of technical equipment was calculated according to the methodology proposed by the Department of Economics and Operation of Agricultural Machines of the Institute of Building Construction, Mechanisation and Electrification of Agriculture (IBMER) in Warsaw (MUZALEWSKI 2003). Technical parameters of the tractors and other machines for the analyses of costs and profitability of willow cultivation were taken from the Agroma information brochure, data from IBMER, and an information brochure issued by the Industrial Institute of Agricultural Machines.

Human labour was valued at PLN 10.23 for an hour. The calculation was based on the mean monthly remuneration assuming, that a full time agricultural worker works 168 hours in a month. Data for the year 2003 were taken for calculations.

The profitability analysis was based on the assumption that the price of a tonne of fresh wood chips amounted to PLN 80, i.e. PLN 10 GJ^{-1}. The

profitability analysis of willow cultivation loco plantation was based on the difference between the income and costs. The calculation did not include the profit earned by the service provider. The whole cost (PLN ha^{-1}) was divided into two stages. The first one included the establishment of the plantation, and the second – willow production in particular harvest cycles. The production cost did not include the cost of biomass transport from the field to the heat generating plant. The cost of establishing the plant was presented as a whole and divided into 16 years of use (1 introductory work + 5 3-year cycles).

Results and Discussion

The mean yield of fresh biomass of *Salix* spp. in the experiment amounted to 65.91 $Mg \cdot ha^{-1}$ (Table 1). The highest yield was obtained from the *Salix viminalis* JORR clone (77.73 $Mg \cdot ha^{-1}$), slightly less – the *Salix viminalis* (1023) and *Salix viminalis* (1033) clones: 72.55 and 70.67 $Mg \cdot ha^{-1}$, respectively. Significantly lower yields of biomass were obtained from the *Salix viminalis* x *Salix purpurea* (54.25 $Mg \cdot ha^{-1}$) and *Salix cordata* (51.89 $Mg \cdot ha^{-1}$). The mean biomass yield in a one-year cycle amounted to 24.65 $Mg \cdot ha^{-1}$, in a two-year cycle – 63.14 $Mg \cdot ha^{-1}$; it was the highest in a three-year cycle: 109.94 $Mg \cdot ha^{-1}$. Calculated for one year of production it gives 31.57 $Mg \cdot ha^{-1} \cdot year^{-1}$ in a two-year cycle and 36.65 $Mg \cdot ha^{-1} \cdot year^{-1}$ in a three-year cycle. The highest yield in a one-year cycle was obtained from *Salix viminalis* 1023: 29.30 $Mg \cdot ha^{-1}$. A high yield was also produced by the hybrid *Salix viminalis* x *Salix purpurea* (27.21 $Mg \cdot ha^{-1}$), which suggests the possibility of cultivating the

Table 1
The yield of fresh biomass of seven clones of *Salix* spp. in relation to harvest frequency ($Mg \cdot ha^{-1}$)

Botanic name and clone number (a)	Harvest frequency (b)			Mean
	every year	every 2 years	every 3 years	
Salix viminalis x *Salix purpurea* (1001)	27.21	55.20	80.35	54.25
Salix cordata (1019)	22.21	56.55	76.90	51.89
Salix viminalis (1023)	29.30	79.20	109.16	72.55
Salix viminalis (1033)	25.02	56.93	130.05	70.67
Salix viminalis var. *gigantea* (1047)	23.03	42.01	134.07	66.37
Salix viminalis JORR	25.31	74.26	133.61	77.73
Salix viminalis ULV	20.48	77.82	105.48	67.93
Mean	24.65	63.14	109.94	65.91
Mean $Mg \cdot ha^{-1} \cdot year^{-1}$	24.65	31.57	36.65	30.96
$LSD_{0.05}$	a – 15.82	b – 10.36	axb – 27.40	

clones in this cycle on commercial plantations. A high yield of biomass was obtained in harvesting the clone *Salix viminalis* var. *gigantea* every three years (44.69 Mg · ha^{-1} · year^{-1}); a similarly high yield in the same cycle was obtained from *Salix viminalis* JORR (44.54 Mg · ha^{-1} · year^{-1}). The clones *Salix cordata* and *Salix viminalis x Salix purpurea* should be regarded as unsuitable for cultivation in 3-year cycles; they yielded only: 25.63 and 26.78 Mg · ha^{-1} · year^{-1}., respectively.

The work and financial outlays needed for performing particular agricultural operations necessary to establish a willow plantation are shown in 2. The total cost of establishing a willow plantation on 1 ha with 40 thousand plants was calculated as PLN 8,131.68; calculated for one year of production, the value amounts to PLN 508.23 ha^{-1}. The highest portion of the total cost was that of the seedling purchase – PLN 6,000 (PLN 0.15/seedling), which accounted for 73.78% of the total cost.

In an earlier study by the same authors (STOLARSKI et al. 2002), the cost of seedlings purchase accounted for 71.3% to 80.7% of the total cost incurred in connection with establishing the plantation of willow, depending on the number of plants per hectare. Based on the experiments conducted in Wales, RANDERSON et al. (2000), pointed out that the largest portion oft he cost of seedlings purchase (73.8% of the total cost – £ 1,600 ha^{-1}, PLN 9,280 ha^{-1}) if the number of plants was 20 thousand per hectare.

The cost of employing tractors and other equipment accounted for 4.72%, and the cost of human labour – 17.62% of the total cost (Table 2). The relatively high cost of human labour is a result of planting the willows manually, which amounted to PLN 816.00 per hectare, while the total cost of human labour amounted to PLN 1,433.10 ha^{-1}.

The willow production cost in different harvesting cycles is presented in Table 3. The cost of establishing the plantation was high and accounted for 31% to 30% of the total cost, respectively, for harvesting every three years and every year. The cost of human labour accounted for 3.22% of the total cost when the plants were harvested every two years and 7.21% when the harvest took place every year. A higher proportion of human labour cost in a one-year cycle was caused by the use of a forage harvester coupled with a tractor. The cost of operation of tractors and other equipment in a one-year cycle accounted for 25.51% of the total cost; the proportion was higher in the two-year (39.75%) and three-year cycle (43.08%). The cost of harvesting machine operation in the two- and three-year cycles increased due to the use of a Claas Jaguar 860 combine harvester. The willow harvest with a Claas Jaguar combine harvester was the most costly process in terms of machine operation. The cost of fertilisers decreased with the duration of the production cycle: it accounted for 21.75%, 18.44% and 17.30% in a one-year, two-year and three-year cycle,

Table 2

Work and financial outlay for establishing a plantation of willow *Salix* spp. with 40 thousand plants per hectare at prime costs (regardless of the clone)

Item	Type		Work outlay			Establishing cost (PLN ha^{-1})			
	tractor	accompanying machine	man-hour	tractor hour	machine hour	labour force	tractor	machine or tool	total
Spray (Roundup)	Ursus C-360-3P	Pilmet 412 sprayer	0.50	0.50	0.50	5.10	11.84	5.60	22.54
Autumn ploughing	MTZ 82A	U 036/2 plough	2.00	2.00	2.00	20.40	70.28	29.00	119.68
Harrowing	MTZ 82A	U 358 harrow	1.00	1.00	1.00	10.20	35.14	1.30	46.64
Seedling purchase price – 40 thousand, PLN 0.15 per seedling)	–	–	–	–	–	–	–	–	6000.00
Making marks for planting	Ursus C-360-3P	P 447/1 hiller	0.50	0.50	0.50	5.10	11.84	2.80	19.74
Manual planting	–	–	80.00	–	–	816.00	–	–	816.00
Spray (Bladex)	Ursus C-360-3P	Pilmet 412 sprayer	0.50	0.50	0.50	5.10	11.84	5.60	22.54
Weeding (2x)	Ursus C-360-3P	P 430/2 weeder	2.00	2.00	2.00	20.40	47.36	28.00	95.76
Roundup purchase	–	–	–	–	–	–	–	–	125.00
Bladexu purchase	–	–	–	–	–	–	–	–	105.00
Agricultural tax	–	–	–	–	–	–	–	–	85.00
Manual harvest after the first vegetation period	Stihl chain saw	–	48.00	–	16.00	489.60	–	64.00	553.60
Transport	Ursus C-360-3P	T604 trailer	6.00	2.00	2.00	61.20	47.36	11.62	120.18
Total	–	–	140.50	8.50	24.50	1433.10	235.66	147.92	8131.68
Per one year of production 1/16 Σ	–	–	8.78	0.53	1.53	89.57	14.73	9.25	508.23

respectively. The cost of production of willow harvested in a one-year cycle was the lowest: PLN 1,303.27 ha^{-1}. Extending the harvesting cycle increased the production costs to PLN 1,537.41 per hectare per year in two-year cycle and PLN 1,639.13 per hectare per year in a three-year cycle. ROSENQVIST and DAWSON (2005) established the cost of willow cultivation in Northern Ireland at €445 per hectare per year (PLN 1,780 per hectare per year). The findings were similar in Sweden: €499/ha (PLN 1,996 ha^{-1}) (ROSENQVIST, NESS 2004). The slightly lower production cost is Poland is a result of the lower cost of human labour than in Sweden and Northern Ireland[1].

Table 3
The cost of willow production loco plantation in three harvest cycles at prime costs (mean values for the clones) (PLN ha^{-1}, %)

Item	Harvest frequency						Mean	
	every year		every two years		every three years			
	(PLN ha^{-1})	%	(PLN ha^{-1})	%	(PLN ha^{-1})	%	(PLN ha^{-1})	%
Cost of establishing the plantation	508.23	39.00	1016.46	33.06	1524.69	31.01	1016.46	32.87
Labour force	94.01	7.21	99.10	3.22	168.96	3.44	120.69	3.92
Tractors	249.64	19.15	187.36	6.09	317.87	6.46	251.62	8.22
Machines	82.89	6.36	1034.89	33.66	1800.38	36.61	972.72	31.15
Fertilisers	–	–	–	–	–	–	–	–
N	91.50	7.02	183.00	5.95	274.50	5.58	183.00	5.92
P and K	192.00	14.73	384.00	12.49	576.00	11.71	384.00	12.42
Agricultural tax	85.00	6.52	170.00	5.53	255.00	5.19	170.00	5.50
Total	1303.27	100.00	3074.81	100.00	4917.39	100.00	3098.49	100.00
Total (PLN · ha^{-1} · year^{-1})	1303.27	–	1537.41	–	1639.13	–	1493.27	–

The cost of production of 1 tonne of wood chips was established based on the total cost of production and mean biomass yield in various harvest cycles (Table 4). The profitability analysis was performed for the price level of PLN 80 Mg^{-1} of fresh chips (PLN 10 GJ^{-1}). It should be emphasised that the price of chips in Poland – PLN 80 Mg^{-1} – are lower than in most countries of Western Europe. Probably, the price of wood chips in Poland will rise in the nearest future to the level close to that charged in the European Union (EU-15).

[1] foreign currencies were converted into Polish zloty according to the average exchange rate (£ 1 = PLN 5.80, € 1 = PLN 4.00).

Table 4
Profitability of willow production loco plantation in three harvest cycles, at the price of fresh chips of PLN 80 Mg^{-1} (mean values for the clones)

Item	Harvest frequency			Mean
	every year	every two years	every three years	
Biomass yield ($Mg \cdot ha^{-1}$)	24.65	63.14	109.94	65.91
Production cost ($PLN \cdot ha^{-1}$)	1303.27	3074.81	4917.39	3098.49
Cost of production of 1 Mg (PLN)	52.87	48.70	44.73	48.77
Price for 1 Mg of chips (PLN)	80.00	80.00	80.00	80.00
Profit from 1 Mg (PLN)	27.13	31.30	35.27	31.23
Profit from 1 ha (PLN)	668.73	1976.39	3877.81	2174.31
Profit from 1 ha per year (PLN)	668.73	988.19	1292.60	983.18

The lowest cost of chips production were determined for the three-year harvest cycle: PLN 44.73 t^{-1} (Table 4). The profit earned from the production of 1 tonne of chips loco plantation amounted to PLN 35.27.

The profit achieved from 1 ha of willow cultivation calculated for one year of production loco plantation amounted to PLN 668.73 ha^{-1} in a one-year cycle, PLN 988.19 per hectare per year in the two-year cycle, and PLN 1,292.60 per hectare per year in the three-year cycle. The calculations made in this experiment show that extending the harvesting cycle from one to two and three years increases the profit by 47 and 93%, respectively. From the point of view of the profitability, the best option is to harvest willow in three-year cycles.

The profit from the production of particular clones of *Salix* spp. loco plantation in different harvesting cycles is shown in Figure 1. When the plants were harvested every year, high profitability was achieved for the *Salix viminalis* 1023 and *Salix viminalis* x *Salix purpurea* clones: PLN 967.51 and PLN 833.22, respectively. The cultivation of the *Salix viminalis* x *Salix purpurea* clone has been shown to be profitable only in a one-year harvesting cycle – extending the cycle reduces the profit. As in the one-year harvesting cycle, the profit earned in the two-year harvest cycle for the *Salix viminalis* 1023 clone was the highest – PLN 1,474.75. High profitability in a three-year harvest cycle was achieved in the cultivation of the *Salix viminalis* var. *gigantea, Salix viminalis* JORR and *Salix viminalis* 1033 clones: PLN 1,779.97; 1,770.68 and 1,698.77 per hectare per year, respectively.

The analysis shows that the expenses incurred for the cultivation and harvesting of the willow in a one-year cycle are balanced by the yield of 16.3 Mg of fresh biomass per hectare per year (i.e. ca 8.2 Mg of dry substance per

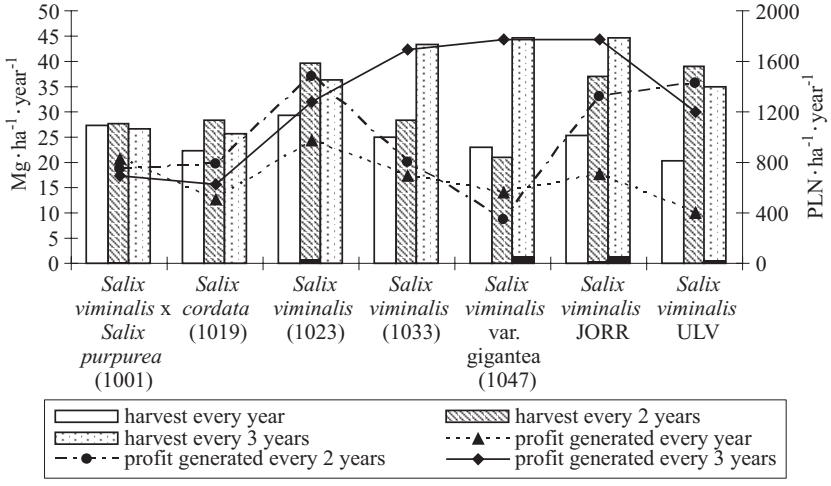

Fig. 1. The yield of fresh biomass and profit loco plantation generated in the production of seven clones of *Salix* spp. in three harvest cycles at the price of fresh chips of PLN 80 Mg^{-1}

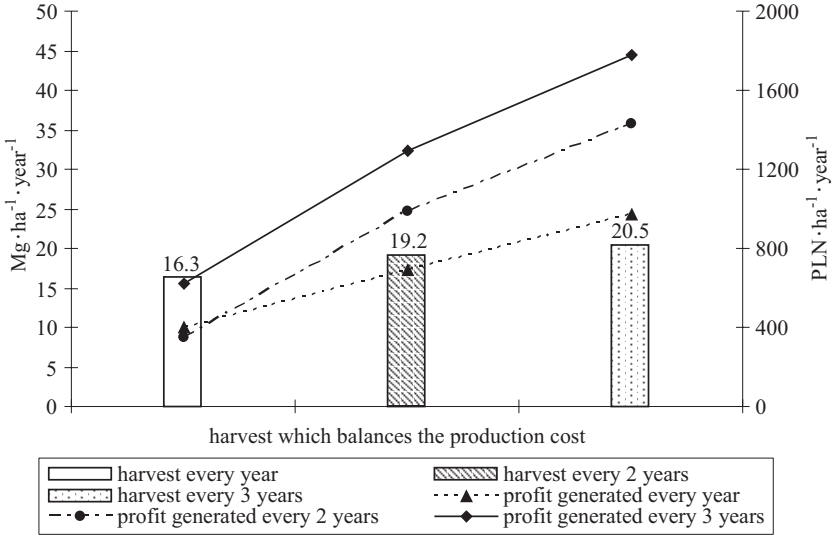

Fig. 2. The range of profit from willow cultivation loco plantation and the yield of fresh biomass which balances the production cost in 3 harvest cycles

hectare per year – Figure 2). It means that a higher yield than the above value will provide a profit, while with a lower productivity, a loss will be incurred. In the willow production in two- and three-year cycles, the cost was balanced by the yield of fresh biomass of 19.2 Mg per hectare per year (i.e. ca 9.6 Mg of dry

substance of wood per 1 hectare per year) and 20.5 Mg per hectare per year (i.e. ca 10.2 Mg of dry substance of wood per 1 hectare per year).

It was shown in earlier studies by the same authors (STOLARSKI et al. 2002) that with the price of PLN 80 for 1 Mg of fresh wood chips, the profit earned from willow cultivation in a one-year harvesting cycle amounted to PLN 1.350 per hectare per year, in a two-year cycle – PLN 1.316 per hectare per year, and in a three-year cycle – PLN 1.767 per hectare per year. ROSENQVIST and DAWSON (2005) showed that willow cultivation in Northern Ireland generated profit at the minimum yield of 18.4 Mg of fresh biomass per hectare per year and at the price of £ 20 for 1 Mg (PLN 116 for 1 Mg). On the other hand, the simulation run for the study showed that if the price of fresh chips decreased to £ 17.5 Mg^{-1} (PLN 101.5 Mg^{-1}), profitability would be achieved at the yield of 24 Mg of fresh biomass per hectare per year. ROSENQVIST and NESS (2004) showed that regardless of the price of wood chips, the application of sludge on willow plantations is always profitable. ROSENQVIST et al. (2000) found that extensively cultivated willow is more competitive on medium and low quality soils, whereas on fertile soils, wheat is more competitive. As part of the EC--RECOVER project (VANDENHOVE et al. 2002) an assessment was conducted of cultivation of willow in Belarus as an alternative plant in contaminated areas. It was shown that with the very low prices of wood as a fuel in Belarus €10.2 Mg^{-1} (PLN 40.8 Mg^{-1}) of dry substance (PLN 20.40 Mg^{-1} of fresh wood), cultivation of willow is not profitable, even with lower costs of human labour and machine operation. Cultivation of willow would become profitable only if the price was as high as €40 Mg^{-1} (PLN 160 Mg^{-1}) of dry chips with a yield of 12 Mg per hectare per year.

Conclusions

1. The economic analysis showed that the cultivation of willow in the Kwidzyn Lowland was profitable.

2. The cost of establishing a plantation of willow as a source of energy strongly depends on the number of willows planted on an area unit and the price of seedlings, and accounts for a large portion of the total cost of willow production.

3. The mean profit earned from 1 ha of willow production loco plantation, calculated for one year of cultivation amounted to PLN $668.73 \cdot ha^{-1}$ in a one-year cycle, PLN $988.19 \cdot ha^{-1} \cdot year^{-1}$ in a two-year cycle and PLN $1{,}292.60 \cdot ha^{-1} \cdot rok^{-1}$ in a three-year cycle.

4. It has been shown that the financial outlays incurred for the cultivation and harvest of willow in a one-year cycle are balanced by a yield of

16.3 Mg · ha^{-1} · year^{-1} of fresh biomass. For willow production in 2- and 3-year cycles, the cost was balanced with the fresh biomass yield of 19.2 and 20.5 Mg · ha^{-1} · year^{-1}.

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Reference

JEŻOWSKI S. 2003. *Rośliny energetyczne – produktywność oraz aspekt ekonomiczny, środowiskowy i socjalny ich wykorzystania jako ekobiopaliwa*. Post. Nauk Rol., 3: 61-73.

KISIEL R., STOLARSKI M., SZCZUKOWSKI S., TWORKOWSKI J. 2004. *Costs of bushy willow coppice production at a plantation established for production of energy*. Econ. Sci., 7: 177-184.

KISIEL R., STOLARSKI M., SZCZUKOWSKI S., TWORKOWSKI J. 2006. *Biomasa pozyskiwana z gruntów rolniczych źródłem energii*. Zag. Ekon. Rol., 4: 90-101.

MUZALEWSKI A. 2003. *Koszty eksploatacji maszyn*. IBMER, Warszawa.

RANDERSON P.F., HEATON R.J., SLATER F.M. 2000. *Economic prospects for short rotation coppice in Wales*: *The need for subsidy in a new agricultural industry*. The 7th Polish-Danish Workshop on „Biomass for Energy", December 7-10-2000, Starbienino, Poland, 135-142 pp.

ROSENQVIST H., DAWSON M. 2005. *Economics of willow growing in Northern Ireland*. Biomass and Bioenergy, 28: 7-14.

ROSENQVIST H., NESS B. 2004. *An economic analysis of leachate purification through willow-coppice 1vegetation filters*. Biores. Technol., 94: 321-329.

ROSENQVIST H., ROOS A., LING E., HEKTOR B. 2000. *Willow growers in Sweden*. Biomass and Bioenergy, 18 (2): 137-145.

STOLARSKI M., KISIEL R., SZCZUKOWSKI S., TWORKOWSKI J. 2002. *Opłacalność produkcji wierzb krzewiastych na gruntach rolniczych w krótkich rotacjach i przy różnym zagęszczeniu roślin*. Rocz. Nauk Rol., Ser. G, 89(2): 96-105.

STOLARSKI M., KISIEL R., SZCZUKOWSKI S., TWORKOWSKI J. 2003. *Koszty produkcji peletów z biomasy wierzb krzewiastych*. Rocz. Nauk Rol., Ser. G, 90(2): 211-216.

STOLARSKI M., SZCZUKOWSKI S., TWORKOWSKI J., KWIATKOWSKI J. GRZELCZYK M. 2005. *Charakterystyka zrębków oraz peletów (granulatów) z biomasy wierzby i ślazowca jako paliwa*. Probl. Inż. Rol., 1(47): 13-22.

STOLARSKI M., TWORKOWSKI J., SZCZUKOWSKI S. KOPACZEL. 2006. *Production of willow (Salix spp.) biomass on arable land in short-term harvesting cycles*. Pol. J. Natur. Sci., (20)1: 53-65.

SZCZUKOWSKI S., TWORKOWSKI J. 2003. *Produkcja wieloletnich roślin energetycznych w regionie Warmii i Mazur – stan aktualny i perspektywy*. Post. Nauk. Rol., 3: 75-84.

VANDENHOVE H., GOOR F., O'BRIEN S., GREBENKOV A., TIMOFEYEV S. 2002. *Economic viability of short rotation coppice for reuse of caesium-contaminated land in Belarus*. Biomass and Bioenergy, 22: 421-431.

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MICROORGANISM COUNT IN THE SOIL CONTAMINATED WITH ZINC

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K e y w o r d s: zinc, soil contamination, microorganism count, ED_{50}.

A b s t r a c t

The effect of soil contamination with zinc on the count of selected taxonomic and physiologic groups of microorganisms has been studied in a laboratory experiment. Samples taken from brown soil originating from light clay with pH_{KCl} 6.9 were contaminated with $ZnSO_4 \cdot 7H_2O$ in the following amounts (mg of $Zn^{2+} \cdot kg^{-1}$ of soil; 0, 5, 500, 1000, 1500, 2000. The experiment was conducted for 120 days at 25°C, in two series: soil with cellulose (15,0 $g \cdot kg^{-1}$ of dry soil) and without cellulose.

The experiment showed that the count of oligotrophic, copiotrophic, ammonifying, cellulolytic and nitrogen immobilising bacteria as well as actinomyces and fungi were positively correlated with the zinc dose while the correlation was negative for *Azotobacter* spp.. The positive effect of zinc on nitrogen immobilising bacteria, actinomyces and fungi persisted throughout the experiment. ED_{50} Zn^{+2} ($mg \cdot kg^{-1}$) equalled for: fungi – 240, oligotrophic bacteria – 584, ammonifying – 803, copiotrophic bacteria – 1016, nitrogen immobilising bacteria – 1620, cellulolytic bacteria – 2000 and actinomyces – 2000. When applied in the amount of 15 $g \cdot kg^{-1}$ of soil, cellulose stimulated the bacteria multiplication in soil contaminated with zinc. It had the most positive effect on actinomyces and fungi.

LICZEBNOŚĆ DROBNOUSTROJÓW W GLEBIE ZANIECZYSZCZONEJ CYNKIEM

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S ł o w a k l u c z o w e: cynk, zanieczyszczenie gleby, liczebność drobnoustrojów, ED_{50}.

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Abstrakt

W doświadczeniu laboratoryjnym badano wpływ zanieczyszczenia gleby cynkiem na liczebność wybranych grup systematycznych i fizjologicznych drobnoustrojów. Próbki pobrane z gleby brunatnej typowej, wytworzonej z gliny lekkiej o pH_{KCl} 6,9, zanieczyszczano $ZnSO_4 \cdot 7H_2O$ w następującej ilości (mg $Zn^{+2} \cdot kg^{-1}$ gleby): 0, 5, 500, 1000, 1500, 2000. Badania prowadzono przez 120 dni w temp. 25°C, w dwóch seriach: gleba z celulozą – 15,0 g $\cdot$ kg^{-1} s.m. gleby i bez celulozy.

Stwierdzono, że liczebność bakterii oligotroficznych, kopiotroficznych, amonifikacyjnych, immobilizujących azot i celulolitycznych oraz promieniowców i grzybów była dodatnio skorelowana z dawką cynku, a liczebność *Azotobacter* spp. – ujemnie. Pozytywny wpływ cynku na liczebność bakterii immobilizujących azot, promieniowców i grzybów utrzymywał się przez cały okres trwania doświadczenia. ED_{50} Zn^{+2} (mg $\cdot$ kg^{-1}) wynosiło dla: grzybów – 240, bakterii oligotroficznych – 584, amonifikacyjnych – 803, kopiotroficznych – 1016, immobilizujących azot – 1620, celulolitycznych – 2000 i promieniowców – 2000. Celuloza, zastosowana w ilości 15 g $\cdot$ kg^{-1}, stymulowała namnażanie drobnoustrojów w glebie zanieczyszczonej cynkiem. Najkorzystniej oddziaływała na promieniowce i grzyby.

Introduction

Zinc is a metal which is indispensable in small amounts for bacteria growth and metabolism. Along with the ions of such metals as iron, copper and cobalt, it is necessary to maintain the gradient of the ion concentrations on both sides of the cell membrane and to maintain the rigidity of its structure. It is also one of the factors responsible for the synthesis and function of nucleic acids and ribosome aggregation (STOHS, BAGCHI 1995).

On the other hand, however, if its concentration in the environment is high, it is known to be a toxic heavy metal with a negative impact on organisms (BÄÄTH 1989, BARABASZ et al. 1997, ROST et al. 2001, YEATES et al. 1994, WYSZKOWSKA, KUCHARSKI 2003, ZABOROWSKA et al. 2006). It slows down biological processes, causes protein denaturation and destroys cell membranes – in effect, reducing the size and activity of bacteria biomass (BÄÄTH 1989). The accumulation of heavy metals in bacteria cells depends primarily on the permeability of the cell membrane and the presence of the immunological factor in plasmides. An important role is also played by bacterial mucus. The effect of metal ions on bacteria is also affected by the type of metal, its concentration and the number of coexisting ions (CABRERO et al. 1997). According to SŁABY and DŁUGOŃSKI (2002), heavy metal toxicity manifests itself in blocking enzyme functional groups by replacing ions which are necessary for the correct cell metabolism (cadmium can replace zinc, zinc can replace magnesium) and inducing conformation changes in polymers.

Zinc bioavailability and, consequently, its toxicity, are affected by the solubility of the compound which it is a part of, the soil reaction and its humidity, the organic matter content and the presence of clay minerals in soil

(ANGELOVA, IVANOV 2000, VIG et al. 2003, ZABOROWSKA et al. 2006). Due to the wide range of factors which determine zinc toxicity to microorganisms, an experiment was conducted to determine the effect of soil contamination with zinc on bacteria counts. The reaction of bacteria to zinc was studied in natural soil and in soil with an addition of cellulose.

Material and Methods

The experiment was conducted in a laboratory. Samples of brown soil, originating from light clay soil, with pH_{KCl} 6.9, were taken from the natural soil humus level (1.0-0.1 mm – 60%, 0.1-0.02 mm – 12%, < 0.02 mm – 28%). It contained 7.8 g Corg · kg^{-1}. Its hydrolytic acidity was 13.6 mmol · kg^{-1} and the total basic exchangeable cations – 113.2 mmol · kg^{-1}.

The variable factors in the experiment included:

1) increasing doses of zinc (mg Zn · kg^{-1} of soil): 0, 5, 500, 1000, 1500 and 2000;

2) the addition of cellulose (g · kg^{-1} of soil): 0 and 15;

3) soil incubation time (days): 15, 30, 60, 90 and 120.

The experiment was performed in 6 replications. 50 g (dry substance) of soil was placed in each of the 100 cm^3 beakers. The soil samples were contaminated with zinc (factor 1), which was applied as an aqueous solution of $ZnSO_4 \cdot 7H_2O$, and cellulose was then added in the appropriate combinations (factor 2). The samples were then thoroughly mixed and their humidity established at 60% of the capillary water capacity. The prepared samples were stored in an incubator at 25°C (factor 3) and the evaporated water was then replenished by sterile distilled water. On established dates, i.e. on day 15, 30, 60, 90 and 120 of the incubation period, the counts of the following bacteria were determined: copiotrophic and oligotrophic bacteria – on ONTA and HATTORY'S broth (1983), ammonifying, nitrogen immobilising and cellulolytic bacteria – on the substrate described in a paper by WYSZKOWSKA (2002), actinomyces – on Kuster and Williams; substrate with an addition of nystatin and actidion (PARKINSON et al. 1971), fungi on MARTIN'S broth (1950) and *Azotobacter* spp. by the method developed by FENGLEROWA (1965). The microorganisms were cultured on Petri dishes at 28°C for the period ranging from 2 (*Azotobacter* spp.) to 21 days (oligotrophic bacteria). The cfu number was determined with the use of a colony count. The results were analysed statistically with the use of a multiple Duncan gap test, using three-factor variation analysis. Taking into account all the replications, the Pearson simple correlation coefficients between the variables were calculated. Based on the regression equations, zinc doses were determined which cause a 50% stimulation of the microorganisms

multiplication (ED_{50}). A statistical analysis was performed with a Statistica software package (StatSoft, Inc.... 2004).

Results and Discussion

The average oligotrophic bacteria count in zinc-contaminated soil increased after the application of cellulose (Figure 1). The other groups of microorganisms did not react significantly to the addition of cellulose. However, the situation was different when cellulose was added to zinc contaminated soil – no differences were found to exist between oligotrophic and cellulolytic bacteria count in the soil with or without cellulose, while the counts of copiotrophic, ammonifying and nitrogen immobilising bacteria, as well as actinomyces and fungi, were considerably higher in the soil with cellulose. But it was not only cellulose which determined the microorganism count; this was also affected by zinc in doses of 500 to 2000 mg $Zn^{+2} \cdot kg^{-1}$ of soil. *Azotobacter* spp. reacted negatively to contamination with zinc. Doses of zinc above 500 mg $Zn^{+2} \cdot kg^{-1}$ of soil totally eliminated the bacteria from the soil system. The other groups of microorganisms reacted differently than *Azotobacter* spp. to increased zinc concentration in soil. The count of these bacteria in the contaminated soil increased considerably. Fungi and ammonifying were particularly positively affected by contamination with zinc. The effect was slightly less intense for actinomyces as well as oligotrophic, copiotrophic and nitrogen immobilising bacteria. Cellulolytic bacteria proved to be the most resistant to zinc. Obviously, the effect of zinc on microorganisms was not constant throughout the experiment (120 days). It fluctuated (Table 1 – Table 8) and was not always the same for each group of microorganisms. Oligotrophic bacteria (Table 1) were much more strongly affected (with stimulated growth) by zinc in the soil without cellulose than with it. This upsetting of the balance was particularly significant on days 15 and 30. On subsequent days it decreased considerably and on day 120 it was only observable for the highest doses (1000 mg – 2000 mg $Zn^{+2} \cdot kg^{-1}$ of soil). The stimulating effect of zinc on copiotrophic bacteria (Table 2) persisted in the soil with cellulose from day 15 to day 120, with periodical fluctuations, regardless of the zinc dose. The effect of zinc was much weaker in the soil without cellulose; initially (day 15, 30 and 60) it was totally unnoticeable, and later (day 90 and 120) it significantly stimulated the microorganism multiplication, but this was only in the highest doses (1500 mg and 2000 mg $Zn^{2+} \cdot kg^{-1}$ of soil). The reaction of *Azotobacter* spp. to soil contamination with zinc was different than that of oligotrophic and copiotrophic bacteria (Table 3). Irrespective of whether cellulose had been applied or not, doses of more than 500 mg of zinc $\cdot kg^{-1}$ of soil completely eliminated the

bacteria from the soil as early as on day 15; this persisted throughout the experiment. In the case of the dose of 500 mg $Zn^{2+} \cdot kg^{-1}$ of soil, the inhibiting effect on the growth of *Azotobacter* spp. lasted for 90 days and disappeared completely on day 120, whereas bacteria growth was stimulated by the lowest dose (5 mg $Zn^{2+} \cdot kg^{-1}$ of soil).

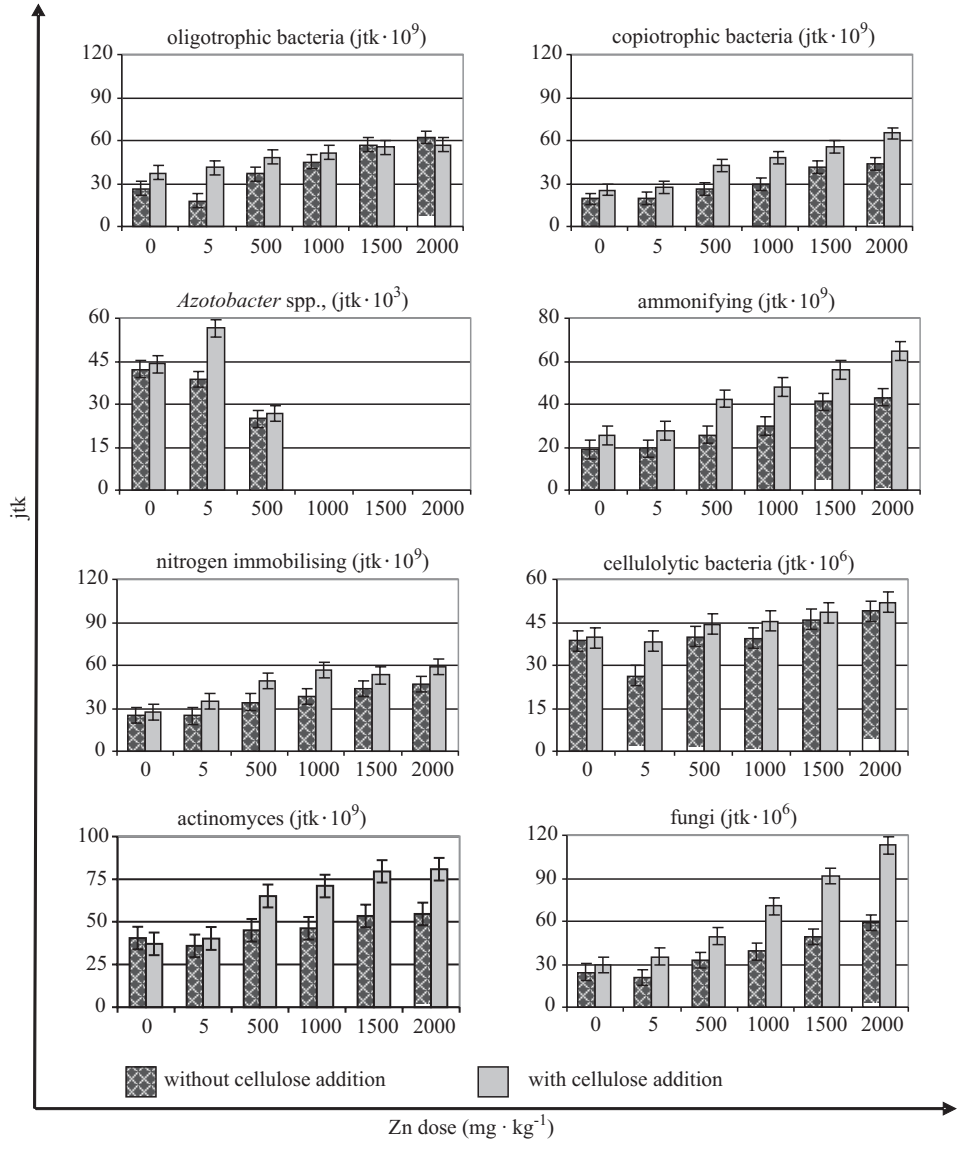

Fig. 1. Average microorganism count in 1 kg of dry soil contaminated with zinc

Table 1

Oligotrophic bacteria count in relation to zinc contamination, cellulose addition and time of incubation in soil (jtk · 10^9 · kg^{-1} d.m.)

Cellulose dose (g · kg^{-1} d.m. soil)	Zn dose (mg · kg^{-1} d.m. soil)	Soil incubation time (days)					*r*
		15	30	60	90	120	
		(jtk · 10^9 · kg^{-1} d.m. soil)					
0	0	13.09	13.50	19.22	65.44	21.68	0.49*
	5	23.72	15.13	24.13	86.71	26.58	0.43
	500	23.93	23.52	25.36	88.75	23.52	0.35
	1000	30.06	28.83	25.56	100.82	38.65	0.45
	1500	31.70	43.76	26.18	111.86	70.35	0.66**
	2000	40.90	46.01	31.70	113.91	78.32	0.68**
	r	0.92**	0.99**	0.86**	0.91**	0.95**	
15	0	20.45	27.81	19.63	82.00	36.20	0.52
	5	25.36	21.68	21.88	95.30	42.13	0.55
	500	27.40	41.10	27.40	99.59	47.03	0.51
	1000	29.65	45.81	30.47	100.41	50.72	0.52
	1500	30.67	50.92	35.99	107.77	52.56	0.50
	2000	33.33	52.35	36.20	113.91	48.47	0.43
	r	0.92**	0.93**	0.97**	0.91**	0.77**	
$LSD_{p = 0.01}$		*a* – 3.45; *b* – 1.99; *c* – 3.15; *a* · *c* – 7.71; *a* · *b* – 4.88; *b* · *c* – 4.45; *a* · *b* · *c* – 10.90					

LSD for: *a* – zinc dose. *b* – cellulose addition. *c* – soil incubation time
r – correlation co-efficient significant at: $*p = 0.05$, $**p = 0.01$

Table 2

Copiotrophic bacteria count in relation to zinc contamination, cellulose addition and time of incubation in soil (jtk · 10^9 · kg^{-1} d.m.)

Cellulose dose (g · kg^{-1} d.m. soil)	Zn dose (mg · kg^{-1} d.m. soil)	Soil incubation time (days)					*r*
		15	30	60	90	120	
		(jtk · 10^9 · kg^{-1} d.m. soil)					
0	0	16.56	10.43	37.22	17.79	19.63	0.20
	5	14.72	15.34	30.47	23.52	20.45	0.45
	500	13.50	16.77	33.74	23.93	21.47	0.43
	1000	14.93	20.65	37.01	24.54	26.38	0.47
	1500	15.13	20.86	44.17	32.11	45.19	0.82**
	2000	22.90	20.45	26.79	34.76	50.72	0.94**
	r	0.63**	0.84**	-0.03	0.93**	0.95**	
1	0	21.27	13.91	38.24	22.90	23.72	0.25
	5	32.31	12.27	36.81	23.11	39.47	0.42
	500	39.26	22.49	40.49	28.02	40.49	0.22
15	1000	41.92	28.83	46.42	31.29	47.44	0.30
	1500	41.31	20.86	56.03	32.92	48.47	0.35
	2000	53.37	20.65	43.56	35.79	50.10	0.20
	r	0.89**	0.55*	0.69**	0.98**	0.81**	
$LSD_{p = 0.01}$		*a* – 3.07; *b* – 1.77; *c* – 2.80; *a* · *b* – 4.34; *a* · *c* – 6.87; *b* · *c* – 3.96; *a* · *b* · *c* – 9.68					

Explanation disclose under the Table 1

Table 3

Azotobacter spp. count in relation to zinc contamination, cellulose addition and time of incubation in soil (jtk · 10^3 · kg^{-1} d. m.)

Cellulose dose (g · kg^{-1} d.m. soil)	Zn dose (mg · kg^{-1} d.m. soil)	Soil incubation time (days)					*r*
		15	30	60	90	120	
		(jtk · 10^9 · kg^{-1} d.m. soil)					
0	0	16.36	47.24	51.74	74.44	20.86	0.17
	5	20.25	55.62	61.96	60.74	56.24	0.62
	500	2.25	44.38	13.91	39.67	24.13	0.28
	1000	0.00	0.00	0.00	0.00	0.00	–
	1500	0.00	0.00	0.00	0.00	0.00	–
	2000	0.00	0.00	0.00	0.00	0.00	–
	r	-0.82**	-0.91**	-0.86**	-0.91**	-0.80**	
15	0	43.97	51.53	41.31	43.56	39.67	-0.64
	5	34.56	96.73	48.26	49.90	52.97	-0.16
	500	3.27	52.56	11.66	31.70	34.56	0.27
	1000	0.00	0.00	0.00	0.00	0.00	–
	1500	0.00	0.00	0.00	0.00	0.00	–
	2000	0.00	0.00	0.00	0.00	0.00	–
	r	-0.81**	-0.86**	-0.87**	-0.92**	-0.91**	
$LSD_{p = 0.01}$		*a* – 2.04; *b* – 1.18; *c* – 1.86; *a* · *b* – 2.89; *a* · *c* – 4.56; *b* · *c* – 2.63; *a* · *b* · *c* – 6.44					

Explanation disclose under the Table 1

Table 4

Ammonifying count in relation to zinc contamination, cellulose addition and time of incubation in soil (jtk · 10^9 · kg^{-1} d.m.)

Cellulose dose (g · kg^{-1} d.m. soil)	Zn dose (mg · kg^{-1} d.m. soil)	Soil incubation time (days)					*r*
		15	30	60	90	120	
		(jtk · 10^9 · kg^{-1} d.m. soil)					
0	0	26.58	15.95	18.20	15.54	19.22	-0.45
	5	26.79	16.36	25.77	20.45	27.81	0.28
	500	33.74	16.56	27.20	23.93	27.40	-0.04
	1000	34.97	18.20	32.31	33.74	28.63	0.14
	1500	39.06	43.35	45.19	37.42	40.49	-0.23
	2000	40.70	50.31	50.72	33.54	40.90	-0.42
	r	0.97**	0.91**	0.97**	0.89**	0.91**	
15	0	27.81	29.86	27.61	18.40	24.34	-0.67**
	5	27.81	33.33	25.56	23.72	27.40	-0.50*
	500	29.65	54.81	62.99	34.76	30.27	-0.29
	1000	30.47	65.24	74.85	34.76	34.56	-0.26
	1500	43.56	65.64	91.00	44.17	35.58	-0.34
	2000	54.60	70.55	111.04	50.10	38.45	-0.36
	r	0.92**	0.92**	0.98**	0.96**	0.97**	
$LSD_{p = 0.01}$		*a* – 3.01; *b* – 1.74; *c* – 2.75; *a* · *b* – 4.26; *a* · *c* – 6.73; *b* · *c* – 3.88; *a* · *b* · *c* – 9.50					

Explanation disclose under the Table 1

Table 5

Nitrogen immobilising bacteria count in relation to zinc contamination, cellulose addition and time of incubation in soil (jtk · 10^9 · kg^{-1} d.m.)

Cellulose dose (g · kg^{-1} d.m. soil)	Zn dose (mg · kg^{-1} d.m. soil)	Soil incubation time (days)					*r*
		15	30	60	90	120	
		(jtk · 10^9 · kg^{-1} d.m. soil)					
0	0	14.52	9.61	16.16	28.02	56.44	0.90**
	5	15.13	11.04	17.38	30.06	79.35	0.87**
	500	17.18	15.34	17.79	40.29	80.37	0.90**
	1000	20.65	15.95	19.02	52.15	83.64	0.91**
	1500	36.61	18.40	24.74	54.81	82.21	0.84**
	2000	45.40	21.47	24.54	54.40	87.12	0.76**
	r	0.95**	0.97**	0.94**	0.94**	0.68**	
15	0	12.68	14.72	21.68	24.34	61.15	0.88**
	5	34.15	17.79	24.95	28.02	70.14	0.70**
	500	60.74	18.20	28.22	52.15	84.66	0.58*
	1000	67.69	23.11	31.29	75.87	86.09	0.59*
	1500	67.08	24.54	31.08	79.75	62.37	0.39
	2000	94.27	26.18	33.33	85.28	57.06	0.01
	r	0.91**	0.96**	0.92**	0.95**	-0.30	
$LSD_{p = 0.01}$		*a* – 3.98; *b* – 2.30; *c* – 3.63; *a* · *b* – 5.63; *a* · *c* – 8.90; *b* · *c* – 5.14; *a* · *b* · *c* – 12.56					

Explanation disclose under the Table 1

Table 6

Cellulolytic bacteria count in relation to zinc contamination, cellulose addition and time of incubation in soil (jtk · 10^6 · kg^{-1} d.m.)

Cellulose dose (g · kg^{-1} d.m. soil)	Zn dose (mg · kg^{-1} d.m. soil)	Soil incubation time (days)					*r*
		15	30	60	90	120	
		(jtk · 10^6 · kg^{-1} d.m. soil)					
0	0	21.06	26.38	50.31	50.51	44.17	0.78**
	5	23.31	27.61	37.01	51.53	43.97	0.88**
	500	24.54	27.20	48.88	52.97	46.22	0.82**
	1000	24.34	26.58	51.12	54.60	40.70	0.67**
	1500	34.15	32.52	57.87	61.35	43.97	0.56*
	2000	41.31	40.70	66.67	46.63	49.90	0.32
	r	0.93**	0.85**	0.89**	0.08	0.44	
15	0	21.06	34.36	36.40	56.24	50.72	0.90**
	5	20.04	38.24	32.52	47.44	52.97	0.89**
	500	23.52	42.54	39.67	52.97	62.99	0.93**
	1000	38.24	46.01	41.51	42.74	58.28	0.75**
	1500	38.45	48.47	59.51	42.94	52.97	0.39
	2000	44.38	50.72	74.03	41.51	49.69	-0.03
	r	0.96**	0.96**	0.95**	-0.80**	-0.21	
$LSD_{p = 0.01}$		*a* – 2.54; *b* – 1.47; *c* – 2.34; *a* · *b* – 3.59; *a* · *c* – 5.68; *b* · *c* – 3.28; *a* · *b* · *c* – 8.01					

Explanation disclose under the Table 1

Table 7

Actinomyces count in relation to zinc contamination, cellulose addition and time of incubation in soil (jtk · 10^9 · kg^{-1} d.m.)

Cellulose dose (g · kg^{-1} d.m. soil)	Zn dose (mg · kg^{-1} d.m. soil)	Soil incubation time (days)					*r*
		15	30	60	90	120	
		(jtk · 10^9 · kg^{-1} d.m. soil)					
0	0	36.20	37.01	34.36	35.17	59.30	0.70**
	5	43.97	40.08	34.97	25.97	60.33	0.28
	500	40.49	42.33	39.26	42.54	60.33	0.76**
	1000	41.10	46.22	39.47	43.56	60.74	0.70**
	1500	47.85	46.83	40.08	57.46	74.64	0.80**
	2000	51.33	52.56	42.74	50.72	75.66	0.64**
	r	0.82**	0.97**	0.94**	0.87**	0.90**	
15	0	35.38	29.45	39.26	41.92	39.26	0.70**
	5	49.69	41.31	40.90	26.79	42.33	-0.51*
	500	101.02	46.63	42.54	86.09	49.08	-0.30
	1000	115.34	60.12	45.60	80.78	52.97	-0.51*
	1500	151.33	61.15	49.28	78.94	57.06	-0.57*
	2000	155.42	65.44	49.90	75.05	58.49	-0.60*
	r	0.96**	0.93**	0.98**	0.68**	0.96**	
$LSD_{p = 0.01}$		*a* – 4.55; *b* – 4.16; *c* – 2.63; *a* · *b* – 6.44; *a* · *c* – 10.18; *b* · *c* – 5.87; *a* · *b* · *c* – 14.35					

Explanation disclose under the Table 1

Table 8

Fungi bacteria count in relation to zinc contamination, cellulose addition and time of incubation in soil (jtk · 10^6 · kg^{-1} d.m.)

Cellulose dose (g · kg^{-1} d.m. soil)	Zn dose (mg · kg^{-1} d.m. soil)	Soil incubation time (days)					*r*
		15	30	60	90	120	
		(jtk · 10^6 · kg^{-1} d.m. soil)					
0	0	18.20	26.79	17.59	35.17	24.95	0.45
	5	20.04	27.61	18.61	36.40	37.63	0.79**
	500	20.04	30.27	21.88	52.56	37.83	0.68**
	1000	35.58	36.20	25.97	56.03	40.08	0.43
	1500	49.28	30.67	43.15	60.53	61.55	0.74**
	2000	82.82	37.63	54.81	65.64	54.40	-0.18
	r	0.94**	0.82**	0.96**	0.95**	0.87**	
15	0	23.31	19.43	40.90	40.08	24.54	0.35
	5	42.94	20.04	41.31	43.35	28.63	-0.02
	500	43.56	58.49	54.81	56.03	34.76	-0.40
	1000	89.37	65.03	89.16	59.92	48.88	-0.74**
	1500	130.88	83.03	132.92	61.55	49.28	-0.74**
	2000	169.12	91.21	177.51	63.39	63.60	-0.65**
	r	0.98**	0.96**	0.98**	0.91**	0.98**	
$LSD_{p = 0.01}$		*a* – 4.06; *b* – 2.35; *c* – 3.71; *a* · *b* – 5.74; *a* · *c* – 9.08; *b* · *c* – 5.24; *a* · *b* · *c* – 12.81					

Explanation disclose under the Table 1

Ammonifying were stimulated (Table 4) by high concentrations of zinc insoil, irrespective of whether cellulose had been added or not. In the initial period of the experiment (day 15), this was observable for the highest doses (1500 mg and 2000 mg $Zn^{2+} \cdot kg^{-1}$ of soil), and on day 90 for the other contaminating doses (500 mg and 1000 mg $Zn^{2+} \cdot kg^{-1}$ of soil); however, the effect of 500 mg of $Zn^{2+} \cdot kg^{-1}$ of soil again considerably weakened on day 120.

Zinc-stimulated multiplication of nitrogen immobilising bacteria (Table 5) persisted throughout the whole period of the experiment (120 days). The effect was observable with various intensity on various days. The increase of the microorganism count was the highest, particularly in the soil with cellulose, on day 15 and was the lowest on day 30 and 60. The lowest number of nitrogen immobilising bacteria were isolated from the soil on day 30, and the highest was on day 120. On the latter day, the count increased several-fold and zinc in each of the applied doses raised it, although the increase caused by the metal was not as large as on day 15.

Cellulolytic bacteria have proved to be nearly insensitive to soil contamination with zinc (Table 6). Initially (day 15), the highest doses (1000 mg – 2000 mg $Zn^{2+} \cdot kg^{-1}$ of soil) increased their count, but later the multiplication stimulating effect decreased, particularly in the soil without cellulose, and disappeared completely on day 90.

Similar to cellulolytic bacteria, actinomyces (Table 7) were relatively resistant to zinc action in the soil without the addition of cellulose. On day 15 and 30, their count increased only in the soil with the highest doses of zinc (2000 mg $Zn^{2+} \cdot kg^{-1}$), while on days 90 and 120 the increase was caused by 1500 mg and 2000 mg $Zn^{2+} \cdot kg^{-1}$. A higher microorganism count was observed in the soil with cellulose for 90 days when the doses of zinc were 500 to 2000 mg $\cdot kg^{-1}$; on day 120 the effect was only observed for the two highest (1500 mg and 2000 mg $Zn^{2+} \cdot kg^{-1}$).

As with the other microorganisms, the fungi count (Table 8) fluctuated during the experiment and depended on the level of contamination with zinc and the cellulose content. On day 15, all doses of zinc in the soil with cellulose had a stimulating effect on fungi multiplication, while in the soil without the carbohydrate the effect was only observed for the highest doses (1000 mg – 2000 mg $Zn^{2+} \cdot kg^{-1}$). No significant effect of zinc on fungi was observed on day 30 in the soil without cellulose. On subsequent days of observation, the reaction of fungi to zinc contamination returned and was the highest on day 120. The stimulating effect of zinc on fungi in the soil with cellulose decreased as the experiment progressed.

Leaving aside the clearly negative effect of zinc on *Azotobacter* spp. bacteria and assuming that each sudden change in the bacteria count affected by any

factor is proof of upsetting the microbiological balance in the soil, ED_{50} coefficients were calculated which refer to the dose of zinc decreasing the microorganism count by 50%. With this assumption, and taking day 120 as the reference point, the conclusion was drawn that the greatest disturbance (except in *Azotobacter* spp.) was caused by zinc in the population of fungi (ED_{50} = 240 mg), followed by oligotrophic bacteria (ED_{50} = 584 mg), ammonifying (ED_{50} = 803 mg), copiotrophic bacteria (ED_{50} = 1016 mg) and nitrogen immobilising bacteria (ED_{50} = 1620 mg); the count of cellulolytic bacteria (ED_{50} > 2000 mg) and actinomyces (ED_{50} > 2000 mg) was affected to the least extent. The addition of cellulose to the soil changed the ED_{50} values for particular groups of microorganisms, but they were still the highest for actinomyces, nitrogen immobilising and cellulolytic bacteria.

According to numerous authors (KUCHARSKI 1994, NIKLIŃSKA et al. 2006, NOWAK et al. 2004a, b, WYSZKOWSKA, KUCHARSKI 2003), heavy metals decrease the microorganism count and inhibit their activity. Some studies (DAHM et al. 1997, HEMIDA et al. 1997, KHAN, SCULLITON 1999) also indicate that the problem may be more complex and ambiguous. A stimulating or inhibiting effect of heavy metals of microorganisms multiplication varies over time. It can be different in the initial period (soon after the soil is contaminated) than after several weeks of the experiment. The correlation between the time of exposure to heavy metals and their toxicity to microorganisms has been described by GILLER et al. (1998). The reaction of microorganisms to heavy metals may be linked to the granulometric composition and pH of soil. HEMIDA et al. (1997) showed that the doses of 200 mg and 2000 mg $\cdot$ kg^{-1} of zinc per 1 kg of soil inhibit the growth of fungi, bacteria and actinomyces in clayey soil, however, its effect in sandy soil is stimulating – especially in week 12 of the study. In addition, as in this experiment, the intensity of the zinc effect on particular groups of microorganisms varied over the 12 weeks of the experiment. KHAN and SCULLITON (1999) showed that copper and nickel have a negative effect on microorganisms, while zinc may stimulate their growth. Earlier research (ZABOROWSKA et al. 2006) has indicated that zinc, present in soil in doses of 1000 mg and 2000 mg $Zn^{+2} \cdot kg^{-1}$, is strongly toxic to *Azotobacter* spp., as was observed in this study, and its effect on other groups of microorganisms depended on the soil pH value. In the soil with neutral pH, it stimulated the multiplication of oligotrophic, copiotrophic and nitrogen immobilising bacteria, actinomyces and fungi, while in acidic soil it inhibited the growth of all microorganisms except for fungi. As in the current study, the count of cellulolytic bacteria remained relatively stable.

Conclusions

1. Contamination of soil with zinc in the dose of 500 to 2000 mg $Zn^{+2} \cdot kg^{-1}$ stimulated the multiplication of oligotrophic, copiotrophic, cellulolytic and nitrogen immobilising bacteria as well as actinomyces and fungi; it inhibited the growth of *Azotobacter* spp.

2. Although fluctuating over time, the effect of zinc on microorganisms was persistent. On day 120, the stimulation of multiplication (increasing the microorganism count by 50%) was observed for the following doses of Zn^{2+} ($mg \cdot kg^{-1}$ of soil): 240 – fungi, 584 – oligotrophic bacteria, 803 – ammonifying, 1016 – copiotrophic bacteria and 1620 – nitrogen immobilising bacteria. The effect of zinc on cellulolytic bacteria and actinomyces, particularly in the soil without cellulose, was very low on day 120 of the experiment.

3. Added to the soil (15 $g \cdot kg^{-1}$ of soil), cellulose stimulated microorganism multiplication in zinc-contaminated soil, but had no effect on the soil without zinc.

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References

Angelova V., Ivanov K. 2000. *Distribution and forms of lead, copper, zinc, cadmium and magnese in industrially polluted soil*. Zesz. Probl. Post. Nauk Rol., 472: 53-59.

Bääth E. 1989. *Effects of heavy metals in soil on microbial processes and populations* (*a review*). Water, Air Soil Pollut., 47: 335-379.

Barabasz W., Galus A., Opalińska-Piskorz J., Sepoił J., Tomasik P. 1997. *Wpływ jonów metali na wzrost oraz akumulacje Cd, Ni i Li w biomasie grzybni Aspergillus flavus LINK*. Zesz. Probl. Post. Nauk Rol., 448a: 15-20.

Cabrero A., Fernandez S., Mirada F, Garcia J. 1997. *Effects of copper and zinc on the activated sludge bacteria growth kinetics*. Wat. Res., 32(5): 1355-1362.

Dahm H., Li CH.Y., Januszek K. 1997. *Development of microorganisms and oxidation of some organic compounds in soil polluted with heavy metals*. Pol. J. Soil Sci., 30(2): 55 -63.

Fenglerowa W. 1965. *Simple method for counting Azotobacter in soil samples*. Acta Microb. Pol., 14(2): 203-206.

Giller K.E., Witter E., McGrath S.P. 1998. *Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review*. Soil Biol. Biochem., 30(10/11): 1389-1414.

Hemida S.K., Omar S.A. Abdel-Mallek A.Y. 1997. *Microbial populations and enzyme activity in soil treated with heavy metals*. Water, Air, Soil Pollut., 95: 13-22.

Khan M., Sculliton J. 1999. *Microbial activity in grassland soil amended with sewage sludge containing varying rates and combinations of Cu, Ni and Zn*. Biol. Fertil. Soils, 30: 202-209.

Kucharski J. 1994. *Wpływ cynku na aktywność Rhizobium leguminosarum* (biov. *viciae*) *oraz innych grup drobnoustrojów glebowych*. Acta Acad. Agricult. Tech. Olst. Agricult., 58: 69-76.

Martin J. 1950. *Use of acid rose bengal and streptomycin in the plate method for estimating soil fungi*. Soil Sci., 69: 215-233.

Niklińska M., Chodak M., Laskowski R. 2006. *Pollution-induced community tolerance of microorganisms from forest soil organic layers polluted with Zn or Cu*. Appl. Soil Ecol., 32: 265-272.

NOWAK A., BŁASZAK M., SZOPA E. 2004a. *Wpływ jonów rtęci na amylolityczne, proteolityczne i lipolityczne mikroorganizmy glebowe*. Acta Agr. Silv., 42: 327-334.

NOWAK A., SZOPA E., BŁASZAK M. 2004b. *Wpływ metali ciężkich (Cd, Cu, Pb, Hg) na ilość biomasy żywych mikroorganizmów w glebie*. Acta Agr. Silv., 42: 335-339.

ONTA H., HATTORI T. 1983. *Oligotrophic bacteria on organic debris and plant roots in paddy field*. Soil Biol. Biochem., 1: 1-8.

PARKINSON D., GRAYF R.G., WILLIAMS S.T. 1971. *Methods for studying the ecology of soil microorganisms*. Blackweel Sci. Publ. Oxford and Einburg, IBP Handbook, 19: 116.

ROST U., JOERGENSEN R.G., CHANDER K. 2001. *Effects of Zn enriched sewage sludge on microbial activities and biomass in soil*. Soil Biol. Biochem., 33: 633-638.

SŁABA M., DŁUGOŃSKI J. 2002. *Mikrobiologiczne usuwanie i odzyskiwanie metali ciężkich*. Post. Mikrobiol., 41(2): 167-183.

Statsoft, Inc. 2004. *Statistica* (*data analysis software system*), *version 6.0*. www.statsoft.com.

STOHS S.J., BAGCHI D. 1995. *Oxidative mechanisms in the toxicity of metal ions*. Free Radical Biol. Med., 182: 321-336.

VIG K., MEGHARAJ M., SETHUNATHAN N., NAIDU R. 2003. *Bioavailability and toxity of cadmium to microorganisms and their activities in soil: a review*. Adv. Environ. Res., 8: 121-135.

WYSZKOWSKA J. 2002. *Biologiczne właściwości gleby zanieczyszczonej chromem sześciowartościowym*. Wyd. UWM, Rozpr. i Monogr., 65: 1-134.

WYSZKOWSKA J., KUCHARSKI J. 2003. *Liczebność drobnoustrojów w glebie zanieczyszczonej metalami ciężkimi*. Zesz. Probl. Post. Nauk Rol., 492: 427-433.

YEATES G.W., ORCHARD V.A., SPEIR T.W., HUNT J.L. HERMANS M.C.C. 1994. *Impact of pasture contamination by copper, chromium, arsenic timber preservative on soil biological activity*. Biol. Fert. Soils, 18(3): 200-208.

ZABOROWSKA M., WYSZKOWSKA J., KUCHARSKI J. 2006. *Microbial activity in zinc contaminated soil of different pH*. Pol. J. Environ. Stud., 15(2A): 569-574.

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HEAVY METALS-INDUCED HYDROGEN PEROXIDE PRODUCTION IN TOBACCO CELLS

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K e y w o r d s: heavy metals, oxidative burst, stress, tobacco cell suspension culture.

Abbreviations: FW – fresh weight; $O_2^{\cdot-}$ – superoxide radical.

A b s t r a c t

In this work the reaction of *Nicotiana tabacum* cv. Bright Yellow 2 cells on the presence of 500 μM zinc, 500 μM cadmium, 10 μM copper and 5 μM iron was studied. It was confirmed that all tested heavy metals induced in tobacco cell suspension culture early stress response manifested by increased production of hydrogen peroxide. The highest level of hydrogen peroxide was marked in samples containing cadmium ions. This metal was also the most toxic for tested plant material. Less toxic were ions of copper and zinc. Iron ions gave the weakest stress response (probably because of the plant cell peroxidase inhibition).

PRODUKCJA NADTLENKU WODORU W KOMÓRKACH TYTONIU POD WPŁYWEM METALI CIĘŻKICH

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S ł o w a k l u c z o w e: metale ciężkie, stres, wybuch oksydacyjny, zawiesina komórek tytoniu.
Skróty: FW – świeża masa; $O_2^{\cdot-}$ – anionorodnik ponadtlenkowy.

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A b s t r a k t

W pracy badano reakcje komórek *Nicotiana tabacum* cv. Bright Yellow 2 na obecność 500 μM cynku, 500 μM kadmu, 10 μM miedzi i 5 μM żelaza. Stwierdzono, że wszystkie testowane metale ciężkie indukowały w zawiesinie tytoniu wczesną odpowiedź stresową, manifestującą się zwiększoną produkcją H_2O_2. Najwyższy poziom nadtlenku wodoru oznaczono w próbach zawierających jony kadmu. Metal ten był również najbardziej toksyczny dla materiału badawczego. Mniejszą toksyczność przejawiały jony miedzi i cynku, najsłabszą – jony żelaza. Było to, najprawdopodobniej, efektem hamującego oddziaływania jonów żelaza na aktywność peroksydaz ścian komórkowych.

Introduction

"Heavy metals" is a common name of the group of metals with density higher then 6 g cm^{-1} and atomic number higher then 20. Some of them (e.g. iron, copper, zinc) in low concentrations are essential for normal growth to most living organisms. However, in sufficient high concentrations they have toxic effects (ALI et al. 1999, BROWN et al. 1999). Cadmium belongs to a group of unnecessary elements, without important biochemical functions. This metal causes the toxic effects after exceeding the tolerance verge of the organism, although in low concentrations it can stimulate plant cells metabolism (SOBKOWIAK et al. 2004).

It is common knowledge that the earliest plants response on the stress causing factors is oxidative burst, what means temporary and extracellular increased production of reactive oxygen species. This process is well-studied only for biotic stressors (WOJTASZEK 1997). In the case of heavy metals it is known that their overabundance can lead to oxidative stress in plants (BRIAT, LEBRUN 1999, CUYPERS et al. 1999, SHÜTZENDÜBEL, POLLE 2002). However, although it has been intensively studied during last years, the ability of these elements to induce oxidative burst is very little known (e.g. PIQUERAS et al. 1999, OLMOS et al. 2003, RAEYMAEKERS et al. 2003).

The main interest of the authors was to examine if heavy metals such as iron, copper and zinc (micronutrients) and cadmium (unnecessary element) are able to induce an oxidative burst in tobacco cell suspension culture.

Materials and Methods

Plant Material

The tobacco (*Nicotiana tabocum* cv. Bright Yellow 2) cell suspension culture described by NAGATA et al. (1992) was used as the plant material. Cells were grown in MS medium (MURASHIGE, SKOOG 1962), supplemented

with 0.2 g of KH_2PO_4, 30 g of sucrose, 0.2 mg of 2.4-dichlorophenoxyacetic acid (2.4-D), 1 mg of thiamine-HCl and 100 mg of myo-inositol per liter. Every seventh day 2 ml of cells in stationary-growth phase was transferred into 50 ml of fresh MS medium. The cells were grown in 300 ml Erlenmeyer flasks placed on a rotary shaker, at 130 rpm in the dark at 27°C. For the H_2O_2 measurements 5-day old cells after transfer were used. The cells were collected by centrifugation at 3000 · g for 90 s. The pellet of cells was washed once with fresh MS medium, repelleted by centrifugation and resuspended in MES buffer (5 mM; pH 6.7). The final cell concentration in the reaction mixtures was 20 mg of fresh weight per ml of reaction mixture, which at time 0 the proper values of heavy metals water solutions were adds to. Experiments were done in a plastic spectrophotometer cuvettes which were incubated by rotating head-over-tail at 45 rpm. Every 1 hour the cuvettes were centrifuged at 1100 · g for 2 min to pellet the cells and the absorbance at 510 nm was measured in the supernatant medium.

Detection Of Hydrogen Peroxide Production

The concentration of hydrogen peroxide in samples was marked with DCHBS/AAP method, using the reaction of oxidative connection of 1 mM of 3.5-dichloro-2-hydroxybenzenesulfonic acid and 0.1 mM of 4-aminoantipyrine (VAN GESTELEN et al. 1998). The hydrogen peroxide was estimated by a calibration curve H_2O_2 (0-20 μM) and commercial horseradish peroxidase (1 U ml^{-1}, EC 1.11.1.7) in 5 mM MES buffer pH 6.7.

Measurement Of Cells Mortality

In experiments the technique of coloring dead cells by Evans blue modified by REICHHELD et al. (1999) was used. After particular time of stirring, Evans blue solution at a final concentration of 0.05% in culture medium was added to tested mixtures and incubated in room temperature for fifteen minutes. Next the samples were centrifuged at 9900 · g for five minutes, and the pellet of cells was four times washed with fresh culture medium. Then the pellet of cells was incubated in 1% solution of sodium dodecyl sulfate for thirty minutes in worm (50°C) water bath. After that, the samples were centrifuged at 9900 · g for five minutes and the absorbance at 600 nm was measured in the supernatant medium.

Chemical reagents used in the studies were bought from following companies: POCh, Polska; Sigma-Aldrich, Germany; Merck, Germany; Bio-Rad, Canada. All chemicals were analytical grade.

Statistical Analysis

All experiments were made at least in 4 series with 3 replications in each series. For each series the average and standard error were calculated (± SE). Results were analyzed using Student's test at $P < 0.05$ and Lavene's test and Brown and Forsyth's test for analysis of variance homogeneity. The Microsoft Excel 2003 and Statistica 6.0 computer programs were used.

Results and Discussion

Cadmium, copper and zinc count among group of elements with very high level of danger for biologic environment (BROWN et al. 1999). It is also known that they can disturb normal growth and development of plants (GWÓŹDŹ et al. 1997, ALI et al. 1999, LOMBARDI, SEBASTIANI 2005). One of the mechanisms of disadvantageous impact of metals on plant cell is creating excessive quantities of reactive oxygen species and free radicals (BRIAT, LEBRUN 1999, CHEN et al. 2000, WANG et al. 2004), also through oxidative burst way, which has not been studied properly yet. PIQUERAS et al. (1999) and OLMOS et al. (2003) proved that treating the suspension of tobacco cells with 5 mM cadmium caused oxidative burst. RAEYMAEKERS et al. (2003) observed this phenomenon in experiments with 10 μM copper. The most common are the reports about oxidative stress caused by heavy metals in whole plants.

Basing on literature, for own studies were chosen concentrations of copper, iron and zinc ions ranging between 0 mM and 3 mM (FANG, KAO 2000, RAEYMAEKERS et al. 2003). In the case of cadmium, the scope was widened up to 5 mM (PIQUERAS et al. 1999). The main experiments were preceded by pilot experiments describing the level of interactions between heavy metals and other ingredients of reactive mixture without cells (so called illusory hydrogen peroxide production). The presence of copper ions in concentration above 50 μM resulted in illusory hydrogen peroxide production (data not presented), thus analyzed copper concentration was limited to 50 μM. The results of own experiments show specific, higher than control level, and depending on used concentration of stress factors accumulation of hydrogen peroxide in tobacco cells culture (Figure 1). On presented graphs it is shown that concentration of metals below 20 μM Cu^{2+} and 1000 μM Cd^{2+} and Zn^{2+} resulted in rapid growth of H_2O_2 production, but above these values the linear dependency appeared. In samples with ions of iron there were negative correlation among concentration of metal and quantity of hydrogen peroxide detected in reactive mixture. According to above presented reasons, in further studies ions of zinc and cadmium were used in concentration 500 μM, copper ions – 10 μM and iron

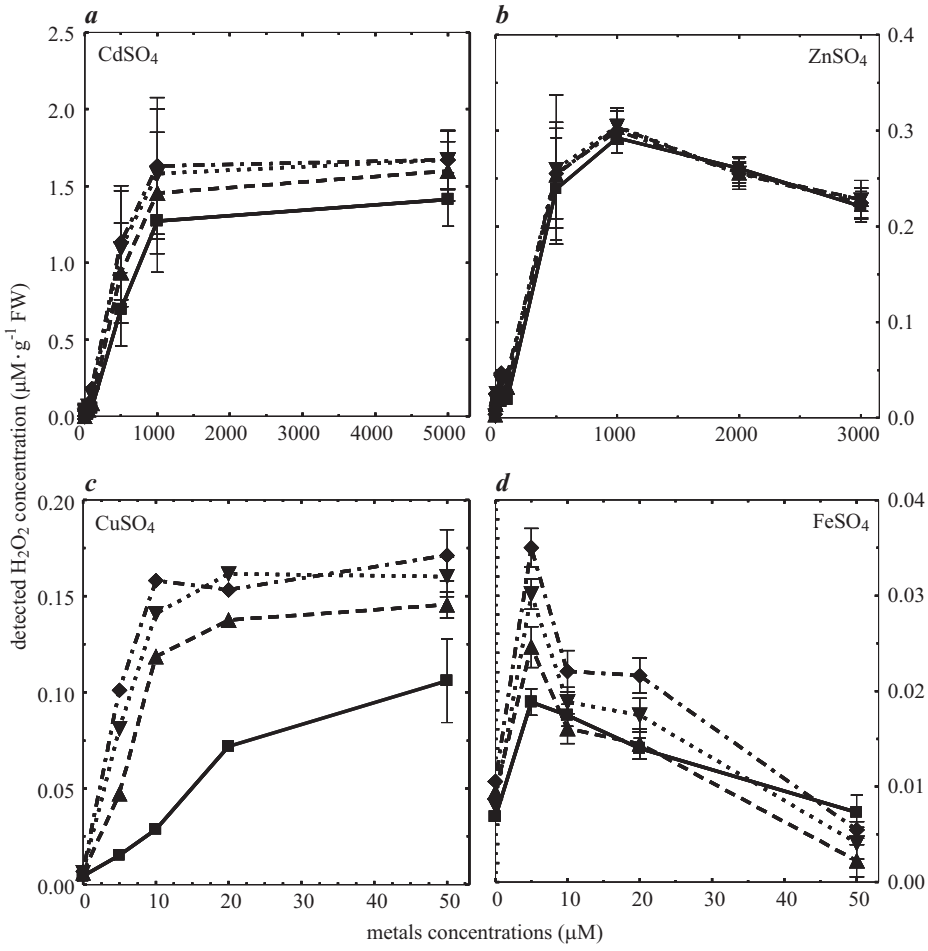

Fig. 1. The effect of different (*a*) $CdSO_4$, (*b*) $ZnSO_4$, (*c*) $CuSO_4$, (*d*) $FeSO_4$ concentrations on H_2O_2 production. The measurements were made after first (■), second (▲), third (▼) and fourth (◆) incubation's hours. Values are the means with standard errors

ions – 5 μM. From Table 1 it results that the strongest hydrogen peroxide production was generated by cadmium ions (1.138 μM H_2O_2 g^{-1} FW cells in 210 minute of incubation). Zinc ions induced about five times smaller production of hydrogen peroxide (0.262 μM H_2O_2 g^{-1} FW cells in 210 minute of incubation) than cadmium ions. It can be assumed that cadmium ions were more stressful for cells than zinc ions. Comparison of maximum H_2O_2 concentrations gained in samples containing 500 μM Zn^{2+} and 10 μM Cu^{2+} (0.160 μM H_2O_2 g^{-1} FW cells in fourth hour of incubation) showed that the difference between them was only twofold. It testifies that cells are more sensitive to action of copper

ions on them than zinc ions. The lowest H_2O_2 concentrations (0.016 μM H_2O_2 g^{-1} FW cells in fourth hour of incubation) were obtained in experiments with iron ions, what was probably the result of inhibition of non-specific peroxidase activity by iron ions (APOSTOL et al. 1989), and negative on the impact effectiveness of chosen method of oxidative burst measurement. Observed production of hydrogen peroxide was comparable with the level of reaction caused by various stressors in plant cell cultures (WOJTASZEK 1997, VAN GESTELEN et al. 1998, RAEYMAEKERS et al. 2003). It can testify that reactions on biotic and abiotic stresses, including heavy metals, can engage similar (or analogical) paths of signal transduction, enzymes producing reactive forms of oxygen and mechanisms defending from oxidative damages (MITHÖFER et al. 2004).

Table 1

Comparison of heavy metals-inducted hydrogen peroxide production in tobacco cell suspension culture. Data are the means with standard errors

Stressor	Detected H_2O_2 concentration (μM g^{-1} FW)				
	incubation time (h)				
	0	1	2	3	4
Control (H_2O)	0.000±0.005	0.003±0.000	0.004±0.000	0.005±0.000	0.007±0.000
500 μM Cd^{2+}	0.000±0.000	0.697±0.239	0.935±0.326	1.093±0.377	1.132±0.371
500 μM Zn^{2+}	0.004±0.001	0.239±0.053	0.254±0.055	0.260±0.078	0.255±0.047
10 μM Cu^{2+}	0.040±0.014	0.029±0.001	0.118±0.002	0.141±0.003	0.158±0.003
5 μM Fe^{2+}	0.005±0.002	0.010±0.005	0.014±0.006	0.019±0.007	0.022±0.001

The presence of copper, iron and zinc ions did not influence negatively on the cells vitality (Table 2). Significant toxicity showed cadmium ions, and mortality of cells on the level of 20% after 2 hours and 50% after 4 hours of incubation was observed. Stress respond (manifested by hydrogen peroxide production) induced by copper, iron or zinc ions was not related to tobacco cells mortality increase. However, cadmium had lethal impact on plant material. Obtained results present that copper and iron were able not only to direct production of reactive forms of oxygen through the Fenton or the Haber-Weiss reaction (URBAŃSKI, BERĘSEWICZ 2000), but also through oxidative burst. This fact was also confirmed for copper by RAEYMAEKERS et al. (2003). Cadmium is probably not able to direct production of reactive oxygen species (SANITA DI TOPPI, GABBRIELLI 1999). It is assumed that toxic influence of this compound on cells membranes can, similar to iron, cause the activation of plasma membrane NADPH-$O_2^{\cdot-}$ oxidase responsible for oxidative burst (YAMAMOTO et al. 1997, PIQUERAS et al. 1999). KAWANO et al. (2002) used ions of zinc as "defenders" slowing down stress response of the cells. On the other hand, many scientists pointed that zinc disturbs processes of normal growth and

development of the plants, including oxidative stress appearance (FANG, KAO 2000, CUYPERS et al. 2001). It can be assumed that surplus of zinc is toxic for plants and leads to early stress reaction.

Table 2

The morality of TBY-2 cells exposed to heavy metals. Data are the means with standard errors

The kind and concentration of metal ion	Death rate of tobacco cells (%)		
	incubation time (h)		
	0	2	4
Control (H_2O)	2.00 ± 0.19	2.00 ± 0.41	3.00 ± 0.52
500 μM Cd^{2+}	3.00 ± 0.49	19.00 ± 0.95	49.00 ± 1.43
500 μM Zn^{2+}	4.00 ± 0.48	4.00 ± 0.48	6.00 ± 0.51
10 μM Cu^{2+}	3.00 ± 0.15	5.00 ± 0.36	6.00 ± 0.08
5 μM Fe^{2+}	3.00 ± 0.24	4.00 ± 0.29	6.00 ± 0.30

From presented article result also further aims to reach. In our further work we will make efforts to answer questions concerning kinetics and characteristics of oxidative burst induced by heavy metals. It will be also necessary to study the impact of this process on cells system of antioxidative defense.

Conclusions

On the base of outcomes it can be stated that the presence of heavy metals induced early stress reaction in cell suspension of *Nicotiana tabacum* cv. Bright Yellow 2. As the result, reactive forms of oxygen were produced – hydrogen peroxide and probably superoxide radical. The most toxic appeared cadmium ions and afterwards ions of copper, zinc and iron.

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References

ALI G., SRIVASTAVA P.S., IQBAL M. 1999. *Morphogenic and biochemical responses of Bacopa monniera cultures to zinc toxicity.* Plant Sci., 143: 187-193.

APOSTOL J., HINSTEIN P.F., LOW P.S. 1989. *Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. Role in defense and signal transduction*. Plant Physiol., 90: 109-116.

BRIAT J.F., LEBRUN M. 1999. *Plant response to metal toxicity*. Life Sci., 322: 43-54.

BROWN G.E., FOSTER A.L., OSTERGERN J.H. 1999. *Mineral surfaces and bioavailability to heavy metals: A molecular-scale perspective*. PNAS, 96: 3388-3395.

CHEN L.-M., LIN C.C., KAO C.H. 2000. *Copper toxicity in rice seedlings: Changes in antioxidative enzyme activities, H_2O_2 level, and cell wall peroxidase activity in roots.* Bot. Bull. Acad. Sin., 41: 99-103.

CUYPERS A., VANGRONSVELD J., CLIJSTERS H. 1999. *The chemical behavior of heavy metals plays a prominent role in the induction of oxidative stress*. Free Rad. Res., 31: 39-43.

CUYPERS A., VANGRONSVELD J., CLIJSTERS H. 2001. *The redox status of plant cells (AsA and GSH) is sensitive to zinc imposed oxidative stress in roots and primary leaves of Phaseolus vulgaris*. Plant Physiol. Biochem., 39: 657-664.

FANG W.C., KAO C.H. 2000. *Enhanced peroxidase activity in rice leaves in response to excess iron, copper and zinc*. Plant Sci., 158: 71-76.

GESTELEN VAN P., LADEGANCK P., WAYNANT I., CAUBERGES R.J., ASARD H. 1998. *The cantharidin-induced oxidative burst in tobacco BY-2 cell suspension cultures*. Protoplasma, 205: 83-92.

GWÓŹDŹ E.A., PRZYMUSIŃSKI R., RUCIŃSKA R., DECKERT J. 1997. *Plant cell responses to heavy metals: molecular and physiological aspects*. Acta Physiol. Plant., 19: 459-465.

KAWANO T., KAWANO N., MUTO S., LAPERYRIE F. 2002. *Retardation and inhibition of the cation-induced superoxide generation in BY-2 tobacco cell suspension culture by Zn^{2+} and Mn^{2+}*. Physiol. Plant., 114: 395-404.

LOMBARDI L., SEBASTIANI L. 2005. *Copper toxicity in Prunus cerasifera: growth and antioxidant enzymes responses of in vitro grown plants*. Plant Sci., 168: 797-802.

NAGATA T., NEMOTO Y., HASEZAWA S. 1992. *Tobacco BY-2 cell line as the "HeLa" cell in the cell biology of higher plants*. Int. Rev. Cytol., 132: 1-30.

MITHÖFER A., SCHULTZE B., BOLAND W. 2004. *Biotic and heavy metal stress response in plants: evidence for common signals*. FEBS Lett., 566: 1-5.

MURASHIGE T., SKOOG F. 1962. *A revised medium for rapid growth and bioassays with tobacco tissue culture*. Physiol. Plant., 15: 437-497.

OLMOS E., MARTINEZ-SOLANO J.R., PIQUERAS A., HELLIN E. 2003. *Early steps in the oxidative burst induced by cadmium in cultured tobacco cells (BY-2 line)*. J. Exp. Bot., 381: 291-301.

PIQUERAS A., OLMOS E., MARTINEZ-SOLANO J.R., HELLIN E. 1999. *Cd-induced oxidative burst in tobacco BY-2 cells: time course, subcellular location and antioxidant response*. Rev. Rad. Res., 31: 933-938.

RAEYMAEKERS T., POTTERS G., ASARD H., GUISEZ Y., HOREMANS N. 2003. *Copper-mediated oxidative burst in Nicotiana tabacum L. cv. Bright Yellow 2 cell suspension cultures*. Protoplasma, 221: 93-100.

REICHHELD J., VERMOUX T., LARDON F., VAN MONTAGU M., INZË D. 1999, *Specific checkpoints regulate plant cell cycle progression in response to oxidative stress*. Plant J., 17: 101-108.

SANITA DI TOPPI L., GABBRIELLI R. 1999. *Response to cadmium in higher plants*. Environ. Exp. Bot., 41: 105-130.

SHÜTZENDÜBEL A., POLLE A. 2002. *Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrihzation*. J. Exp. Bot., 53: 1351-1365.

SOBKOWIAK R., RYMER K., RUCIŃSKA R., DECKERT J., GWÓŹDŹ E.A. 2004. *Cdamium-induced changes in antioxidant enzymem in suspension culture cells of soybean*. Acta Biochim. Pol., 51: 219-222.

URBAŃSKI N.K., BERĘSEWICZ A. 2000. *Generation of $^{\cdot}OH$ initiated by interaction of Fe^{2+} and Cu^{+} with dioxygen; comparison with the Fenton chemistry*. Acta Biochim. Pol., 47: 951-962.

WANG Y., FANG J., STEPHEN L.S., RAO K., MURALI K. 2004. *Cadmium inhibits the electron transfer chain and induces reactive oxygen species*. Free Rad. Biol. Med., 36: 1434-1443.

WOJTASZEK P. 1997. *Oxidative burst: an early plant response to pathogen infection*. Biochem. J., 322: 681-692.

YAMAMOTO Y., HACHIYA A., MATSUMOTO H. 1997. *Oxidative damage to membranes by a combination of aluminum and iron in suspension-cultured tobacco cells*. Plant Cell Physiol., 38: 1333-1339.

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EFFECT OF QUANTITATIVELY RESTRICTED FEEDING ON FEED CONSUMPTION AND SLAUGHTER QUALITY OF YOUNG GEESE

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K e y w o r d s: geese, feed restriction, growth parameters, slaughter quality.

A b s t r a c t

The experiment was performed on Koluda geese (96 ♂ and 96 ♀) allocated to four feeding groups. The geese were fed starter diets (from 1 to 5 weeks of age) and grower/finisher diets (from 6 weeks of age), containing 20.16% and 19.14% of total protein, and 12.34 MJ and 12.10 MJ of metabolizable energy, respectively. The birds were raised to 12 weeks of age. In the 1st week geese of all four groups were fed *ad libitum*. In subsequent weeks the ration was restricted by 20% (in relation to the control group): from week 2 until the end of the rearing period (group IV) or until week 5 (group III) and from week 6 until week 12 (group II).

The best economic results were achieved when geese were fed *ad libitum* for the first six weeks, and a restricted ration from week 7 until the completion of the experiment (group 2). Birds of this group, compared to those of the control group, had similar body weights (5151 g and 5311 g, respectively), but were characterized by significantly lower feed consumption per kg of body weight gain (3.34 kg vs. 3.95 kg). Their slaughter quality was also better, due to a higher meat content (50.64% vs. 48.11%) and a lower proportion of undesirable fat with skin in the carcass (27.18% vs. 31.25%). Birds of group 3 (feed restriction between week 2 and week 6) and birds of group 1 had comparable body and carcass weights, but feed intake was significantly higher in the former. Feed consumption was at a comparable level in group 4 (feed restriction from week 2 until the end of the rearing period) and in group 1, but the carcasses in group 4 contained less skin with fat (29.77% vs. 31.25%) and more lean (51.79% vs. 48.11%). On the other hand, birds of group 4 had lower body weights and lower meat weight in the carcass, in comparison with the control group (4678 g vs. 5311 g and 1448 g vs. 1571 g, respectively).

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WPŁYW ILOŚCIOWO OGRANICZONEGO ŻYWIENIA NA ZUŻYCIE PASZY I WARTOŚĆ RZEŹNĄ MŁODYCH GĘSI

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S ł o w a k l u c z o w e: gęsi, restrykcje żywieniowe, parametry wzrostowe, wartość rzeźna.

A b s t r a k t

Gęsi kołudzkie (96 ♂ i 96 ♀), rozmieszczone w 4 grupach żywieniowych, żywiono mieszankami Starter (do 5. tyg.) i Grower / Finisher (od 6. tyg.), o zawartości odpowiednio 20,16 i 19,14% białka ogólnego oraz 12,34 i 12,10 MJ energii metabolicznej. Odchów prowadzono do wieku 12. tyg. W 1. tyg. ptaki ze wszystkich grup otrzymywały mieszankę do woli, a w następnych żywienie ograniczono ilościowo o 20% (w stosunku do ilości paszy pobranej przez ptaki z grupy kontrolnej): od 2. tyg. do końca odchowu (IV) lub tylko do 5. tyg. odchowu (III) oraz od 6. tyg. do końca odchowu (II).

Najlepsze wyniki osiągnięto stosując żywienie *ad libitum* do 6. tyg., a od 7. tyg. do końca odchowu – dawkę ograniczoną (grupa II). Ptaki z tej grupy, w porównaniu z ptakami z grupy kontrolnej, miały zbliżoną masę ciała (odpowiednio 5311 i 5151 g), ale statystycznie istotnie zużywały mniej paszy (3,95 i 3,34) kg na 1 kg przyrostu masy ciała. Wartość rzeźna tych ptaków była również lepsza – większy udział mięsa (48,11 i 50,64%), mniejszy zaś udział niepożądanego tłuszczu ze skórą w tuszce (31,25 i 27,18%). Ptaki z grupy III, w porównaniu z ptakami z grupy I, miały zbliżoną masą ciała i tuszki, ale zużywały istotnie więcej paszy. Gęsi z grupy IV, w porównaniu z ptakami z grupy kontrolnej, zużywały zbliżone ilości paszy. Wykazano u nich mniejszą zawartość skóry z tłuszczem (odpowiednio 31,25 i 29,77%), natomiast większy udział mięsa w tuszce (48,11 i 51,79%). Cechami ujemnymi były ich mniejsza masa ciała (odpowiednio 5311 i 4678 g) i związany z nią mniejszy uzysk mięsa z tuszki (1571 i 1448 g).

Introduction

Results of experiments on broiler chickens show that quantitative feed restriction, compared with *ad libitum* feeding, enables to decrease carcass fatness and reduce feed consumption levels (JANISZEWSKA et al. 1999, PLAVNIK, HURVITZ 1991). Studies on ducks have also brought positive results. SZEREMETA et al. (2002) found that daily ration restriction by 20% applied in the 2nd week of rearing contributed to lower feed intake and lower carcass fatness in male ducks. BOCHNO et at. (1989) observed a decrease in feed consumption per kg of body weight or edible part weight in groups of male and female ducks in response to 25% ration restriction initiated at 4 weeks of age. The carcasses of feed-restricted birds were lighter, but characterized by a more desirable lean-to-fat ratio. Similar results were obtained in an experiment on hybrid (Muscovy x Pekin) ducks (WILKIEWICZ-WAWRO 1994).

Results of studies on feed restriction in geese also seem promising. BOCHNO and BRZOZOWSKI (1992) reported that geese whose daily ration was restricted by 20% in relation to the control group had lower body weights, but their

carcasses contained less fat and showed a higher meat-to-fat ratio. The ration restricted by 30% during the 3rd and 4th week of rearing allowed to improve the slaughter quality traits of geese as well as to reduce feed intake (JANISZEWSKA et al. 2000). On the other hand, the skip-a-day feeding program, in which birds were given reduced amounts of feed every other day, had no significant influence on production results (JANISZEWSKA et al. 2002).

The aim of the present study was to determine the effect of quantitatively restricted feeding, applied from week 2 or week 7, on the growth rate and slaughter quality of White Italian geese.

Materials and Methods

The experiment was performed on White Italian geese (Koluda line, 96 ♂ and 96 ♀). Day-old goslings, were marked individually, weighed and randomly allocated to four groups (1-4). Each group consisted of two pens of males and two pens of females (12 birds per pen). The birds were raised to 12 weeks of age. They were fed starter diets (from 1 to 5 weeks of age) and grower/finisher diets (from 6 to 12 weeks of age), containing 20.16% and 19.14% of total protein, and 12.34 MJ and 12.10 MJ of metabolizable energy, respectively.

In the 1st week geese of all four groups were fed *ad libitum*. In subsequent weeks particular groups were fed as follows:

group 1 (control) – feeding *ad libitum* over the entire experimental period;

group 2 – feeding *ad libitum* during the first six weeks, between week 7 and 12 the ration was restricted by 20%, compared with group 1;

group 3 – feeding *ad libitum* during the 1st week, between week 2 and 6 the ration was restricted by 20%, compared with group 1, from week 7 until the end of the experimental period the average ration per bird was the same as in group 1;

group 4 – feeding *ad libitum* during the 1st week, from week 2 until the end of the experimental period the ration was restricted by 20%, compared with group 1.

During the experimental period, at one- or two-week intervals, the birds were weighed and their body weight gains and feed intake were determined on an individual and group basis, respectively. At 12 weeks of age 10 males and 10 females were selected randomly of each feeding group, fasted for about 12 hours and slaughtered. The birds were weighed, sacrificed, plucked and eviscerated. Next the weight of a warm carcass, giblets and abdominal fat was determined. The carcass dressing percentage was calculated as the weight of a carcass with giblets, expressed as a percentage of live body weight. The carcasses were chilled (for about 18 hours at 4°C) and divided into primary cuts

which were then dissected into meat, bones, skin with subcutaneous and intermuscular fat.

The statistical analysis of the collected numerical material included:

– feed consumption: per bird, per kg of body weight gain, per kg of carcass, per kg of dissected meat,
– statistical characteristics of the tested traits: arithmetic means ($\bar{x}$) and coefficients of variation (v),
– significance of differences between birds of particular feeding groups and between males and females with respect to feed consumption and slaughter quality traits: two-factorial analysis of variance, with two elements in subgroups (two pens) for feed consumption or with ten elements in subgroups for slaughter quality traits (individual treatment).

Results and Discussion

1. *Body weight gains*

In the control group (*ad libitum* feeding) mean daily weight gains recorded for both males and females increased from 31 g in the first week to 115 g at four weeks, to decrease to 27.5 g during the last two weeks (Figure 1). Birds of group 4 (feed restriction from week 2 until the end of the rearing period) were characterized by a slower growth rate than geese of the control group over the entire experiment. Daily gains in group 2, fed *ad libitum* to 6 weeks of age,

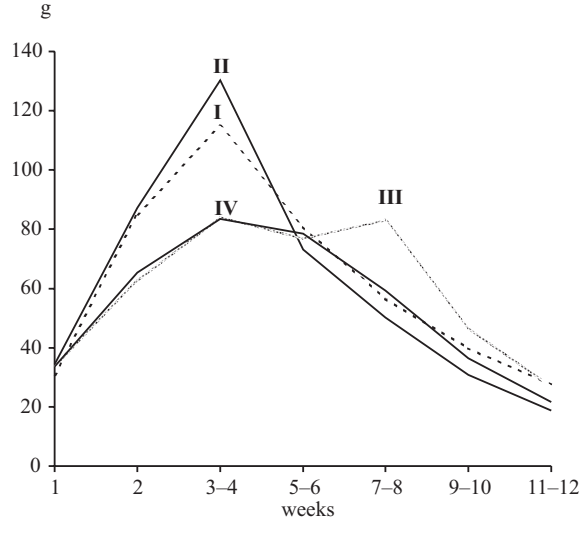

Fig. 1. Daily gains body weight ♂ ♀ (g)

were comparable to daily gains in the control group. Following feed restriction initiated at week 7 daily gains in group 2 and in group 4 were at a similar level. Between week 2 and week 6 daily gains in group 3, fed a restricted ration over this period, were comparable to daily gains observed in group 4. *Ad libitum* feeding, applied from 7 weeks of age in group 3, resulted in an increase in body weight gains (77 g from week 5 to week 6, 83 g from week 7 to week 8). In subsequent weeks daily gains in group 3 decreased, but still were slightly higher than in the other groups (Figure 1). The phenomenon of accelerated daily gains following feed restriction is referred to as compensatory growth. Compensatory growth has been observed in chickens and turkeys (PLAVNIK, HURVITZ 1991) as well as in ducks (SZEREMETA et al. 2002) and geese (BOCHNO, BRZOZOWSKI 1992, JANISZEWSKA et al. 2000).

Figure 2 shows changes in the body weights of birds during the experimental period. 20% feed restriction initiated at week 2 resulted in a decrease in daily gains in groups 3 and 4. The body weights of geese in these groups were lower, compared to groups 1 and 2, fed *ad libitum* over this period. This tendency was observed until week 6, when the body weights of geese were as follows: group 1 – 3663 g, group 2 – 3811 g, group 3 – 3040 g and 3075 g. The difference between groups 1 and 2 and groups 3 and 4 exceeded 600 g (Figure 2). At 7 weeks of age the average ration per bird in group 3 was the same as in group 1, which resulted in an increase in daily gains. As soon as at 8 weeks of age the body weights of geese in groups 3 and 1 (control) were comparable

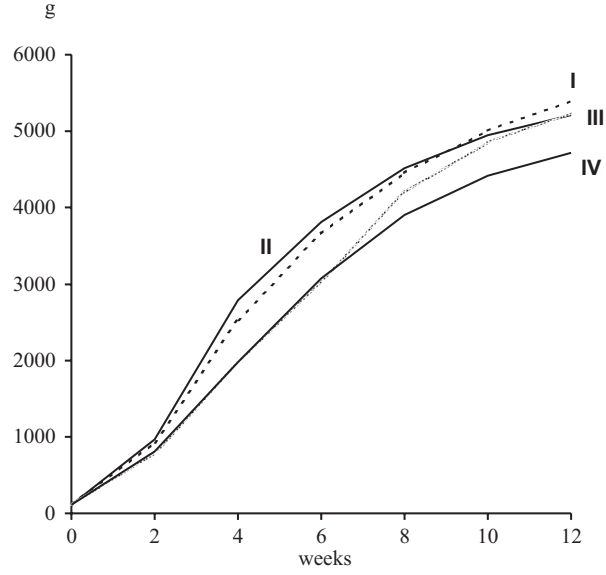

Fig. 2. Body weight ♂ ♀ (g)

(4201 g vs. 4454 g); at 12 weeks their body weights were 5231 g and 5396 g, respectively. Feed restriction applied from week 7 in group 2 caused a slight decrease in daily gains. At 12 weeks of age the body weights of geese in groups 2 and 1 were comparable (5208 g vs. 5396 g).

2. *Slaughter quality*

The mean body weights of birds selected randomly for slaughter in particular groups (Table 1) were similar to the mean body weight determined for all birds in a given group (Figure 2). As already mentioned, geese of group 4 had significantly lower body weights (4678 g) than birds of the other groups (> 5129 g), which resulted from 20% feed restriction applied in this group from week 2 until the end of the rearing period. This concerned also carcass weight and abdominal fat weight. However, geese of group 4, compared with the control group, had more desirable carcass tissue composition. The proportion

Table 1

Arithmetic means and coefficients of variation for the tested traits

Specification	Statistical measures	Group				Sex	
		I	II	III	IV	♂	♀
Live weight (g)	$\bar{x}$	5311.5^{A}	5151.0^{A}	5129.5^{A}	4678.0^{B}	5210.8**	4924.3
	v	8.31	8.76	7.63	7.76	8.81	8.95
Giblet weight (g)	$\bar{x}$	302.7^{A}	358.2^{A}	345.5^{A}	341.8^{B}	350.5**	323.7
	v	9.80	12.84	12.44	12.45	13.57	11.94
Abdominal fat weight (g)	$\bar{x}$	201.0^{A}	124.4^{B}	145.1^{B}	79.6^{C}	126.1	148.6**
	v	27.16	29.34	24.60	29.27	41.10	42.20
Carcass dressing percentage (%)	$\bar{x}$	68.82	68.74	68.56	68.77	68.69	68.76
	v	3.31	2.74	3.13	2.58	3.02	3.16
Cold carcass weight (g)	$\bar{x}$	3269.7^{Aa}	3095.7^{Ab}	3087.1^{A}	2794.9^{B}	3143.4**	2980.4
	v	318.16	278.92	274.04	210.82	305.04	313.67
Content in the carcass (g):							
meat	$\bar{x}$	1571.1^{A}	1566.3^{A}	1509.3^{AB}	1448.2^{B}	1584.2**	1463.2
	v	10.39	8.74	10.48	8.67	8.43	10.08
fat with skin	$\bar{x}$	1023.9^{Aa}	843.2^{B}	918.6^{ABb}	696.7^{C}	848.0	893.2
	v	17.54	15.59	12.78	11.53	20.37	20.11
bones	$\bar{x}$	490.6	494.9	476.3	471.6	510.9**	455.9
	v	12.88	11.73	11.28	12.20	10.40	10.81
Meat-to-fat ratio	$\bar{x}$	1.57^{A}	1.89^{B}	1.66^{A}	2.10^{C}	1.93**	1.69
	v	18.34	14.98	14.47	11.97	16.49	18.20
Percentage in the carcass:							
meat	$\bar{x}$	48.11^{A}	50.64^{BCa}	48.86^{ABb}	51.79^{C}	50.50**	49.20
	v	6.21	3.90	4.60	2.95	4.27	5.97
fat with skin	$\bar{x}$	31.25^{A}	27.18^{Ba}	29.77^{A}	24.95^{Bb}	26.79	29.79**
	v	12.81	10.96	10.18	9.94	13.35	12.62
bones	$\bar{x}$	15.00^{A}	16.00^{BCa}	15.43^{AB}	16.85^{Cb}	16.30**	15.34
	v	8.71	7.87	6.85	7.59	8.76	7.74

Means followed by different letters (feeding) or * (sex) differ significantly;
capital letters or ** – at $\alpha = 0.01$
small letters or * – at $\alpha = 0.05$

of meat, expressed as a percentage of carcass weight, was by 3.68% higher in group 4 (51.79 vs. 48.11), while the proportion of fat with skin was by as much as 6.30% lower. The meat-to-fat ratio was 2.10:1 in group 4 and 1.57:1 in group 1 (significant difference at $\alpha = 0.01$).

Birds of group 2, fed *ad libitum* during the first six weeks and a restricted ration from week 7 until the completion of the experiment, compared to those of the control group, had similar body weights, but were characterized by more desirable ratios between tissue components in the carcass, including a lower proportion of fat with skin (27.18% vs. 31.25%), a higher meat content (50.64% vs. 48.11%), a higher meat-to-fat ratio (1.89:1 vs. 1.54:1) and a lower fat content of breast muscles (2.09% vs. 2.66%).

The feed restriction program applied in the reversed order than in group 2, i.e. restricted feeding between week 2 and 6, followed by *ad libitum* feeding from week 7 to week 12, resulted in lower carcass weights in group 3. However, percentage carcass tissue composition was comparable to that in the control group (Table 1). It should be noted that feed restriction initiated not at two weeks of age (like in this study) but on day 1, and continued for six weeks, enabled to achieve slaughter quality comparable to the slaughter quality of birds fed a ration restricted by 20% over the entire experimental period (BOCHNO, BRZOZOWSKI 1992).

Females, compared to males, had lower body and carcass weights, lower weights of individual tissue components as well as a less desirable meat-to-fat ratio, which is in agreement with results of other experiments performed on geese (BOCHNO, BRZOZOWSKI 1992, JANISZEWSKA et al. 2000).

Feed restriction applied in this experiment had a slight effect on the chemical composition and water-holding capacity of meat. The concentrations of total protein and crude fat in breast muscles of geese in groups 2 and 3 were

Table 2

Chemical composition and water-holding capacity of breast muscles

Specification	Statistical measures	Feeding group				Sex	
		I	II	III	IV	♂	♀
Dry matter (%)	$\bar{x}$	26.10^{A}	24.72^{B}	25.42	24.92^{B}	25.43	25.15
	v	3.53	3.89	3.30	3.44	4.01	4.07
Total protein (%)	$\bar{x}$	20.93	20.42	19.70	20.19	20.62	20.00
	v	8.43	5.52	6.15	9.80	6.81	8.52
Crude fat (%)	$\bar{x}$	2.66^{Aa}	2.09^{ABb}	2.43^{ABb}	1.84^{C}	2.42*	2.09
	v	29.53	23.11	23.67	17.60	26.44	28.33
Crude ash (%)	$\bar{x}$	1.24	1.18	1.20	1.21	1.19	1.22
	v	4.39	7.66	3.53	4.09	6.85	2.83
Water-holding capacity (cm^2)	$\bar{x}$	5.77	5.93	5.45	6.60	5.68	6.19
	v	16.57	20.77	22.35	18.27	19.93	19.87

Explanations – see Table 1

comparable to their content recorded in the control group. The water-holding capacity of muscles was also at a similar level. Only the fat content of muscles in these two experimental groups was lower when compared to birds fed *ad libitum* (Table 2). 30% ration restriction over a short period during the raising of young geese had no considerable influence on the chemical composition of breast muscles, either (SOBINA et al. 1999). However, restricted feeding applied from week 2 until the completion of the experiment (group 4) caused a significant decrease in the percentages of dry matter and fat.

3. *Feed consumption*

Feed consumption per bird over the entire experimental period was the highest in the control group (22.42 kg) and the lowest in group 4 (18.16 kg), which is consistent with our methodological assumptions. In groups 2 and 3 the amounts of feed consumed were lower than in the control group and higher than in group 4, i.e. 19.73 kg and 20.65 kg, respectively (Table 3).

Table 3

Feed consumption

Specification	Feeding groups				Sex	
	I	II	III	IV	♂	♀
Feed consumption per bird (kg)	22.419^{A}	19.734^{Ba}	20.646^{Bb}	18.158^{C}	20.632*	19.847
Feed consumption (kg) per kg of body weight gain:	3.955^{ABa}	3.336^{C}	4.030^{Ab}	3.893^{B}	3.787	3.820
Utilization per kg of body weight gain:						
protein (g)	767.64^{A}	647.38^{B}	780.33^{Aa}	760.57^{Ab}	734.80	743.16
metabolizable energy (MJ)	47.122^{A}	40.021^{B}	47.741^{A}	46.639^{A}	45.147	45.614
Feed consumption (kg) per kg						
cold carcass	6.283^{Aa}	5.394^{B}	6.546^{Ab}	6.403^{A}	6.131	6.182
meat	13.090^{Aa}	10.663^{B}	13.224^{Aa}	12.367^{Ab}	12.073	12.599*

Explanations – see Table 1

There were significant differences in feed intake per kg of body weight gain during the experimental period. Birds fed *ad libitum* from week 1 to 6, and then fed a restricted ration (group 2), consumed the least feed per kg of body weight gain (3.34 kg). On the other hand, the highest feed intake per kg of body weight gain (4.03 kg/kg) was recorded in geese given a restricted ration between week 2 and 6, and then fed *ad libitum*. Similar tendencies were also observed in the utilization of total protein and metabolizable energy.

Feed consumption per kg of carcass weight (without giblets) was significantly lower in group 2 (5.39 kg) and significantly higher in group 3 (6.55 kg), in comparison with the control group (6.28 kg). Feed intake in groups 4 and

1 was comparable. Similar trends were observed in the case of feed consumption per kg of dissected meat.

Conclusions

1. The best economic results were achieved when geese were fed *ad libitum* for the first six weeks, and a restricted ration from week 7 until the completion of the experiment (group 2). Birds of this group, compared to those of the control group, had similar body weights (5151 g and 5311 g, respectively), but were characterized by significantly lower feed consumption per kg of body weight gain (3.34 kg vs. 3.95 kg), kg of carcass weight (5.39 kg vs. 6.28 kg) and kg of dissected meat (10.66 kg vs. 13.09 kg). Their slaughter quality was also better, due to a higher meat content (50.64% vs. 48.11%), a lower proportion of undesirable fat with skin in the carcass (27.18% vs. 31.25%), a higher meat-to-fat ratio (1.89:1 vs. 1.57:1) and a lower fat content of breast muscles (2.09% vs. 2.66%).

2. Birds of group 3 (feed restriction between week 2 and week 6) and birds of group 1 had comparable slaughter quality traits as well as body and carcass weights, but feed intake per kg of body weight gain and carcass weight gain was significantly higher in the former (4.03 kg vs. 3.34 kg and 6.55 kg vs. 5.39 kg, respectively).

3. Geese of group 4 (feed restriction from week 2 until the end of the rearing period) were characterized by better slaughter quality traits, compared with those of group 1: their carcasses contained less skin with fat, more lean and showed a more desirable ratio between meat and fat with skin. Feed consumption was at a comparable level in these groups when expressed per kg of body weight gain, while lower in group 4 when expressed per kg of dissected meat (12.37 kg vs. 13.09 kg). On the other hand, birds of group 4 had lower body weights and lower meat weight in the carcass, in comparison with the control group (4678 g vs. 5311 g and 1448 g vs. 1571 g, respectively).

4. The results of this experiment (*ad libitum* feeding from one to six weeks, followed by feed restriction from 7 weeks in group 2) should be verified under production conditions. In case they were confirmed, quantitatively restricted feeding could be recommended for intensively farmed geese, starting from 7 weeks of age.

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References

BOCHNO R., LEWCZUK A., WAWRO K. 1989. *Porównanie wyników odchowu i wartości rzeźnej kaczek żywionych do woli i dawką ograniczoną*. Acta Acad. Agricult. Tech. Olst., Zoot., 32: 195-204.

BOCHNO R., BRZOZOWSKI W. 1992. *Wpływ ilościowego ograniczenia dawki pokarmowej w różnych okresach wzrostu na zużycie paszy i wartość rzeźną gęsi białych włoskich*. Acta Acad. Agricult. Tech.. Olst., Zoot., 37: 131-141.

JANISZEWSKA M., BOCHNO R., LEWCZUK, A., MICHALIK D. 1999. *Effect of periodical feed restriction on the body weight, tissue composition of carcass and feed conversion in broiler chickens*. Natur. Sc., 2: 121-134

JANISZEWSKA M., BOCHNO R., LEWCZUK A., RYMKIEWICZ J. 2000. *Changes in the body weight and slaughter value of White Koluda geese fed ad libitum or subjected to feed restriction*. Pol. J. Natur. Sc., 4: 147-159.

JANISZEWSKA M., BOCHNO R., LEWCZUK A, RYMKIEWICZ J. 2002. *Masa ciała i wartość rzeźna młodych gęsi żywionych do woli lub ilościowo ograniczoną dawką paszy*. Biul. Nauk. UWM, 17: 245-252.

PLAVNIK J., HURVITZ S. 1991. *Response of broiler chickens and turkey poults to food restriction of varied severity during early life*. Brit. Poult. Sci., 32: 343-352.

SOBINA I., JANISZEWSKA M., KONDRATOWICZ J. 1999. *Effect of periodical feed restriction on some physicochemical and sensory parameters of meat quality in White Italian geese*. Pol. J. Natur. Sc., 5: 199-210.

SZEREMETA J., BOCHNO R., BRZOZOWSKI W. 2002. *Influence of restricted feeding on growth performance and slaughter value of young ducks*. Pol. J. Natur. Sc., 10 (1): 153-162.

WILKIEWICZ-WAWRO E. 1994. *Wyniki odchowu oraz wartość rzeźna kaczek mieszańców międzygatunkowych żywionych do woli lub dawką ograniczoną*. Acta Acad. Agricult. Tech. Olst. Zoot., 40, Suppl. B:3-37.

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SOME PLASMA BIOCHEMICAL PARAMETERS OF YOUNG SLAUGHTER TURKEYS RAISED UNDER VARIED ENVIRONMENTAL CONDITIONS

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K e y w o r d s: slaughter turkeys, housing system, age, biochemical parameters.

A b s t r a c t

The study was conducted in a period from May to November on young slaughter turkey-toms of the heavy (Big 6) and medium-heavy type (BUT 9), raised from 7 to 22 weeks of age indoor – in a brooder house (control group) or under a shelter with access to open-air runs (experimental group). The stocking density was 35 kg b.w. per m^2 of area in the house or under the shelter. The surface area of the runs was twofold larger than that of the shelter.

Blood for biochemical analysis was collected from turkey-toms aged 6, 10, 14, 18 and 22 weeks. The values of plasma biochemical parameters indicated the occurrence of periodical differences between the types of turkeys and management systems. At 22 weeks of age, turkeys raised under a shelter had higher plasma levels of glucose (by 64 mg dl^{-1}), total cholesterol (by 11 mg dl^{-1}) and uric acid (by 0.83 mg dl^{-1}), in comparison to birds raised indoor. At 14 and 18 weeks the plasma levels of total lipids were also higher in the experimental group, by 107 and 125 mg dl^{-1} respectively. The plasma activity of lysozyme and alanine aminotransferase was affected by the housing system and showed higher individual variation. Differences dependent on the type of turkeys were observed in some weeks only, and concerned the plasma levels of total protein and alkaline phosphatase activity at 10 weeks, HDL cholesterol and lysozyme activity at 6 weeks, total cholesterol at 6 and 18 weeks, alanine aminotransferase activity at 6 and 10 weeks.

The results of the present study can extend our knowledge with regard to some plasma biochemical parameters of two types of slaughter turkeys with no health problems, fed identical diets and raised under varied environmental conditions during five periods of their life. No significant changes were recorded in blood plasma biochemistry of turkey-toms kept under a shelter, as compared to those raised traditionally in a brooder house.

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WYBRANE PARAMETRY BIOCHEMICZNE OSOCZA KRWI MŁODYCH INDYKÓW RZEŹNYCH ODCHOWYWANYCH W ZRÓŻNICOWANYCH WARUNKACH ŚRODOWISKOWYCH

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S ł o w a k l u c z o w e: indory rzeźne, warunki utrzymania, wiek, wskaźniki biochemiczne.

A b s t r a k t

Badania przeprowadzono w miesiącach od maja do listopada na młodych indorach rzeźnych typu ciężkiego (Big 6) i średniociężkiego (BUT 9), odchowywanych od 7. do 22. tygodnia życia tradycyjnie w budynku zamkniętym (grupa kontrolna) lub w wiacie z dostępem do wybiegów (grupa doświadczalna). Ustalając obsadę, przyjęto 35 kg m.c. na 1 m^2 powierzchni budynku lub wiaty. Wybiegi były 2-krotnie większe od powierzchni wiaty.

Krew do badań wskaźników biochemicznych pobierano od indorów w: 6., 10., 14., 18. i 22. tygodniu życia.

Na podstawie wskaźników biochemicznych osocza krwi stwierdzono okresowe różnice tak między typami użytkowymi indorów, jak i warunkami ich utrzymania. Oceniając wpływ warunków utrzymania indorów na wskaźniki biochemiczne krwi 22-tygodniowych ptaków, wykazano wyższą (o 64 mg dl^{-1}) zawartość glukozy w osoczu krwi indyków odchowywanych w wiacie (289 mg dl^{-1}), wyższą zawartość cholesterolu całkowitego (o 11 mg dl^{-1}) i kwasu moczowego (o 0,83 mg dl^{-1}), a także w 14. i 18. tygodniu wyższy poziom lipidów całkowitych, odpowiednio o 107 i 125 mg dl^{-1}. W aktywności lizozymu i aminotransferazy alaninowej w osoczu krwi, w przypadku większej zmienności osobniczej, wykazano różnice w zależności od warunków utrzymania ptaków.

Różnice zależne od typu użytkowego odnotowano tylko w niektórych tygodniach życia indorów: w przypadku zawartości białka całkowitego i aktywności AP w 10. tyg., zawartości cholesterolu HDL i aktywności lizozymu w 6. tyg., zawartości cholesterolu całkowitego w 6. i 18. tyg., aktywności ALAT w 6. i 10. tygodniu.

Wyniki niniejszych badań poszerzają wiedzę naukową w zakresie niektórych wskaźników biochemicznych osocza krwi dwóch typów użytkowych indorów rzeźnych w 5 okresach ich życia, jednakowo żywionych, odchowywanych bez zakłóceń zdrowotnych, w różnych warunkach utrzymania. Wychów w wiacie nie powodował wyraźnych zmian w kształtowaniu się wartości parametrów osocza krwi indorów, w porównaniu z ptakami utrzymywanymi tradycyjnie w wychowalni.

Introduction

Prior to our accession to the European Union, the production of young slaughter turkeys used to develop dynamically in Poland due to, among others, the fact that the carcass dressing percentage is higher in turkeys than in other farm animals, ranging from 79 to 85%, including about 30% of breast

muscles and about 22% of leg muscles (MAYER et al. 1997, PUCHAJDA et al. 1997). Since the moment Poland became a EU member state, poultry producers have been obliged to implement the EU directives on animal health and welfare as well as on the production of high-quality branded foodstuffs, which requires the modification of the existing systems of turkey management, husbandry and housing. One of alternative turkey production technologies, discussed in this paper, involves raising older birds under a shelter with free access to outdoor runs.

It is a well-known fact that intensive turkey farming, aimed at increasing the body weight of birds, is associated with a higher incidence of serious health problems that have to solved by producers and veterinary service. Plasma biochemical parameters may help to diagnose the physiological condition and pathological state of turkeys (*Poultry diseases* 2005). However, the main obstacle to the broad-scale practical application of the above indices is a lack of sufficient literature and reference data (KONCICKI, KRASNODĘBSKA-DEPTA 2005). Blood biochemical indices of birds are affected by a variety of factors, particularly age, sex, species, breed, nutrition, physiological condition and management technology (KONCICKI, KRASNODĘBSKA-DEPTA 2005). The currently available data on blood biochemical indices of turkeys are generally limited to females only (KONCICKI, KRAZNODĘBSKA-DEPTA 2005), with no information on their breed, type and environmental conditions. In another publication (*Poultry diseases* 2005) the values of blood biochemical indices are given for turkeys of unknown sex and age.

The aim of the present study was to extend our knowledge with regard to some plasma biochemical parameters determined in two types of slaughter turkey-toms at 6, 10, 14, 18 and 22 weeks of age, fed identical diets and raised under varied environmental conditions.

Materials and Methods

The study was conducted in a period from May to November on young slaughter turkey-toms of the heavy (Big 6) and medium-heavy type (BUT 9), raised on an experimental farm of the University of Warmia and Mazury in Olsztyn. 136 Big 6 and 149 BUT 9 turkey-toms stayed in a brooder house for the first 6 weeks (8 replications), under controlled conditions, in accordance with the recommendations of the Turkey Testing Station (FARUGA 1997). At 7 weeks of age the birds were divided into two groups and raised under varied environmental conditions, as shown in the diagram below:

Age (weeks)	Type – Housing system			
	Big 6		But 9	
1-6	brooder house			
	136 birds (8 replications)		149 birds (8 replications)	
7-22	brooder house	shelter	brooder house	shelter
	60 birds (4 replications)	69 birds (3 replications)	67 birds (4 replications)	78 birds (3 replications)

The stocking density (number of birds per pen) was 35 kg b.w. per m2 of floor area. The ratio between the surface area of the runs and the shelter was 2:1. The turkeys were fed complete standard diets whose nutritive value was modified every three weeks. The health status of the flock was monitored on a regular basis by a veterinary doctor, a specialist on bird diseases.

Blood for biochemical analysis was collected in heparin from the wing veins of the same 10 turkey-toms of each group (2-3 birds of each replication), aged 6, 10, 14, 18 and 22 weeks. Lysozyme activity was determined in blood samples taken from another 10 birds. The turkeys were tagged for easier identification. The plasma levels of total protein, glucose, total cholesterol, HDL cholesterol, total lipids and activity of alkaline phosphatase (AP) were determined by standard methods using Biochemtest diagnostic kits. The plasma activity of alanine aminotransferase (ALAT) and the plasma concentration of uric acid were determined using Alpha Diagnostics kits. Absorbance was measured with a Beckman DU-640 spectrophotometer. Quantitative immunoglobulin levels were determined as described by BRZEZIŃSKA-SLEBODZIŃSKA and SLEBODZIŃSKI (1982). Serum lysozyme activity was determined by a turbidimetric method (PARRY et al. 1965).

The results were verified statistically by one- and two-factorial analysis of variance and Duncan's test, using Statistica 6.0 software.

Results and Discussion

Highly significant differences in the plasma levels of total protein (Table 1) between heavy and medium-heavy type turkey-toms were observed only at 10 weeks of age (41.41 vs. 34.06 g l^{-1}, a difference of 17.7%). No significant type-dependent differences in total protein content were recorded in the other periods analyzed (week 14, 18 and 22). The effect of environmental conditions on the plasma protein concentrations was first noted 8 weeks after the change of the housing system, i.e. at the age of 14 weeks, when a significantly higher (by 10.9%) total protein content was recorded in birds raised indoor, as

compared to those kept under a shelter (37.14 g l^{-1}). In the other periods no significant differences in plasma protein levels, resulting from the housing system, were observed between birds. It should be noted that the values of this index within groups were comparable. Age-related changes in plasma protein concentrations of turkeys were reported by other authors (KONCICKI et al. 1990, 2000). According to SOWIŃSKA (2002), the serum levels of total protein may be affected not only by the age of birds, but also by their type and management system. However, the results of our study fail to unequivocally support this hypothesis.

Table 1

Plasma levels of total protein (g l^{-1})

Age (weeks)	Statistical measures	Housing system		Type	
		brooder house	shelter	heavy	medium-heavy
6	x	37.03	36.34	36.34	37.03
	v%	8.76	8.76	9.06	8.46
10	x	38.21	37.26	41.41^{a}	34.06^{b}
	v%	14.06	14.06	10.85	8.55
14	x	41.69^{a}	37.14^{b}	39.80	39.14
	v%	19.06	8.36	10.44	20.76
18	x	36.01	34.56	35.74	34.77
	v%	9.77	11.16	10.61	10.57
22	x	36.03	38.62	37.73	36.96
	v%	13.51	11.91	14.56	11.31

Note: heavy type – Big 6, medium-heavy type – But 9
Means followed by different letters differ significantly: *A, B* – $P \leq 0.01$, *a, b* – $P \leq 0.05$

The plasma levels of glucose over the experimental period are presented in Table 2. No significant differences dependent on the type of turkeys and housing system were found in this parameter. The only exception was plasma glucose concentration measured at 22 weeks, which was found to be substantially higher (by 22.1%) in turkeys raised under a shelter, which could result from their increased energy demand to keep warm when air temperature dropped to 3°C (weekly mean). Similarly as in our study (Table 2), also KONCICKI et al. (1990) and SOWIŃSKA (2002) observed a tendency towards age-related changes in glucose content in turkeys. These authors reported an increase in glucose to week 16, followed by a decrease between week 23 and 24. According to SOWIŃSKA (2002), such changes can be also related to the management system. The above relationships were also noted in this experiment in birds aged 22 weeks.

Table 2

Plasma levels of glucose (mg dl^{-1})

Age (weeks)	Statistical measures	Housing system		Type	
		brooder house	shelter	heavy	medium-heavy
6	x v%	203.61 12.15	193.20 16.11	198.35 12.87	198.45 15.81
10	x v%	210.82 12.99	209.99 22.94	209.85 12.28	210.96 23.25
14	x v%	250.26 12.22	237.60 11.27	248.90 11.99	239.43 11.86
18	x v%	276.38 12.03	269.16 12.70	276.95 9.68	268.24 14.75
22	x v%	225.75^{b} 10.37	289.88^{a} 8.53	252.28 13.86	265.13 16.99

Table 3

Plasma levels of total cholesterol (mg dl^{-1})

Age (weeks)	Statistical measures	Housing system		Type	
		brooder house	shelter	heavy	medium-heavy
6	x v%	168.57^{b} 10.28	182.13^{a} 15.08	169.64^{b} 14.61	181.06^{a} 11.95
10	x v%	98.07 17.37	112.01 27.50	102.26 24.99	107.82 24.03
14	x v%	118.93 15.68	112.40 18.29	118.70 14.92	112.90 18.92
18	x v%	111.99 18.39	116.73 14.07	107.50^{b} 16.66	121.64^{a} 13.65
22	x v%	116.61^{b} 13.89	127.57^{a} 13.93	120.01 12.36	124.54 16.38

The plasma levels of total cholesterol (Table 3) showed greater individual variation, as confirmed by distinctly higher coefficients of variations. Thus, it would be difficult to attribute these changes to the type of turkeys or environmental conditions after the first 6 weeks of rearing. A certain regularity was noted in this study, namely that cholesterol content was higher (168-182 mg dl^{-1}) in the youngest turkey-toms (aged 6 weeks), especially in medium-heavy ones. At 10 weeks no tendency towards a decrease in the plasma levels of total cholesterol along with the age of birds was observed, contrary to the findings of other authors (KONCICKI, KRASNODĘBSKA-DEPTA 2005, *Poultry diseases* 2005). In our previous experiment (FARUGA et al. 2003), conducted on heavy-type 18-week-old turkey-toms, total cholesterol concentra-

tion was much lower, i.e. 82.31-89.54 mg dl^{-1}. SOWIŃSKA (2002) analyzed alternative housing systems (litter, slatted floor) and found that serum cholesterol levels increased until 16 weeks of age. This author also demonstrated that a slatted floor affected higher serum concentrations of total

Table 4

Plasma levels of HDL cholesterol (mg dl^{-1})

Age (weeks)	Statistical measures	Housing system		Type	
		brooder house	shelter	heavy	medium-heavy
6	x	107.53	118.15	104.06^{b}	121.62^{a}
	$v\%$	23.68	15.19	22.33	14.91
10	x	69.62	73.04	69.41	73.26
	$v\%$	23.10	23.57	24.90	21.78
14	x	114.93	85.19	84.69	115.07
	$v\%$	126.02	19.32	19.12	125.07
18	x	83.99^{b}	95.74^{a}	87.59	92.51
	$v\%$	13.51	12.30	17.60	10.77
22	x	88.38	80.97	82.48	86.80
	$v\%$	18.29	18.39	18.01	19.35

cholesterol. KONCICKI et al. (2000) described similar age-related changes in the values of this index in turkeys for the first 16 weeks. In another study KONCICKI et al. (1990) noted an increase in serum cholesterol in turkey-toms aged 16 to 24 weeks. The interpretation of the above results is difficult since serum cholesterol values were determined by various methods, on turkeys of different origin, and are given in various units. At 6 weeks of age the plasma levels of HDL cholesterol (Table 4) were higher in medium-heavy birds, and remained at a comparable level within this population. HDL cholesterol concentrations were lower in older birds, with greater individual variation at 14 weeks of age. A significant effect of the housing system on the plasma levels of HDl cholesterol was demonstrated only in turkey-toms aged 18 weeks.

Distinct, type-related differences in the plasma levels of immunoglobulins were observed only in turkeys aged 10 weeks (Table 5). The effect of environmental conditions on immunoglobulin content showed relatively low variation (v%), but was not confirmed statistically, and plasma immunoglobulin concentrations were at a comparable level. It is difficult to interpret these results, since available literature on the subject provides no reference data for turkeys. It is common knowledge that increased immunoglobulin concentrations may indicate a pathological state. According to STRZEŻEK and WOŁOS (1997), in hens the mean plasma level of gamma-globulins, of which the vast majority are

immunoglobulins, is 29.4% of total protein. In the present study immunoglobulins accounted for about 27% of total protein in turkeys, which is comparable to the physiological limits for hens.

Table 5

Plasma levels of immunoglobulins (g l^{-1})

Age (weeks)	Statistical measures	Housing system		Type	
		brooder house	shelter	heavy	medium-heavy
6	x v%	10.81 9.72	10.74 9.11	10.89 9.27	10.66 9.47
10	x v%	9.56 7.85	9.73 7.56	9.41^{b} 7.04	9.88^{a} 7.61
14	x v%	9.33 13.44	9.86 8.63	9.86 9.67	9.32 12.64
18	x v%	9.49 11.14	9.49 10.26	9.22 10.70	9.76 9.90
22	x v%	9.76 13.22	9.92 7.59	9.84 10.24	9.83 11.27

Noticeable, confirmed statistically, differences in uric acid content were observed at 22 weeks in turkeys of both types, raised under different environmental conditions (Table 6). Considerable differences in the plasma levels of uric acid within groups makes it difficult to determine the influence of the experimental factors on this parameter. The values obtained in the study are close to physiological values for turkeys (KONCICKI, KRASNODĘSKĄ-DEPTA 2005, *Poultry diseases* 2005).

Table 6

Plasma levels of uric acid (mg dl^{-1})

Age (weeks)	Statistical measures	Housing system		Type	
		brooder house	shelter	heavy	medium-heavy
6	x v%	3.21 36.17	2.62 46.39	2.83 40.86	3.00 42.87
10	x v%	4.14 39.81	4.04 41.04	4.33 30.16	3.84 50.26
14	x v%	5.28 29.55	4.59 62.44	5.16 32.94	4.73 58.54
18	x v%	3.35 28.62	2.74 49.60	3.27 33.94	2.80 45.86
22	x v%	3.46^{b} 27.05	4.26^{a} 30.13	4.36^{a} 29.62	3.40^{b} 25.42

The plasma levels of total lipids (Table 7) also showed very high individual variation within groups, which makes it impossible to directly relate them to the experimental factors. The correlation between the plasma concentrations of total lipids and the type of turkeys was found to be statistically non-significant, and so was the correlation between total lipids and the housing system (brooder house or shelter), except for week 14 and 18, when higher values of this parameter (by 16.9% and 21.8%, respectively) were recorded in turkeys raised under a shelter.

Table 7

Plasma levels of total lipids (mg dl^{-1})

Age (weeks)	Statistical measures	Housing system		Type	
		brooder house	shelter	heavy	medium-heavy
6	*x*	534.86	563.27	540.86	557.27
	v%	23.39	27.53	17.62	31.54
10	*x*	343.14	376.54	346.16	373.51
	v%	28.52	22.00	24.12	26.31
14	*x*	524.45*b*	631.50*a*	578.63	575.12
	v%	21.05	19.54	22.12	22.62
18	*x*	489.21*b*	614.43*a*	524.81	582.91
	v%	18.30	16.30	23.62	16.29
22	*x*	633.75	593.56	603.93	622.88
	v%	29.52	24.34	28.18	26.47

At 6 weeks of age ALAT activity was by 15.6% higher in heavy-type turkeys, as compared with medium-heavy ones (22.15 vs. 18.69 U l^{-1}; Table 8). After another 4 weeks considerable differences in the values of this index were found within groups, and a difference of 46.8% in ALAT activity between turkeys of both types was highly significant. In the other periods there were no significant differences in ALAT activity between heavy and medium-heavy type turkeys. Differences in ALAT activity related to environmental conditions were recorded in older birds, from 14 weeks until the end of the experiment (Table 8). However, high variation in this parameter within groups precludes far-reaching conclusions. The values of ALAT activity recorded in this study are lower than those reported by FARUGA et al. (2003) – 20.91-24.28 U l^{-1}. Significant changes in ALAT activity were reported by FITKO et al. (1992) who studied the effects of various feeding levels and lighting conditions on turkey-hens. The activity of this enzyme increased in turkey-hens fed *ad libitum* in response to shorter periods of light. The opposite tendency was noted in our experiment, since ALAT activity decreased in turkey-toms that stayed under

a shelter to 18 weeks of age as the natural daylight period became shorter. In the last month of the experiment (22 weeks), when the natural daylight period was still shorter, the activity of this enzyme increased, particularly in birds raised under a shelter. The activity of alanine aminotransferase determined in our study (Table 8) is close to the values considered physiological (*Poultry diseases* 2005), and higher than the results obtained by KONCICKI et al. (1990). A tendency towards considerable fluctuations in ALAT activity over the growth period of turkeys, observed in our experiment, is similar to that described by KONCICKI et al. (2000) and SOWIŃSKA (2002).

Table 8

Activity of alanine aminotransferase (ALAT) in blood plasma (U l^{-1})

Age (weeks)	Statistical measures	Housing system		Type	
		brooder house	shelter	heavy	medium-heavy
6	*x*	20.51	20.33	22.15^{a}	18.69^{b}
	v%	29.08	25.09	27.18	23.37
10	*x*	16.88	15.81	11.36^{b}	21.34^{a}
	v%	42.84	52.09	31.89	34.98
14	*x*	16.03^{a}	12.06^{b}	13.61	14.55
	v%	35.82	28.65	38.20	35.16
18	*x*	8.99^{b}	11.76^{a}	10.48	10.33
	v%	27.44	42.42	48.58	29.16
22	*x*	17.67^{b}	22.12^{a}	19.67	20.341
	v%	6.92	24.83	21.66	27.86

Differences in the activity of alkaline phosphatase were noticeable only at 10 weeks of age (Table 9). Higher (by 9.3%) AP activity was recorded in heavy--type turkey-toms, in comparison to medium-heavy ones (104.74 U l^{-1}). There was no relation between AP activity and the turkey management systems tested in the study. As expected, age-related changes in AP activity were observed in particular experimental periods. In physiological states, alkaline phosphatase present in blood plasma comes primarily from bones, small intestinal mucosa, kidneys, epithelium of biliary canaliculi, while in pathological states also from neoplastic tissues. The activity of this enzyme is the highest in young birds and mammals, when their skeleton grows dynamically, and then decreases with age. Such a relationship was also observed in turkey-toms KONCICKI et al. (1990, 2000, 2005). KACZANOWSKA-TARASZKIEWICZ (2001) also demonstrated that there is a close correlation between mineral balance and skeletal system development, and serum phosphatase activity. The activity of this enzyme decreases with age, when the ossification process is

completed. According to OSTROWSKI (1995), this enzyme is a good indicator of bone tissue formation. The activity of alkaline phosphatase recorded in this study are substantially lower (by one order of magnitude) than that obtained by KONCICKI and KRASNODĘBSKA-DEPTA (2005) and described in *Poultry diseases* (2005). However, the method used by these authors to determine AP activity remains unknown. AP activity depends on the pH of a reaction mixture and buffer quality. Additional research performed using a standard method described for the first time by BESSY et al. (1946) confirmed the values of AP activity shown in Table 9. It seems that the results obtained by KONCICKI and KRASNODĘBSKA-DEPTA (2005) as well as those included in *Poultry diseases* (2005) are given in nanokatals (nkat l^{-1}), units that are seldom used in professional biochemical literature, and not in standard international units (U l^{-1}). Since 1 U l^{-1} = 16.67 ncat l^{-1}, the results are comparable to those reported here.

Table 9

Activity of alkaline phosphatase (AP) in blood plasma (U l^{-1})

Age (weeks)	Statistical measures	Housing system		Type	
		brooder house	shelter	heavy	medium-heavy
6	x v%	117.82 15.91	120.16 12.42	119.92 15.56	118.06 12.76
10	x v%	106.48 12.08	113.67 14.46	115.41^{a} 11.79	104.74^{b} 14.09
14	x v%	84.27 18.00	88.52 15.41	85.84 13.36	86.82 19.64
18	x v%	73.56 23.86	84.02 23.69	73.97 19.97	84.09 26.94
22	x v%	47.03 16.19	47.44 16.02	46.74 15.78	47.75 16.33

Note: 1 U l^{-1} = 16.67 ncat l^{-1} (unit of activity, International System of Units SI)

Great individual variation within groups was also observed in lysozyme activity (Table 10), so again any general conclusions with regard to the observed differences should be formulated with caution. Type-dependent changes in lysozyme activity in turkey-toms were recorded at 6 weeks of age only. As concerns the effect of environmental conditions on lysozyme activity (at high variation), it should be noted that it decreased in older birds kept under a shelter. RUTHERFORD et al. (1998) and ŚWIĘCICKA-GRABOWSKA et al. (1998) demonstrated that decreased lysozyme activity may indicate immunosuppression or the absence of an agent stimulating non-specific immunity. In the experiments carried out by these authors lysozyme activity ranged

from 1.26 to 4.34 mg dl^{-1}. According to MAJEWSKA et al. (2004), feeding oat-supplemented diets to turkeys may stimulate their non-specific immunity.

Table 10

Lysozyme activity in blood plasma (mg l^{-1})

Age (weeks)	Statistical measures	Housing system		Type	
		brooder house	shelter	heavy	medium-heavy
6	x	19.57	20.52	16.96^{b}	23.13^{a}
	v%	28.13	36.63	21.20	31.88
10	x	15.88	21.02	19.05	17.86
	v%	21.81	56.18	41.99	56.16
14	x	18.89^{a}	14.22^{b}	17.23	15.96
	v%	22.77	21.56	27.61	25.06
18	x	26.07^{a}	20.65^{b}	23.22	23.50
	v%	24.90	25.12	28.29	27.30
22	x	17.53	15.66	16.16	17.08
	v%	25.66	47.08	27.46	44.76

Conclusions

1. The following limit values were obtained for plasma biochemical parameters of slaughter turkey-toms aged 6 to 22 weeks:
- total protein 34.06 – 41.69 g l^{-1},
- glucose 193.20 – 289.88 mg dl^{-1},
- total cholesterol 98.07 – 182.13 mg dl^{-1},
- HDL cholesterol 69.41 – 121.62 mg dl^{-1},
- immunoglobulins 9.32 – 10.89 g l^{-1},
- uric acid 2.74 – 5.28 mg dl^{-1},
- total lipids 346.16 – 633.75 mg dl^{-1},
- ALAT activity 8.99 – 22.15 U l^{-1},
- AP activity 46.74 – 120.16 U l^{-1},
- lysozyme activity 14.22 – 26.07 mg l^{-1}.

2. No regular age-related changes were observed in plasma biochemical parameters of slaughter turkey-toms, except for AP activity.

3. A significant effect of the type of turkeys on the values of plasma biochemical parameters was noted only in the levels of total protein, total cholesterol, immunoglobulins and AP activity at 10 weeks of age, HDL cholesterol, activity of ALAT and lysozyme at 6 weeks as well as uric acid at 22 weeks.

4. A significant effect of the housing system on the values of plasma biochemical parameters was recorded in the levels of total protein at 14 weeks

of age, total cholesterol, glucose, uric acid and ALAT activity at 22 weeks, HDL cholesterol at 18 weeks, total lipids at 14 weeks as well as lysozyme activity at 14 and 18 weeks.

5. The plasma biochemical indices of young slaughter broad-breasted turkey-toms (inter-strain hybrids), determined in the study, showed no regular changes dependent on the type and housing system over the growth period.

It may be concluded that in order to ensure their welfare and well-being, it is recommended to raise slaughter turkeys under a shelter with access to outdoor runs from 6 weeks of age, except for the winter months.

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References

BESSY O.A., LOERY O.H., BROCK M.J. 1946. *A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum*. J. Biol. Chem., 164: 321-323.

BRZEZIŃSKA-ŚLEBODZIŃSKA E., ŚLEBODZIŃSKI A.B. 1982. *A combined polyethyleneglycol immunoglobulin precipitation with the serum protein determination as a routine clinical test for estimation of the immune and nutritional status in neonatal calves*. Br. Vet. J., 138: 145-154.

Choroby drobiu (*Poultry diseases*) 2005. Red. M. MAZURKIEWICZ. AXA, Wrocław.

FARUGA A. 1997. *Technologia odchowu indyków rzeźnych*. Mag. Drob., 11: 8-11.

FARUGA A., POLAK M., JĘDRYCZKO R., ŁOWCZAK I. 2003. *Wyniki odchowu i aktywności enzymów surowicy krwi indorów żywionych mieszankami z zawartością Flawomycyny lub preparatu ziołowego*. Biul. Nauk. UWM, 22: 167-171.

FITKO R., ZIELIŃSKI H., ROSZKO E., FARUGA A. 1992. *Wpływ różnego poziomu żywienia i oświetlenia na wskaźniki hematologiczne i biochemiczne krwi indyków*. Acta. Acad. Agricult. Tech. Olst., Vet., 20: 171-178.

KACZANOWSKA-TARASZKIEWICZ E. 2001. *Wpływ wieku i płci na mineralizację kości oraz poziom wapnia, fosforu i aktywności fosfatazy zasadowej w osoczu krwi przepiórek*. Med. Wet., 57(11): 827-831.

KONCICKI A., KRASNODĘBSKA-DEPTA A., MIKULSKI D., FARUGA A. 1990. *Wpływ skarmiania mieszanek pełnoporcjowych ze zwiększonym udziałem surowców krajowych na zachowanie się niektórych wskaźników hematologicznych i biochemicznych krwi oraz na wyniki produkcyjne młodych indyków rzeźnych*. Acta Acad. Agricult. Tech. Olszt. Vet., 19: 243-253.

KONCICKI A., KRASNODĘBSKA-DEPTA A., ZDUŃCZYK Z., JANKOWSKI J., WRÓBLEWSKA M., FALKOWSKA A. 2000. *Biochemical indices in blood and tissue of turkeys fed feed mixtures containing fat of different oxidation degree*. Pol. J. Vet. Sci., 3(2): 81-86.

KONCICKA A., KRASNODĘBSKA-DEPTA A. 2005. *Możliwości wykorzystania wyników badań hematologicznych i biochemicznych w diagnostyce chorób drobiu*. Mag. Wet., Supl. Drób., 5: 20-22.

MAJEWSKA T., MIKULSKI D., ŚWIĘCICKA-GRABOWSKA G., WÓJCIK R. 2004. *Owies w żywieniu młodych indyków rzeźnych*. Med. Wet., 60(6) 657-661.

MEYER H., PETERSEN J., SCHEPERS K.H., THOLEN E. 1997. *Effects of dietary energy and amino acid content on carcass composition and meat quality in two strains of turkey*. Proc. 13th Europ. Symp. Quality of Poult. Meat, Poznań, 82-89 pp.

PARRY R.M., CHANDAU R.C., SHAHANI R.M. 1965. *A rapid and sensitive assay of muramidase*. Proc. Soc. Exp. Biol. Med., 119: 384-386.

OSTROWSKI K. 1995. *Histologia*. PZWL, Warszawa.

PUCHAJDA H., FARUGA A., PUDYSZAK K. 1997. *Effect of turkey on meat quality*. Proc. 13th Europ. Symp. Quality of Poult. Meat, Poznań, 53-57 pp.

RUTHERFORD M., WIŚNIEWSKI J., ŚWIĘCICKA-GRABOWSKA G. 1998. *Immunosupresja u drobiu – próby przeciwdziałania jej skutkom lewamizolem*. Zesz. Nauk. Prz. Hod., 36: 193-198.

SOWIŃSKA J. 2002. *Wpływ systemu utrzymania, typu użytkowego i obrotu przedubojowego na wybrane wskaźniki krwi, obraz morfologiczny mięśnia piersiowego oraz jakość mięsa indyków rzeźnych*. Wyd. UWM Olsztyn, Rozpr. i Monogr., 69: 7-73.

STRZEŻEK J., WOŁOS A. 1997. *Ćwiczenia z biochemii*. Wyd. ART Olsztyn.

ŚWIĘCICKA-GRABOWSKA G., MAJEWSKA T., WÓJCIK R., GAŁECKI W., WIŚNIEWSKI J., KOZŁOWSKI K. 1998. *Wpływ niekonwencjonalnych dodatków paszowych w żywieniu indyków rzeźnych na kształtowanie się wybranych wskaźników odporności nieswoistej*. Zesz. Nauk. Prz. Hod., 36: 177-183.

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RELATIONSHIPS BETWEEN THE LEVELS OF BLOOD INDICES IN THE PERINATAL PERIOD AND THE BODY CONDITION AND PERFORMANCE TRAITS OF COWS

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K e y w o r d s: dairy cows, body condition, negative energy balance, metabolic profile, fertility.

A b s t r a c t

The aim of the present study was to determine relationships between changes in the body condition of cows at the first stage of lactation and their fertility and productivity. The experiment was performed on a group of 243 Holstein-Friesians. Body condition was assessed post-partum, and then at two-week intervals until 20 weeks of lactation. Production traits and fertility were also evaluated. Blood was collected from 21 cows a week pre-partum and two weeks post-partum, to determine the metabolic profile. The highest rate of lipolysis was accompanied by lower serum levels of magnesium and glucose, higher serum concentrations of urea, free fatty acids and β-hydroxybutyric acid as well as an increase in the activity of alanine aminotransferase and aspartate aminotransferase. Cows that had sufficient fat reserves at the beginning of lactation produced the most milk and were characterized by lower fertility.

Blood analysis made in the precalving period is of low diagnostic value with regard to the metabolic rate and potential metabolic disorders post-calving, while the levels of blood indices and changes in the body condition of cows determined at the initial stage of lactation may contribute to improving feeding management as well as support decision-making and planning with respect to reproduction. In high-performance cows, characterized by a fast rate of lipolysis at the beginning of lactation, the return to normal reproductive status after calving should be delayed in order to increase insemination efficiency.

POZIOM WSKAŹNIKÓW KRWI W OKRESIE OKOŁOPORODOWYM I JEGO ZWIĄZEK Z KONDYCJĄ KRÓW I ICH UŻYTKOWOŚCIĄ

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A b s t r a k t

Badano zależności między wielkością zmian kondycji w początkowym okresie laktacji a płodnością i produkcyjnością 243 krów holsztyńsko-fryzyjskich. Oceniano kondycję (BCS) po wycieleniu i – w odstępach 2-tygodniowych – do 20. tyg. laktacji, cechy produkcyjne i płodność. Od 21 krów pobrano krew na 1 tydzień przed porodem oraz 2 tygodnie po porodzie i oznaczono profil metaboliczny. W surowicy krwi krów o najintensywniejszej lipolizie stwierdzono niższy poziom magnezu i glukozy, wyższą zawartość mocznika, wolnych kwasów tłuszczowych i kwasu β-hydroksymasłowego oraz wzrost aktywności aminotransferaz alaninowej i asparaginowej. Krowy najintensywniej mobilizujące tłuszcz ciała na początku laktacji, wyprodukowały najwięcej mleka i charakteryzowały się obniżoną płodnością.

Badanie krwi przed wycieleniem ma niewielkie znaczenie diagnostyczne intensywności przemian materii i zaburzeń po wycieleniu, natomiast wartości wskaźników krwi oraz ocena zmian kondycji w pierwszej fazie laktacji mogą umożliwić ocenę prawidłowości żywienia oraz ułatwić podejmowanie decyzji w rozrodzie. Aby zwiększyć efektywność inseminacji, należy „opóźniać" powrót do rozrodu po wycieleniu krów wysoko produkcyjnych, charakteryzujących się intensywną lipolizą na początku laktacji.

Introduction

A rapid increase in the milk performance of cows, especially Holstein-Friesians, has been observed in recent years. In the leading dairy cow populations the progress in annual milk production is 3 to 4%. Intensive selection directed mainly towards an increase in milk yield per cow resulted in a discrepancy between energy requirements for milk production and energy supply in feed. Cows usually attain peak milk yields 6 to 8 weeks after calving, and maximum feed intake no later than 12 to 14 weeks after calving. Negative energy balance, usually observed during early lactation, leads to increased use of energy and fat reserves (primarily of adipose tissue), followed by a decrease in body weight and changes in body condition. Energy mobilization during lactation is a physiological character of the mammals. PRYCE et al. (1999) found that in the first weeks of lactation about 7 kg of milk is produced daily from body tissue reserves. A chronic and serious energy deficiency results in metabolic stress, which may the cause of health problems, fertility disorders and decreased productivity (DOMECQ et al. 1997, GEARHART, CURTIS 1990).

It is impossible to precisely measure the energy balance of cows in the cowshed. Body condition is an indirect parameter indicating the mobilization or replenishment of energy reserves (particularly of adipose tissue) in cows over a period of positive energy balance. Body condition scoring is a subjective measure of fat reserves, providing a basis for distinguishing differences in nutritional needs of cows as well as for determining their health status (WILDMAN et al. 1982). According to PRYCE et al. (2001), body condition scoring is an easy-to-apply and useful herd management tool. The body condition score is positively correlated with the fertility traits of cows at the beginning of

lactation, and negatively during the first 15 weeks post-partum (VEERKAMP et al. 2001). Similarly as milk performance, both the body condition of cows and changes in body condition score are determined genetically in 25 to 35%, and are positively correlated with fertility traits (VEERKAMP et al. 2001, DECHOW et al. 2002). Fertility traits have a very low heritability. Thus, there is a need to search for traits correlated with fertility that could be used in order to improve reproductive performance.

Polish literature on the subject provides no information on the effect of the body condition of cows on their fertility and productivity. The aim of the present study was to determine relationships between changes in the body condition of cows at the first stage of lactation and their fertility and productivity as well as levels of blood indices.

Materials and Methods

The experiment was conducted under production conditions, during the years 2004-2006, on a dairy cattle farm, on 243 Holstein-Friesian cows. The animals were kept in tying stalls. Milking was performed twice daily using a pipeline milking system. Throughout the year the cows were fed a total mixed ration (TMR), composed of corn-cob mix (CCM), cereal-legume silage, brewer's spent grains (BSG), beet molasses, rapeseed meal, soybean meal, wheat bran and mix B. The proportions of particular components depended on the mean level of herd milk production for which TMR was balanced. The diet was supplemented with vitamins and minerals, depending on the physiological condition of cows.

The body condition of cows was assessed post-partum and then at two-week intervals until 20 weeks of lactation, on a five-point scale with 0.25 unit intervals, where 1 denotes an extremely thin cow and 5 an excessively fat cow, as described by WILDMAN et al. (1982). Based on a decrease in body condition (the difference between BCS post-partum and the lowest BCS at the initial stage of lactation), the cows were divided into the following groups: 1) ≤ 0.5, 2) 0.75-1.0, 3) > 1.0.

Data on the productivity and fertility traits of cows during the first lactation after calving come from breeding records, Symlek databases and direct observations. The following determinations were made:

– ECM (energy corrected milk) yield, mean milk fat yield and mean protein yield for 100 and 305 days of lactation, on an individual basis ECM – milk with a standardized energy content (SJAUNJA et al. 1990),

$$\text{ECM (kg)} = \text{milk (kg)} \cdot \frac{(0.383 \cdot \text{fat (\%)} + 0.242 \cdot \text{protein (\%)} + 0.7832)}{3.140};$$

– conception rate (sum of all inseminations/number of in-calf cows), inter-pregnancy interval (number of days from calving to conception), service period duration (number of days from the first insemination to fertilization).

Blood was collected from the external jugular vein of 21 clinically healthy cows selected randomly, a week pre-partum and two weeks post-partum. The serum levels of total magnesium, urea, glucose, free fatty acids (FFAs), β-hydroxybutyric acid (BHB) as well as the activity of alanine aminotransferase (ALAT) and aspartate aminotransferase (AspAT) were determined using BioSystem diagnostic kits, a Hitachi 902 biochemical analyzer and an Eppol 20 spectrophotometer.

The results were verified statistically using Statistica 6.0 software. Fertility traits and productivity were estimated by one-factor analysis of variance in a non-orthogonal design. The significance of differences between means was determined by Tukey's test for unequal group sizes.

Results and Discussion

The curves of changes in the body condition of cows illustrate the persistence of negative energy balance (Figure 1). The dynamics of a decrease in the fat reserves during early lactation differed depending on the body condition of cows at calving. The most significant body fat loss (0.5 point) was observed in cows with the highest body condition score during the first 2-3 weeks of lactation. The curves of a decrease in the body condition of cows of the other groups show that their energy reserves were used at a slower rate and in a more uniform manner during the first 10-12 weeks of lactation. The most substantial total decrease in body condition was recorded in the group of cows with the highest body condition score at calving. PEDRON et al. (1993) also reported that changes in the body condition of cows are dependent on the degree of fatness of a cow at calving. In our study cows started replenishing fat reserves on average between 10 and 14 weeks of lactation, and the process was delayed in cows with lower body condition scores (positive energy balance). In an experiment conducted by DOMECQ et al. (1997) high-performance Holstein-Friesian cows received the lowest body condition scores between 4 and 8 weeks of lactation.

According to WHITAKER (1997), the values of biochemical blood indices enable to evaluate the nutritional status of a cow and contribute to diagnosing

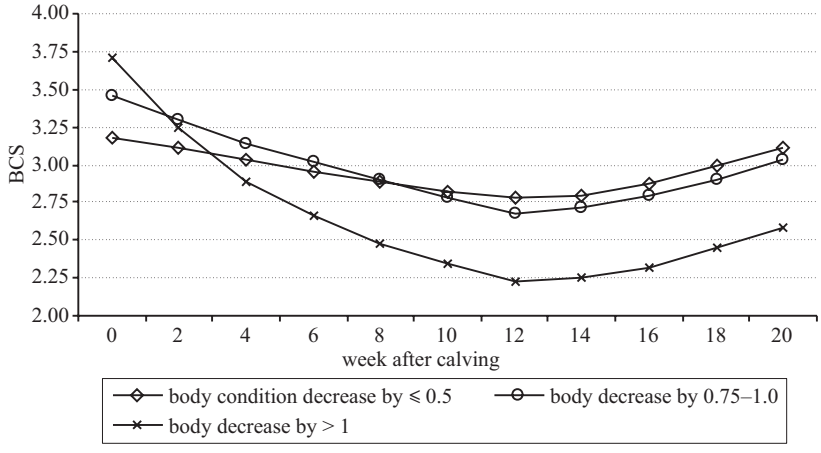

Fig. 1. Body condition scores at calving and during the first 20 weeks of lactation

Table 1

Changes in body condition versus levels of blood indices

Traits	Body condition decrease (calving – the lowest point)						Total	
	≤ 0.5		0.75-1.0		> 1			
	x	SD	*x*	SD	*x*	SD	*x*	SD
Blood collected before calving								
Days before calving	5-10		4-9		5-9		4-10	
Number of cows, head	7		8		6		21	
Mg, mmol l^{-1}	0.74	0.09	0.83	0.09	0.76	0.09	0.78^{A}	0.09
Urea, mmol l^{-1}	2.61	1.00	1.99	0.34	2.52	0.68	2.35^{a}	0.55
Glucose, mmol l^{-1}	3.33	0.17	3.68	0.67	3.68	0.29	3.57^{a}	0.47
BHB, mmol l^{-1}	0.52	0.20	0.65	0.29	0.59	0.34	0.59^{a}	0.27
FFAs, mmol l^{-1}	0.28	0.11	0.40	0.25	0.37	0.15	0.35^{A}	0.19
ALT, U l^{-1}	15.29	2.75	17.63	3.96	15.17	2.14	16.14	3.21
AST, U l^{-1}	68.86	22.09	61.38	7.74	61.33	12.52	63.86^{A}	14.82
Blood collected after calving								
Days after calving	13-15		13-16		13-15		13-16	
Number of cows, head	7		8		6		21	
Mg, mmol l^{-1}	0.63	0.11	0.67	0.11	0.62	0.12	0.64^{A}	0.11
Urea, mmol l^{-1}	2.73	1.02	2.86	0.99	4.48	2.96	3.28^{a}	1.86
Glucose, mmol l^{-1}	3.08	1.10	2.71	0.68	2.11	1.41	2.63^{a}	1.12
BHB, mmol l^{-1}	0.69	0.82	1.69	1.68	1.83	1.47	1.40^{a}	1.36
FFAs, mmol l^{-1}	0.46^{a}	0.26	0.60	0.34	0.83^{a}	0.44	0.62^{A}	0.36
ALT, U l^{-1}	12.14^{A}	1.95	19.37^{a}	9.71	36.67^{Aa}	21.21	22.14	15.71
AST, U l^{-1}	88.00^{a}	22.98	156.75	130.52	251.50^{a}	163.63	160.90^{A}	130.93

Values followed by the same letters differ significantly: capital letters – $P \leq 0.01$; small letters – $P \leq 0.05$

metabolic diseases. In this study the levels of some indices in blood samples collected 7 days pre-partum were within the reference ranges (WINNICKA 2002) – Table 1. There were no significant differences in the levels of biochemical blood indices between particular groups of cows, so their diagnostic value with regard to the metabolic rate after calving and metabolic disorders (quite common in this period) is limited.

Before calving mean blood magnesium concentrations were at the low end of the normal range (WINNICKA 2002). After calving the serum levels of magnesium did not exceed the value of 0.74 mmol l^{-1}, indicating hypomagnesemia. The decrease in blood magnesium concentrations observed in early lactation is aused by a high demand for this macronutrient under conditions of intensively increasing milk production as well as by a poor appetite of cows, followed by reduced feed (and magnesium) intake. Hypomagnesemia intensifies with an increasing rate of lipolysis of energy reserves, which may be accompanied by increased mineral requirements, as well as in the case of impaired nutrient mobilization from the bone reserves (KUREK, STEC 2005).

Increased energy demands observed with the progress of lactation, accompanied by reduced feed intake after calving, result in a decrease in blood glucose levels, an increase in the concentrations of free fatty acids and excessive ketone body production (DALE et al. 1979). In the present study, significantly lower glucose levels and significantly higher concentrations of free fatty acids (FFAs) and β-hydroxybutyric acid (BHB) were recorded in blood samples taken several days after calving, compared to those collected before calving. This trend was particularly strong in cows that most intensively mobilized and used their body reserves post-partum. The glucose concentrations in blood samples taken after calving from cows whose body condition score decreased maximally by 1 point were at the low end of the reference range given by WINNICKA (2002). The glucose levels in blood samples collected from cows with the highest decrease in body condition was below normal (2.11 mmol l^{-1}), which indicated the need for energy supplementation. According to WHITAKER (1997), the optimal serum glucose concentration in dairy cows should exceed 3 mmol l^{-1}.

The blood levels of free fatty acids reflect the amount of energy derived from adipose tissue. A fast rate of reserve fat release leads to excessive production of fatty acids, which – under conditions of low glucose levels – are partially converted into ketone bodies. Intensive lipolysis may be caused by extreme fatness of a cow (KUREK, STEC 2005) and by genetically determined high milk productivity. In this experiment there were statistically significant ($P \leq 0.05$) differences in FFA levels between groups of cows with the highest and lowest body condition scores. Serum BHB concentrations in cows whose body condition score decreased by 0.75 point or more exceeded 1.2 mmol l^{-1},

which – according to DUFFIELD et al. (2006) – is a threshold value for defining subclinical ketosis.

Aminotransferases are responsible for maintaining protein balance, which is of particular importance in the period of intensive metabolism. The activity of aminotransferases increases at a high protein content of feed as well as in the case of parenchymal hepatocyte dysfunction (WHITAKER 1997). In our study the serum levels of alanine aminotransferase (ALAT) and aspartate aminotransferase (AspAT), determined before calving, were within normal ranges in all cows (WINNICKA 2002). KUREK and STEC (2005) obtained comparable results in young cows 7 days pre-calving. In this experiment a significant increase in the activity of both aminotransferases was observed after calving, which was related to a high metabolic rate during intensive lactation. Compared to their serum levels measured before calving, the activity of alanine aminotransferase and aspartate aminotransferase increased after calving over twofold and fourfold, respectively. AST, as a cytoplasmic enzyme, is a labile and sensitive indicator of liver diseases occurring in high-production cows.

The cows whose post-calving body condition score decreased to the highest degree had increased serum urea levels. An increase in blood urea concentrations may be a consequence of a high content of protein, especially rumen-degradable protein, in the ration. Under physiological conditions, the large quantities of toxic ammonia produced in the rumen are converted into microbial protein. However, with no sufficient energy for microbial assimilation, ammonia is detoxified by the liver to urea whose excessive levels are then recorded in the blood, urine and milk. In our study, in the group of cows characterized by the fastest rate of lipolysis, urea concentration exceeded 18 mg dl^{-1} (3 mmol l^{-1}), i.e. a threshold value above which conception rate decreases. BERNHARD (1992) demonstrated that in energy-deficient cows urea concentrations increased in the blood serum and uterine cervical mucus. In the opinion of this author this may decrease the efficiency of both natural mating and artificial insemination due to reduced sperm motility.

ąIn the tested population energy supply was insufficient to meet the requirements of high-production cows at the initial stage of lactation, under conditions of low feed intake. This could result from the fact that the ration was not adjusted to satisfy the individual needs of cows with the highest genetic milk yield potential, predisposed to intensive lipolysis.

In the majority of cows the body condition score decreased by 0.75 – 1.0 point from calving to the moment when cows began to replenish their energy reserves (Table 2). According to BORKOWSKA (2000), it is normal for the body condition score of high-performance cows to decrease by 0.5 – 1.0 point in early lactation. In the present study the cows that mobilized their body fat reserves to the greatest degree were characterized by the highest productivity. Over the

first hundred days of lactation the mean difference in milk yield between cows with the highest and lowest decrease in body condition was 148 kg, and increased to 408 kg of ECM during the standard lactation period. However, this difference was found to be statistically non-significant. The total decrease in body condition was positively correlated with milk yield. A similar relationship was observed by WALTNER et al. (1993), while BERRY et al. (2002) demonstrated that cows that mobilized their body reserves to a lower degree produced more milk on the first 240 days of lactation.

Table 2

Productivity and levels of fertility indices of cows as dependent on body condition decrease

Traits	Body condition decrease (calving – the lowest point)					
	≤ 0.5		0.75-1.0		> 1	
	x	SD	x	SD	x	SD
Number of cows, head	89		101		53	
100-day lactation:						
ECM, kg	2693	567.15	2732	446.12	2841	641.11
Fat, %	3.37	0.79	3.52	0.63	3.40	0.60
Protein, %	3.07	0.30	3.10	0.25	3.12	0.40
305-day lactation:						
ECM, kg	7560	1144.5	7743	1056.1	7968	558.9
Fat, %	3.50	0.75	3.81	0.64	3.71	0.45
Protein, %	3.37	0.27	3.37	0.19	3.37	0.17
Age at calving, months	33.1^{A}	9.55	32.6^{B}	8.94	44.8^{AB}	11.22
Inter-pregnancy interval, days	139.4	56.22	156.7	70.47	180.2	85.63
Conception rate	1.91	1.08	2.42	1.70	2.83	1.63
Service period, days	37.8^{a}	45.71	55.4	61.29	89.0^{a}	63.55

Values followed by the same letters differ significantly: capital letters – $P \leq 0.01$; small letters – $P \leq 0.05$

While evaluating fertility traits, in terms of the rate and extent of fat reserve mobilization, attention should be paid to the age difference between cows of particular groups. The cows whose body condition decreased » 1 point were significantly older at calving than the other ones. The values of fertility indices differed from normal reference ranges, which could be partly related to the high productivity of the tested population. The levels of fertility indices were found to be correlated with body condition scores, and decreased along with increasing intensity of fat reserve mobilization. Service period duration was significantly ($P \leq 0.05$) longer in cows whose body condition decreased by more than 1.0 point, compared to the animals in which body fat reserve loss was the slightest (≤ 0.5). Reproduction disorders could result from too intensive lipolysis at the beginning of lactation. The mean number of services per conception (conception rate) was 2.83 in cows with the highest decrease in body

condition. In a representative sample of cows of this group the mean serum concentration of β-hydroxybutyric was 1.83 mmol l^{-1}. WALSH et al. (2004) reported that insemination efficiency decreased by as much as 50% already at BHB concentration of 1.4 mmol l^{-1}. DOMECQ et al. (1997) found that the first insemination was successful in 53% and 17% of cases in cows whose body condition decreased by 0.5 – 1 points and by more than 1.0 point, respectively, in the first weeks following calving. In cows that are genetically inclined to have a lower BCS post-partum, the rest period is naturally prolonged (DECHOW et al. 2002). According to BRAND (2006), insemination efficiency can be increased provided that the first service is carried out during the period of positive energy balance. Therefore, changes in body condition may be a predictor of fertility, and body condition scoring may support decision-making and planning with respect to reproduction.

Conclusions

The lowest body condition scores were recorded at 10-14 weeks of lactation, and the most substantial decrease in body condition was related to delayed replenishment of body fat reserves. The highest rate of lipolysis at the onset of lactation was accompanied by lower serum levels of magnesium and glucose, higher serum concentrations of urea, free fatty acids and β-hydroxybutyric acid (subclinical ketosis) as well as an increase in the activity of alanine aminotransferase and aspartate aminotransferase. Cows that had sufficient fat reserves at the beginning of lactation produced the most milk and were characterized by lower fertility.

Blood analysis made in the precalving period is of low diagnostic value with regard to the metabolic rate and potential metabolic disorders post-calving, while the levels of blood indices and changes in the body condition of cows determined at the initial stage of lactation may contribute to improving feeding management as well as support decision-making and planning with respect to reproduction. In high-performance cows, characterized by a fast rate of lipolysis at the beginning of lactation, the return to normal reproductive status after calving should be delayed in order to increase insemination efficiency.

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References

BERNHARD A. 1992. *Der Diagnostische Wert der Harnstoffbestummung in der Milch im Hinblick auf die Fruchtbarkeit beim Rind*. Universität Leipzig (praca doktorska).

BERRY D.P., BUCKLEY F., DILLON P., EVANS R.D., RATH M., VEERKAMP R.F. 2003. *Genetic relationships among body condition score, body weight, milk yield, and fertility in dairy cows*. J. Dairy Sci., 86: 2193-2204.

BERRY D.P., BUCKLEY F., DILLON P., EVANS R.D., RATH M., VEERKAMP R.F. 2002. *Genetic parameters for level and change of body condition score and body weight in dairy cows.* J. Dairy Sci., 85: 2030-2039.

BORKOWSKA D. 2000. *Analiza wpływu wybranych czynników na kondycję krów z gospodarstw indywidualnych*. Med. Wet., 56: 743-745.

CHASSAGNE M., BARNOUIN J., CHACORNAC J.P. 1999. *Risk factors for stilbirth in Holstein heifers under field conditions in France: a prospective survey*. Theriogenology, 51: 1477-1488.

CONTRERAS L.L., RYAN C.M., OVERTON T.R. 2004. *Effects of dry cow grouping strategy and prepartum body condition. Score on performance and health of transition dairy cows*. J. Dairy Sci., 87: 517-523.

DALE H., VIK-MO L., JELLHEIM P.F. 1979. *A field survey of fat mobilization and liver function of dairy cows during early lactation: relationship to energy balance, appetite and ketosis*. Nord. Vet. Med., 31: 97-105.

DECHOW C.G., ROGERS G.W., CLAY J.S. 2002. *Heritability and correlations among body condition score loss, body condition score, production and reproductive performance.* J. Dairy Sci., 85: 3062-3070.

DOMECQ J.J., SKIDMORE A.L., LLOYD J.W., KANEENE J.B. 1997. *Relationship between body condition scores and conception at first artificial insemination in a large dairy herd of high yielding Holstein cows*. J. Dairy Sci., 80: 113-120.

DUFFIELD T.T. 2006. *Minimizing subclinical metabolic diseases in dairy cows.* Adv. Dairy Technol., 18: 43-55.

GEARHART M.A., CURTIS C.R. 1990. *Relationship of changes in condition score to cow health in Holsteins*. J. Dairy Sci., 73: 3132-3140.

HAYIRLI A., GRUMMER R.R., NORDHEIM E.V., CRUMP P.M. 2003. *Models for predicting dry matter intake of Holsteins during the prefresh transition period*. J. Dairy Sci., 86: 1771-1779.

KOWALSKI M. 2005. *Wpływ błędów żywieniowych na przemiany energii u krów w okresie przejściowym*. VI Środkowoeuropejski Kongres Bujatryczny.

KUREK Ł., STEC A. 2005. *Wpływ okresu okołoporodowego i wieku na zawartość wybranych makroelementów, wolnych kwasów tłuszczowych oraz wskaźników funkcji narządów miąższowych u zdrowych krów mlecznych*. Ann. UMCS Lublin, sect. DD, 60 (6): 37-52.

MARKUSFELD O., GALON N., EZRA E. 1997. *Body condition score, health, yield and fertility in dairy cows.* Vet. Rec., 141 (3): 67-72.

OLECHNOWICZ J., JAŚKOWSKI J.M. 2005. *Kondycja, zaburzenia rozrodu i produkcja mleka u krów*. Med. Wet., 61 (9): 972-975.

PEDRON O., CHELI F., SENATORE E., BAROLI D., RIZZI R. 1993. *Effect of body condition score at calving on performance, some blood parameters, and milk fatty acid composition in dairy cows*. J. Dairy Sci., 76: 2528-2535.

PRYCE J.E., NIELSEN B.L., VEERKAMP R.F., SIMM G. 1999. *Genotype and feeding system effects and interaction for health and fertility traits in dairy cattle*. Livestock Prod. Sci., 57: 193-201.

PRYCE J.E., COFFEY M.P., SIMM G. 2001. *The relationship between body condition score and reproductive performance*. J. Dairy Sci., 84: 1508-1515.

SJAUNJA L.O., BAEVRE B., JUNKKARINEN L., PEDERSEN J., SETALA J. 1990. *A Nordic proposal for an energy corrected milk (ECM) formula*. Paper presented at the 27th Session of the ICRPMA, July 2-6, Paris, France.

VEERKAMP R.F., KOENEN E.P.C., DE JONG G. 2001. *Genetic correlations among body condition score, yield, and fertility in first- parity coes estimated by random regression models*. J. Dairy Sci., 84: 2327-2335.

WALTNER S.S., MCNAMARA J.P., HILLERS J.K. 1993. *Relationships of body condition score to production variables in high producting Holstein dairy cattle*. J. Dairy Sci., 76: 3410-3419.

WHITAKER M.A. 1997. *Interpretation of metabolic profiles in dairy cows*. Cattle Practice, 5 (1): 57-60.
WILDMAN E.E., JONES G.M., WAGNER P.E., BOMAN L.R., TROUT H.F., LESCH T.N. 1982. *A dairy cow body condition scoring system and its relationship to selected production characteristics*. J. Dairy Sci., 65: 495-501.
WINNICKA A. 2002. *Wartości referencyjne podstawowych badań laboratoryjnych w weterynarii*. Wyd. SGGW, Warszawa.

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POSTEMBRYONIC DEVELOPMENTAL STAGES OF ASP *ASPIUS ASPIUS* (L.)

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K e y w o r d s: asp, larval development, developmental stages.

A b s t r a c t

Fourteen easily recognizable developmental stages (DS) for larval and juvenile asp *Aspius aspius* (L.) were described, beginning from hatching until the time of complete squamation of juveniles. Rearing temperature ranged between 14.0 and 18.7°C (mean 17.2°C). The proposed system of developmental stages for asp can be used both in laboratory studies and field work in order to follow the course and rate of asp early development.

POSTEMBRIONALNE STADIA ROZWOJOWE BOLENIA *ASPIUS ASPIUS* (L.)

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S ł o w a k l u c z o w e: boleń, rozwój larwalny, stadia rozwojowe.

A b s t r a k t

Czternaście łatwo rozpoznawalnych stadiów rozwojowych bolenia, obejmujących cały rozwój larwalny i początek rozwoju juwenilnego, opisano od wyklucia do całkowicie ułuszczonych osobników młodocianych.. Eksperyment wykonano w wodzie o temp. 14,0 – 18,7°C (średnio 17,2°C). Proponowany system stadiów rozwojowych dla bolenia może być z powodzeniem używany zarówno w pracach laboratoryjnych, jaki i terenie.

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Introduction

Numerous papers on early fish ontogeny present systems of developmental periods, phases and steps suggested by BALON (1975, 1991) (for example KORWIN-KOSSAKOWSKI 1988, LAURILA et al. 1987, PEŇÁZ, GAJDUSEK 1979). However, in many cases there are differences in opinions about description of particular intervals (VASNETSOV et al. 1957 and DMITRIEVA 1960). In some papers early fish ontogeny was described using a system of developmental stages (FALKOWSKI et al. 1988, LUCZYNSKI et al. 1988) which may be used as an accurate, easily recognizable description of early fish development. In many research fish were taken from natural conditions, when variable temperatures, food availability, oxygen concentration, etc., may influence developmental rate of fish, thus the duration of periods, phases and steps in their development also varied.

We adopted the system of developmental stages proposed for coregonids by FALKOWSKI et al. (1988) and LUCZYNSKI et al. (1988) and for common bream, *Abramis brama* by KUCHARCZYK et al. (1998). The system has been based on the instantaneous stages of ontogeny, i.e. on the characteristic moments of development. On the other hand, the numeric description of developmental stages gives possibility to using statistical analysis. Asp *Aspius aspius* (L.), is a cyprinid predator fish, which have an important role in fish farm producing stocking material, especially in Poland.

Larval development of asp has been described by, KAZANSKII 1928, BERG 1949, KRYZHANOVSKII (1949), KOBLITSKAYA (1968, 1981). The autors used the systems of developmental periods, phases and steps, which differ in number and duration. However, the fish for which developmental schemes were, had often been caught from lakes and rivers, so the age and growth rate of examined fish were unknown. Also those systems did non allow statistical analysis of the quantitative aspects of fish development.

In this paper we present the system of easily recognizable developmental stages (DSs) for asp reared under controlled conditions. These stages have been based on the instantaneous intervals of ontogeny, recognized as the characteristic moments in fish development (BALON 1975).

Material and Methods

Asp spawners were caught in the Pierzchalskie Lake (North Poland). Fish were transported to the hatchery and kept in 1000 dm^{-3} tanks with controlled light and thermal conditions (KUJAWA et al. 1999). Fish were artificially reproduced in the laboratory based on the method described by KUJAWA (1998). Water temperature was raised from 4 to 10°C during one week. Later females

obtained carp pituitary extract in two doses (0.4 mg and 3.6 mg kg^{-1}) and males in one dose (2.0 mg kg^{-1}). Time between injection was 24 h. Eggs of asp were obtained 30-36 h after the last injection, mixed with pooled milt obtained from few males and then fertilized using "dry" method. Fertilized eggs were incubated at a constant temperature of 14°C. After hatching the larvae were transferred into a 30 dm^3 tank. Initial stocking density was 75 individuals · dm^{-3}. Fish were fed *ad libitum* with *Artemia* sp. nauplii. Rearing temperature ranged between 14 and 18.7°C (mean 17.2°C), the photoperiod was 18 h light and 6 h dark. Fish were reared for 60 days, until complete squamation, so that the experiment comprised the whole larval period, as well as the last stage of larval development and a beginning of a juvenile period.

Samples of 20 fish were taken beginning from the day of first larvae swimming and then on the every 4 days of rearing. All observations were made on the preserved material in 4% formaline. Each fish was measured (total length, ±0.01 mm) and weighed (wet weight, ±0.001 g). The fish were stained with alizarin Red S and microphotographed. Outlines of fish were drawn from projected photographs. Details were drawn when each fish was observed under a binocular microscope (magnification from 8 to 20 x). After dissection of fish their swimbladder was examined.

The arbitrally chosen developmental stages were numbered from 0 to 13. Each fish was classified to the respective DS. Regression equations for the relationship between DS No. of fish and their length (TL), for DS No. versus age of fish (expressed as number of days after hatching), were calculated. In total, 280 fish were examined.

Results and Discussion

Fourteen developmental stages were described (Table 1). The rearing started at the larval stage, described as DS-0 (average TL 9.0 mm). First scales were observed in fish at stage DS-10 (average TL 24.1 mm). Lateral line formation started in two regions, the first one in the tail part, and later another part (light stained) was formed in the head region. Both parts of the lateral line joined together at the time of completion of the squamation. VASNETSOV et al. (1957), EREMEEVA (1960) and KUCHARCZYK et al. (1997, 1998) observed the same pattern of squamation and of lateral line development in another cyprinid fish species.

Developmental advancement of asp was strongly correlated both with length (Figure 1), and with age of fish (Figure 2). Coefficients of determination were 0.984 and 0.977, respectively. Highly significant correlation between developmental stages (DS No.) and total length of fish (Figure 1) provides the possibility of identifying the length class of fish with the respective DS No.,

Table 1

Developmental stages (DS) of asp *Aspius aspius* (L.)

Developmental stages (DS)	Water temperat. (°C)	Age (days)	Length range, mean (mm)	Characteristic feature
0	14.0	1	7.9-9.3 (8.9)	hatching. yolk sack protruding from the body
1	14.0	7	9.0-10.0 (9.7)	beginning of exogenous (mixed) feeding, caudal fin bud present
2	14.0	11	9.9-10.4 (10.3)	fin rays at the bases of caudal fin, dorsal and anal fin buds present
3	15.5	15	10.2-11.9 (11.0)	fin rays at the bases of dorsal and anal fins, fin rays at the whole length of caudal fin (anterior chamber of swim bladder inflated)
4	16.5	19	11.7-12.9 (12.5)	pelvic fin buds present
5	17.5	22	12.8-15.2 (14.2)	fin rays at the bases of pectoral fins
6	15.0	26	14.9-15.8 (15.5)	fin rays at the bases of pelvic fins (curvature of alimentary tract visible)
7	16.0	30	15.3-19.8 (17.8)	pelvic fins protrude out of the embryonic fold, embryonic fold remnant encompasses pelvic fins
8	17.5	38	19.5-22.4 (20.6)	embryonic fold present only between pelvic and anal fin
9	17.5	42	20.7-24.2 (22.1)	scale primordia present
10	18.0	50	22.3-25.8 (24.1)	sparse scales cover body sides from head to tail
11	18.5	54	23.4-27.9 (26.3)	densely packed scales on body sides, lateral line present on the tail part
12	18.7	58	26.3-30.9 (29.4)	lateral line on the head part present
13	18.5	62	over 30.1	squamation and lateral line completed

similarly as described by LUCZYNSKI et al. (1988) for several *Coregonus* species and by KUCHARCZYK et al. (1998) for common bream. For field research such relationship was very important, because in natural environment fish of the same age may differ in size and developmental advancement according to variable environmental conditions, food supply, etc. (DMITRIEVA 1960). Additionally, such numerical system of description gave possibility to do statistical analysis of fish taken from different environments or rearing conditions (KUCHARCZYK et al. 1998).

The presented system of easy recognizable developmental stages might be used for examining influence of temperature or food supply for early ontogeny in asp during intensive rearing. Easily recognizable developmental stages and related to fish length may be used for the comparative observations of fish growing under different conditions or for evaluation of the quality of stocking material produced in hatcheries.

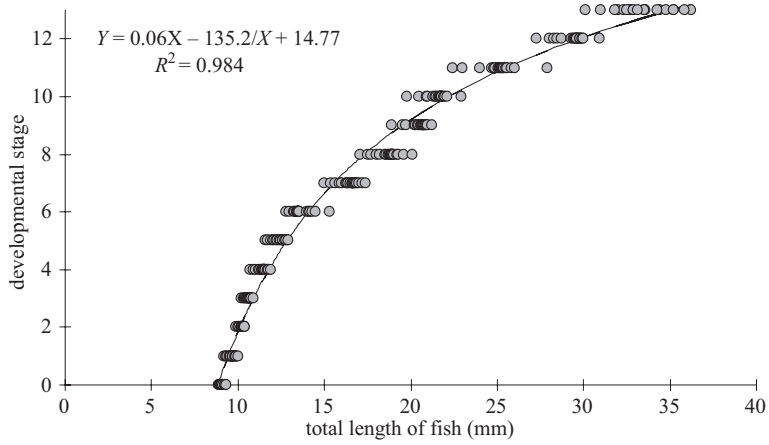

Fig. 1. The relationship between developmental stages (DSs) and length (TL) in asp *Aspius aspius* (L.) larvae and juveniles

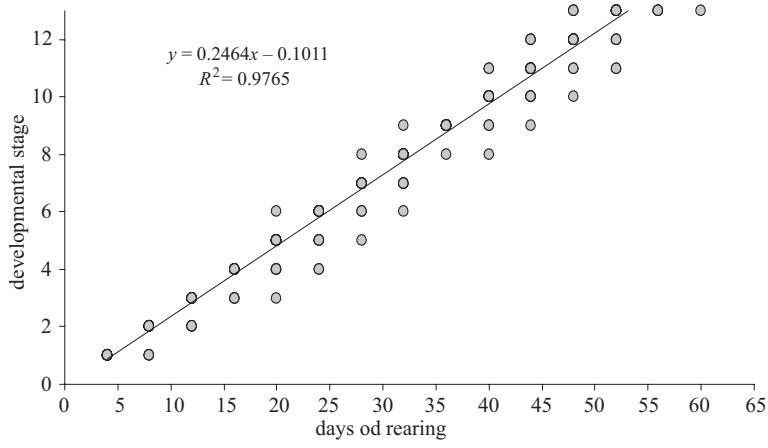

Fig. 2. The relationship between developmental stages (DSs) and age in asp *Aspius aspius* (L.) larvae and juveniles

During analysis of studied larval asp it was found that appearance of some developmental features was stronger correlated with size of fish, than with their age. Such strong relationship resulted from very good conditions of rearing. In nature, the appearance of new feature or group of features in development was always a result of mutual relations between needs of developing larvae and conditions of their environment. In studies, carried out on larval asp, it was found that the rate of morphological development of fish

was strongly related with thermal conditions. The age of larvae has the secondary importance. Asp reared for three weeks at temperature of 10°C reached the weight about 10 mg. In the same time, fish reared at temperature of 31°C weighted on average 135 mg (KUJAWA et al. 1998).

In conclusion we can say that:

1. Reaching of determined stage of morphological development by asp is related with the size of their body independent of age.

2. Strong correlation between developmental stages and fish body length can be used for practical reasons for comparisons between larvae from different conditions.

3. The numerical system of developmental stages for asp may be also used in fishery practice for evaluation of stocking material quality in hatcheries.

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References

BALON E. K. 1975. *Terminology of intervals in fish development*. J. Fish. Res. Board Can., 32: 1663-1670.

BALON E. K. 1991. *Probable evolution of the coelacanth's reproductive style; lecithotrophy and orally feeding embryos in cichlid fishes and in Latimeria chalumnae*. Env. Biol. Biol. Fish, 32: 249-265.

BERG L. S. 1949. *Ryby presnykh vod SSSR i copredel;nykh stron*. Moskva.

DMITRIEVA E. N. 1960. *Developmental steps on the non-migratory bream*. Trud. Inst. Morph. Zhiv., 28: 41-77 (in Russian).

EREMEEVA J. F. 1960. *Developmental steps on the bream from Rybinskoye reservoir*. Trud. Inst. Morph. Zhiv., 28: 79-106 (in Russian).

FALKOWSKI S., LUCZYNSKI M. VUORINEN J. 1988. *Embryonic and larval development of whitefish* (*Coregonus lavaretus* L.) *and peled (C. peled* Gmelin) *hybrids*. Finnish Fish. Res., 9: 71-79.

KAZANSKII V., I. 1928. *K morfologii i sistematike lichinochnykh stadii karpovykh ryb tipa vobly* (*Rutilus rutilus caspicus* Jak). Trudy Astrakh. Nauch. Ryboch., 3: 1-28.

KOBLITSKAYA A., F. 1966. *Opredelitel molodi ryb delty Volgi*. Izd. „Nauka" Moskva, 62-65.

KOBLITSKAYA A.F. 1981. *Opredelitel molodi presnovodnykh ryb*. Legkaya i Pishchevaya Promyshlennost, Moskva, 208 pp.

KORWIN-KOSSAKOWSKI M. 1988. *Larval development of carp*, *Cyprinus carpio* L., *in acidic water*. J. Fish Biol., 32: 17-26.

KRYZHANOVSKII S. G. 1949. *Ekologo-morfologicheskie zakonomernosti razvitiya karpovykh, v'yunovykh i somovykh ryb* (*Cyprinoidei i Siluroidei*). Trud. Inst. Morf. Zhiv., 1: 5-165.

KUCHARCZYK D., ŁUCZYŃSKI M., KUJAWA R., CZERKIES P. 1997. *Effect of temperature on embryonic and larval development of bream* (*Abramis brama* L.). Aquatic Sci., 59: 214-224.

KUCHARCZYK D., ŁUCZYŃSKI M., KUJAWA R., KAMIŃSKI R., ULIKOWSKI D., BRZUZAN P. 1998. *Influence of temperature and food on early development of bream* (*Abramis brama* L.). Arch. Hydrobiol., 141: 243-256.

KUJAWA R. 1998. *Analiza wybranych elementów biologii wczesnych stadiów rozwojowych bolenia*, (*Aspius aspius* L.) *w warunkach kontrolowanych*. ART w Olsztynie (praca doktorska), ss. 80.

KUJAWA R., KUCHARCZYK D., MAMCARZ A. 1998. *Wpływ temperatury na tempo wzrostu larw bolenia Aspius aspius* (L.) *podchowywanych w warunkach kontrolowanych*. Kom. Ryb., 6: 29-31.

KUJAWA R., KUCHARCZYK D., MAMCARZ A. 1999. *A model system for keeping spawners of wild and domestic fish before artificial spawning*. Aquacult. Eng., 20: 85-89.

LAURILLA S., PIIRONEN J., HOLOPAINEN F. 1987. *Notes on egg development and larval and juvenile growth of crucian carp* (*Carassius carassius* (L.)) Ann. Zool. Fennici, 24: 315-321.

LUCZYNSKI M., FALKOWSKI S., KOPECKI T. 1988. *Larval development in four coregonid species* (*Coregonus albula, C. lavaretus, C. muksun* and *C. peled*). Finnish Fish. Res., 9: 61-69.

PEŇÁZ M., GAJDUSEK J. 1979. *Early development of bream*, *Abramis brama from the water reservoir Mostiste, CSSR*. Fol. Zool., 28: 347-360.

VASNETSOV V. V., EREMEEVA E.F., LANGE N. O., DMITRIEVA E. V., BRAGINSKAYA R. YA. 1957. *Developmental steps on the commercial semi-migratory fish from Volga River and Don River – bream, carp, roach and pikeperch*. Trud. Inst. Morph. Zhiv., 16: 7-76 (in Russian).

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BACTERIAL *amoA* AND 16S rRNA GENES EXPRESSION IN ACTIVATED SLUDGE DURING AERATION PHASE IN SEQUENCING BATCH REACTOR

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K e y w o r d s: *amoA*, 16S rRNA, RT-PCR, sequencing batch reactor, nitrogen compounds.

A b s t r a c t

Transcription levels of *amoA* mRNA and 16S rRNA during an aeration phase in SBR (sequencing batch rector) were analysed using reverse transcription PCR (RT-PCR) and a relationship between *amoA* mRNA expression in activated sludge and changes in nitrogen compounds concentrations was examined. Expression of *amoA* gene reached a detectable level two hours after a beginning of the aeration phase and did not disappear until its end. Lack of detectable *amoA* expression at the beginning of the aeration phase was in agreement with nitrite concentration decrease. Gradual increase in *amoA* transcripts level observed during next hours indicated a rise in ammonia-oxidising bacteria activity, a detectable change in nitrite concentration was observed 2 h after the RT-PCR signal was provided for the first time. Changes in 16S rRNA transcription level indicated that metabolic activity of the activated sludge bacterial community increased gradually during the aeration phase.

EKSPRESJA GENÓW amoA ORAZ 16S rRNA W OSADZIE CZYNNYM PODCZAS NAPOWIETRZANIA W REAKTORZE SBR

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S ł o w a k l u c z o w e: *amoA*, 16S rRNA, RT-PCR, reaktor SBR, związki azotu.

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A b s t r a k t

W pracy badano, z wykorzystaniem techniki Reverse-Transcription-PCR (RT-PCR), poziom transkrypcji mRNA genów *amoA* oraz 16S rRNA podczas fazy napowietrzania w reaktorze SBR. Określono zależność między ekspresją mRNA genu *amoA* w osadzie czynnym a zmianami stężenia związków azotu w reaktorze. Ekspresję genu *amoA* po raz pierwszy stwierdzono 2 h po rozpoczęciu napowietrzania, i nie zanikła ona aż do końca fazy napowietrzania. Brak ekspresji genu *amoA* na początku fazy napowietrzania pokrywał się ze spadkiem stężenia azotu azotanowego (III) w reaktorze. Stopniowy wzrost ilości transkryptów genu *amoA*, obserwowany w kolejnych godzinach fazy napowietrzania, wskazywał na zwiększanie aktywności bakterii utleniających amoniak. Wzrost stężenia azotu azotanowego (III) stwierdzono od ok. 5. h fazy napowietrzania. Zmiany w transkrypcji 16S rRNA wskazywały, że aktywność metaboliczna zbiorowisk mikroorganizmów osadu czynnego ulegała stopniowemu zwiększaniu w fazie napowietrzania.

Introduction

In municipal wastewater treatment plants nitrification is commonly a nitrogen removal limiting process. The first nitrification reaction, which is ammonia oxidation to hydroxyloamine, is catalysed by ammonia monooxygenase (AMO), an enzyme exclusive to ammonia-oxidising bacteria (AOB). The physiological abundance and activity of AOB in wastewater processing is critical in designing and operating of wastewater treatment systems, particularly since these organisms display low growth rates and high sensitivity to environmental disturbances and inhibitors.

An important aspect of microorganisms activity relates to reactor design. The SBR accomplishes equalisation, aeration, and clarification in a timed sequence in a single reactor basin. By varying the operating strategy different oxygen conditions can be achieved to encourage the growth of desirable microorganisms. In SBR reactors nitrification occurs simultaneously with removal of carbonaceous oxygen demand (COD); activated sludge in these reactors contains both heterotrophs and nitrifiers that have to compete for resources such as oxygen (ZHU, CHEN 2001). Observation of how *amoA* gene transcription varies with time allows monitoring of ammonia-oxidising bacteria activity in SBR cycle. Obtained information can be applied in order to improve wastewater treatment effectiveness e.g. by introducing additional ammonia dose when AOB activity is the highest.

Gene expression occurs in two steps. In the first step the information encoded in DNA is transcripted into a molecule of RNA and then the information encoded in the nucleotides of mRNA is translated into a defined sequence of amino acids in a protein. By applying molecular techniques that focus on markers of *in situ* metabolism, such as mRNA, metabolic activity of bacteria can be measured.

Molecules of mRNA are typically short-lived, so mRNA can be used as an indicator of living cells or the specific activity in a complex microbial community. The *amo* operon genes are present in all known ammonia oxidisers (SAYAVEDRA-SOTO et al. 1998), therefore the study of the *amo* operon genes targets a whole physiological group of ammonia-oxidising bacteria and *amoA* mRNA oriented transcript analysis may be related to an *in situ* activity of AOB (EBIE et al. 2004).

Many bacterial species are known to vary their ribosome number in accordance with their cellular activity – metabolically active bacteria contain more rRNA than resting or starving cells (WAGNER 1994). Due to a longer half-life of rRNA species and their variable retention following a variety of bacterial stress treatments rRNA is less accurate indicator of viability than mRNA targets (VILLARINO et al. 2000). Nevertheless, 16S rRNA based analysis is best suited for general information on total cellular activity in given environmental conditions.

In this study the expression of *amoA* and 16S rRNA genes during aeration phase in SBR cycle was analysed using RT-PCR and the relationship between *amoA* mRNA expression in activated sludge and changes in nitrogen species concentrations was examined.

Materials and Methods

SBR operation. In the experiment a 3.0 l sequencing batch reactor was employed. Seed sludge was collected from a conventional municipal wastewater treatment plant in Olsztynek (Poland). The SBR operated in 24-hour cycle, with the following operating strategy: aerobic (23 h), settle (0.50 h) decant (0.25 h) and filling (0.25 h). The mean cellular residence time (sludge age) was 25 days and the total suspended solids (TSS) averaged 2 500 mg $TSS \cdot l^{-1}$. Reactor operated at about 2 $g \cdot l^{-1}$ of dissolved oxygen. The temperature was maintained at 20°C and pH was kept between 7 and 8. During the filling period 1.0 l of synthetic wastewater was added to the reactor to make the final working volume of 2.5 l. The artificial wastewater was composed of CH_3COOH (650 mg $COD \cdot l^{-1}$), NH_4Cl (300 mg $N\text{-}NH_4 \cdot l^{-1}$), $Na_2HPO_4 \cdot 12H_2O$ (46.0 mg $\cdot l^{-1}$), NaCl (10.0 mg $\cdot l^{-1}$), KCl (4.7 mg $\cdot l^{-1}$), $CaCl_2 \cdot 2H_2O$ (4.7 mg $\cdot l^{-1}$), $MgSO_4 \cdot 7H_2O$ (16.7 mg $\cdot l^{-1}$), $NaHCO_3$ (240.0 mg $\cdot l^{-1}$), Na_2CO_3 (160.0 mg $\cdot l^{-1}$), $FeCl_3 \cdot 6H_2O$, $MnSO_4 \cdot H_2O$, $ZnSO_4$, $CuSO_4$ (< 0.2 mg $\cdot l^{-1}$), (COEHLO et al. 2000, modified) leading to final concentrations at the beginning of aeration phase of about 260 mg $COD \cdot l^{-1}$ and 120 mg $N\text{-}NH_4 \cdot l^{-1}$.

Analytical measurements. Everyday sampling was made at the influent and effluent of the reactor. During the whole experiment nitrite accumulation was

observed. The adaptation period had lasted 50 days until the range of changes of particular parameters within 7 days' time did not exceed 10-15% (BARBUSIŃSKI, KOŚCIELNIAK 1995). On 65-th day of the experiment, after 15 days of stable reactor operation, samples were taken to describe concentration profiles during the SBR cycle. Sampling was performed every hour at the beginning of SBR cycle, after 8 hours of aeration samples were collected in 2-6-hour intervals. All samples were filtered using 0.2 μm micro-pore filter before being assayed. The following parameters were determined in wastewater: organic compounds expressed as total and dissolved COD (PN-74/C-04578.03], ammonia by direct Nesslerization method [PN-C-04576-4:1994], total suspended solids by drying at 103-105°C [PN-EN 12879:2004], nitrite and nitrate concentrations by colorimetric methods [PN-73/C-04576.06] and [PN-82/C-04576.08], respectively (Polish Committee for Standardisation, 2004).

RNA extraction and purification. All reagents and processes were prepared with sterile, disposable, nuclease-free equipment. For RNA extraction sludge pellets had been stored in RNA Later (Ambion, USA) to maintain the quality of extracted RNA. RNA was extracted in duplicate from approximately 0.02 g of centrifuged sludge sample using a commercial RNA isolation kit (Total RNA, A&A Biotechnology, Poland). To eliminate contaminating genomic DNA, 3 U of RQ1 RNase-Free DNase (Promega, USA) was added to each RNA extract before incubation at 37°C for 30 min. Quality and quantity of isolated RNA was measured spectrophotometrically using Biotech Photometer (WPA, UK). There were no observable variations between RNA extracts. Purified RNA samples from both isolations were immediately used for further analyses.

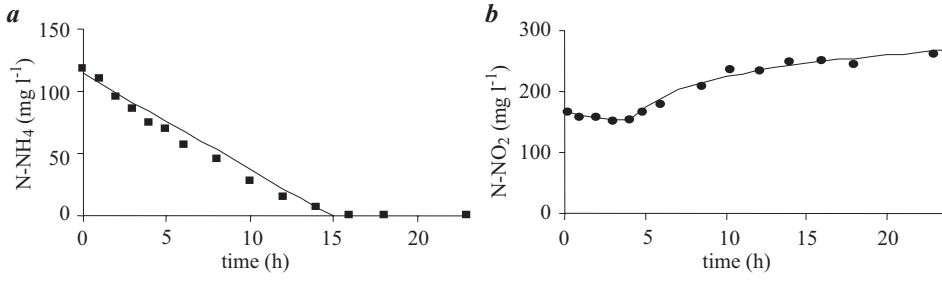
RT-PCR. The reverse transcription (RT) reaction was carried out using the RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas) with random hexamer primers. A half of microgram of total RNA sample, 1 μl of random hexamer primer ($0.2\ \mu g \cdot \mu l^{-1}$) and distilled water to make the final volume of 12 μl was added to PCR tube. After the mixture was incubated at 70°C for 10 min to denature secondary structures of rRNA, the tube was placed on ice and the following mixture was added: 4 μl of 5x reaction buffer [250 mM Tris/HCl (pH 8.3 at 25°C), 250 mM KCl, $MgCl_2$, 50 mM dithiothreitol (DTT)], 1 μl of ribonuclease inhibitor ($20\ U \cdot \mu l^{-1}$) and 2 μl of 10 mM dNTP mix. The mixture was incubated at 25°C for 5 min to allow annealing of random hexamer primers. After adding 1 μl of RevertAid M-MuLV reverse transcriptase ($200\ U \cdot \mu l^{-1}$) reaction was carried out at 25°C for 10 min and at 42°C for additional 60 min.

PCR. PCR was carried out with oligonucleotide primers 301F (5'-GAC--TGGGACTTCTGGCTGGACTGGAA-3') and 302R (5'-TTTGATCCCCTCT--GGAAAGCCTTCTTC-3') (NORTON et al. 2002). This primer set amplifies a core region of 675 bp of *amoA* gene from both pure cultures and soil DNA

templates. The 16S rRNA was amplified with 8F (5'-GTGCTGCAGAGAG--TTTGATCCTGGCTCAG-3') and 536R (5'-CACGGATCCGTATTACCGCGGC--TGCTG-3') primer set (MISKIN et al. 1999) which amplifies the 5' end (~ 530 bp) of bacterial 16S rRNA. Each 30 μl of PCR reaction mixture contained 5 μl of synthesised first strand cDNA, 1 μl of each primer (20 pmol · μl^{-1}), 1 μl of $MgCl_2$ (50 mM), 1 μl of dNTP mixture (10 mM of each dNTP), 0.5 μl of Delta 2 polymerase (DNAGdańsk, Poland), 3 μl of buffer mixture supplied with the DNA polymerase and 17.5 μl of distilled water. PCR was performed using an Eppendorf thermal cycler. For ribosomal cDNAs the following cycling parameters were employed: 95°C for 5 min, 30 cycles of 1 min at 95°C, 1 min at 55°C and 1 min at 72°C followed by a final extension step at 72°C for 5 min. For *amoA* amplification similar cycling parameters were used but annealing temperature of 54°C was employed. Contamination of RNA templates by DNA was controlled by the inclusion of PCR reactions containing RNA preparations that were not reverse-transcribed. PCR reaction products were analysed electrophoretically in a 1% agarose gel stained with ethidium bromide. The gel was visualised with UV light and photographed. *AmoA* and 16S rRNA genes expression was evaluated by densitometry scanning of agarose gels containing RT-PCR-amplified products using KODAK 1D 3.6 Image Analysis Software (Eastman Kodak Company, USA).

Results

The samples were taken on 65-th day of the experiment, after 15 days of stable reactor operation. In this period nitrification effectiveness was 99%, total suspended solids averaged 2 500 mg TSS · l^{-1}, COD/N-NH_4 ratio at the beginning of aeration phase was 3.8, N-NH_4 load to biomass was 0.05 g · · $TSS^{-1} \cdot d^{-1}$ and COD load to biomass was 0.1 g · $TSS^{-1} \cdot d^{-1}$. Figure 1. depicts changes of inorganic nitrogen species concentrations during the aerobic stage in the SBR cycle. A significant decrease in ammonia nitrogen content was observed at the end of the 15-th hour of aeration – the concentration dropped from initial 117 mg · l^{-1} to 0.3 mg · l^{-1}. Over 99% of ammonia nitrogen presented in wastewater was oxidised or used for biomass synthesis, an ammonia decrease rate was 3.08 mg N-NH_4 · $TSS^{-1} \cdot h^{-1}$. After 4 hours of aeration the lowest nitrites concentration was observed – 151.9 mg · l^{-1}. From 4-th hour of aeration concentration of nitrites started to increase with an increasing rate of 2.04 mg N-NO_2 · $TSS^{-1} \cdot h^{-1}$ and reached maximum (266 mg · l^{-1}) after 23 h of aeration. Nitrates were gradually accumulated in reactor (oxidation rate 0.41 mg N-NO_3 · $TSS^{-1} \cdot h^{-1}$) and reached a concentration of 36.6 mg N-NO_3 · l^{-1} at the end of the aeration phase.



Parameter	Constant rate (k)	Initial rate (r)
$N\text{-}NH_4$	7.700 $mg \cdot l^{-1} \cdot h^{-1}$	3.08 mg $N\text{-}NH_4 \cdot g\ TSS^{-1} \cdot h^{-1}$
$N\text{-}NO_2$	0.022 h^{-1}	1.45 mg $N\text{-}NO_2 \cdot g\ TSS^{-1} \cdot h^{-1}$
	0.170 h^{-1}	2.04 mg $N\text{-}NO_2 \cdot g\ TSS^{-1} \cdot h^{-1}$
$N\text{-}NO_3$	0.060 h^{-1}	0.41 mg $N\text{-}NO_3 \cdot g\ TSS^{-1} \cdot h^{-1}$

Fig. 1. Changes of inorganic nitrogen species during the 23-hour aeration phase: *a* – $N\text{-}NH_4$, *b* – $N\text{-}NO_2$, *c* – $N\text{-}NO_3$. Constant rate (k) and initial rate (r) values are given in the table (TSS – total suspended solids)

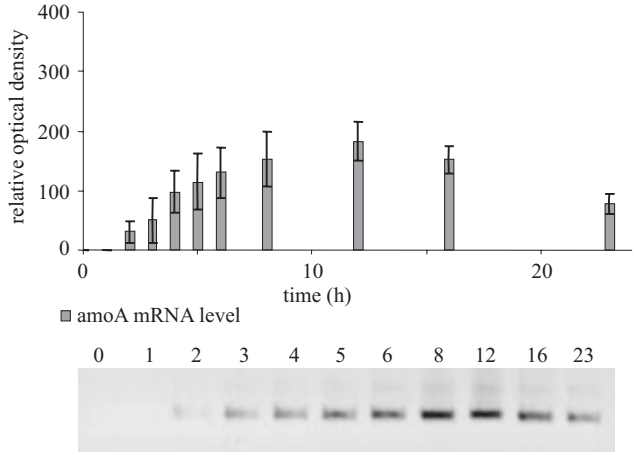


Fig. 2. The graph presents the *amoA* gene expression during SBR cycle evaluated by densitometry scanning of electrophoresed PCR products (10 µl of product per lane) corresponding to level of *amoA* transcripts. Numbers in photograph demonstrate hours during aeration phase in SBR cycle in which activated sludge samples for molecular analysis were taken

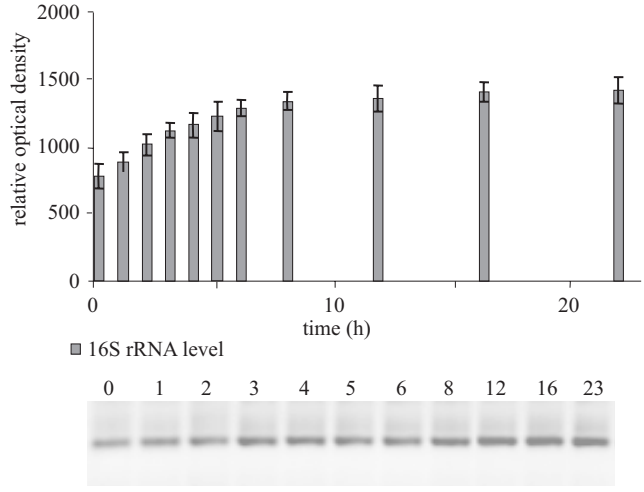


Fig. 3. The graph presents the 16S rDNA gene expression during SBR cycle evaluated by densitometry scanning of electrophoresed PCR products (3 μl of product per lane) corresponding to 16S rRNA level. Numbers in photograph demonstrate hours during aeration phase in SBR cycle in which activated sludge samples for molecular analysis were taken

The RNA extraction protocol described above was found to be applicable to RNA isolation from activated sludge. Total RNA from 0.02 g of centrifuged sludge sample was of a purity and quantity suitable for molecular analysis by RT-PCR procedures. The suitability of extracted RNA for molecular analysis was confirmed by RT-PCR analyses of both *amoA* and 16S rDNA genes transcripts (Figure 2, Figure 3). In case of *amoA,* densitometry scanning showed detectable expression of the gene about 2 hours after the beginning of aeration (Figure 2). The *amoA* expression increased gradually through next hours and reached its maximum 12 h after the beginning of aeration period then it started to decrease. Densitometry scanning of electrophoresed PCR products corresponding with 16S rRNA gene expression indicated that transcription level gradually increased during the aeration phase (Figure 3). As measured by relative optical density at the end of the cycle about twofold increase in bacterial activity was observed in comparison with the start of aeration.

Discussion

In the present study, RT-PCR amplification was used to analyse expression of bacterial *amoA* and 16S rRNA genes during a 23-hour SBR aeration cycle and relationships between *amoA* mRNA expression in activated sludge and changes in nitrogen compounds concentrations were examined.

In the experiment *amoA* primers 301F and 302R (NORTON et al. 2002) were used instead of Rotthauwe primers (ROTTHAUWE et al. 1997) generally used in AOB diversity surveys (PURKHOLD et al. 2000). 301F/302R primer set allows getting much longer PCR product, which is important in case of identifying active species of AOBs, and possesses very good coverage of AOB diversity in the same time.

Expression of *amoA* at detectable level occurred after two hours of aeration and gradually increased. AOI and coworkers (2004b) showed that *amoA* mRNA level increased almost immediately in response to the sudden exposure to ammonia, but in cited experiment no organic substances were present in synthetic wastewater. In our experiment influent COD/N-NH_4 ratio was about 4 so in the reactor simultaneously nitrification and organic carbon removal occurred. Lack of *amoA* expression at the beginning of the aeration phase was probably caused by high organic carbon concentration which stimulated heterotrophic bacteria rather than nitrifying microorganisms. Fast growing heterotrophic microorganisms compete for oxygen with nitrifiers and may produce intermediary metabolic byproducts, which inhibit ammonia oxidising bacteria (GIESEKE et al. 2001). Presented approach provides a "mean *amoA* transcription response" of all AOBs. It might be possible that individual AOB populations show different cellular *amoA* levels during SBR cycle (SAYAVEDRA-SOTO et al. 1998).

Lack of ammonia oxidation activity at the beginning of experiment was in agreement with nitrite concentration decrease. During the first 4 hours of aeration a nitrite concentration slightly decreased. The observed decrease may have resulted from microaerophilic dentrification in activated sludge flocs, which was favored by high organic carbon load. Increase in *amoA* transcripts level depicted gradual increase in ammonia-oxidising bacteria activity but detectable increase in nitrite concentration was observed just 2 h after a signal concerned with ammonia-oxidising bacteria activity was provided for the first time. Gradual increase in *amoA* transcripts level coincided with increase in N-NO_2 concentration during next hours of aeration. The highest *amoA* expression was observed after 12 h of aeration. Despite the fact that from 15-th hour of aeration ammonia was already depleted in reactor and nitrite concentration did not change noticeably the level of *amoA* transcripts decreased slowly till the end of SBR cycle. AOI and coworkers (2004 a) hypothesised that the possible reason for slow decrease of *amoA* mRNA level is that ammonia-oxidising bacteria sacrifice the strict regulation of AMO to conserve energy when cell activity decreases because AMO seems to be the initial key transcript in the cell during the recovery of cell activity.

Changes in 16S rRNA transcription level showed that metabolic activity of the activated sludge bacterial community appeared to increase substantially

during the SBR cycle. Increase in metabolic activity of activated sludge could have been caused by new bacterial cells generation as well as because of increase in other metabolic functions. The research has proved that 16S rRNA oriented analysis may give a general idea about microorganism's activity without possibility to study specific bacterial consortia responses.

Conclusions

– *amoA* expression in the bench-scale activated sludge reactor with nitrification and organic carbon removal can be detected without previous cultivation by RT-PCR with 301F/302R primer set,

– at given artificial wastewater composition *amoA* gene expression reached a detectable level two hours after the beginning of aeration phase and did not disappear until its end, the highest AOB activity was observed after 12 h of aeration,

– changes in nitrite concentration during aeration phase were bounded with AOB activity estimated on *amoA* expression level. Changes in 16S rRNA transcription level showed about twofold increase in bacterial activity in comparison with the start of aeration, this analysis gives a general idea about microorganism's activity.

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References

Aoi Y., Shiramasa Y., Masaki Y., Tsuneda S., Hirata A., Kitayama A., Nagamune T. 2004a. *Expression of amoA mRNA in wastewater treatment processes examined by competitive RT-PCR*. J. Biotech., 111: 111-120.

Aoi Y., Masaki S., Tsuneda S., Hirata A. 2004b. *Quantitative analysis of amoA mRNA expression as a new biomarker of ammonia oxidation activities in a complex microbial community*. Lett. Appl. Microbiol., 39: 477-482.

Barbusiński K., Kościelniak H. 1995. *Influence of substrate loading intensity on floc size in activated sludge process*. Water Res., 29: 1703-1710.

Coehlo M.A.Z., Russo C., Araujo O.Q.F. 2000. *Optimization of sequencing batch reactor for biological nitrogen removal*. Water Res., 34: 2809-2817.

Ebie Y., Noda N., Miura H., Matsumura M., Tsuneda S., Hirata A., Inamori Y. 2004. *Comparative analysis of genetic diversity and expression of amoA in wastewater treatment processes.* Appl. Microbiol. Biotechnol., 64: 740-744.

Gieseke A., Purkhold U., Wagner M., Amann R., Schramm A. 2001. *Community structure and activity dynamics of nitrifying bacteria in a phosphate-removing* biofilm. Appl. Environ. Microbiol., 67: 1351-1362.

Miskin I.P., Farrimond P., Head I.M. 1999. *Identification of novel bacterial lineages as active members of microbial populations in a freshwater sediment using a rapid RNA extraction procedure and RT-PCR*. Microbiology, 145: 1977-1987.

NORTON J.M., ALZERRECA J.J., SUWA J., KLOTZ M.G. 2002. *Diversity of ammonia monooxygenase operon in autotrophic ammonia-oxidizing bacteria*. Arch. Microbiol., 177: 139-149.

Polish Committee for Standardisation, 2004. http://www.pkn.pl

PURKHOLD U, POMMERING-ROSER A., JURETSCHKO S., SCHMID M.C., KOOPS H-P., WAGNER M. 2000. *Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: implications for molecular diversity surveys*. Appl. Environ. Microbiol., 66: 5368-5382.

ROTTHAUWE J.-H., WITZEL K.-P., LIESACK W. 1997. *The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations*. Appl. Environ. Microbiol., 63: 4704-4712.

SAYAVEDRA-SOTO L.A., HOMMES N.G., ALZERRECA J.J., ARP D.J., NORTON J.M., KLOTZ M.G. 1998. *Transcription of amoC, amoA and amoB genes in Nitrosomonas europaea and Nitrosospira sp. NpAV*. FEMS Microbiol. Lett., 167: 81-88.

VILLARINO A., BOUVET O.M., REGNAULT B., MARTIN-DELAUTRE S., GRIMONT P.A.D. 2000. *Exploring the frontier between life and death in Escherichia coli: evaluation of different viability markers in live and heat- or UV-killed cells*. Res. Microbiol., 151: 755-768.

WAGNER R. 1994. *The regulation of ribosomal RNA synthesis and bacterial cell growth*. Arch. Microbiol., 161: 100-106.

ZHU S., CHEN S. 2001. *Effects of organic carbon on nitrification rate in fixed film biofilters*. Aquacult. Engin., 25: 1-11.

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KINETICS OF ORTHOPHOSPHATE RELEASE AND UPTAKE IN THE VOLATILE FATTY ACID-FED SEQUENCING BATCH REACTOR TREATING DAIRY WASTE WATER

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K e y w o r d s: activated sludge, SBR, external carbon source, volatile fatty acids (VFAs), orthophosphate release and uptake, reaction kinetics.

A b s t r a c t

One of the methods to increase the effectiveness of phosphorus removal in Sequencing Batch Reactors (SBR) is the application of Volatile Fatty Acids (VFAs). Effectiveness of their activity changes depending on the application period.

The aim of the study was to determine in the dynamic conditions the effect of acetic, propionic and butyric acids on the kinetics of orthophosphate release and uptake, in the mixing phase and the aeration phase, respectively. Orthophosphate transformations were examined in the laboratory-scale SBR treating dairy waste water in the presence of VFAs.

It was found that VFAs have effect on the rate of orthophosphates release and uptake rate. Along with the increasing quantity of the applied propionic and butyric acids the increased rate of orthophosphate release and uptake was observed in the whole examined range of concentrations (COD increase from 50 mg to 2100 mg $O_2 \cdot dm^{-3}$). Acetic acid dosed in the amount that elevated the COD level from 50 to 1400 mg $O_2 \cdot dm^{-3}$ contributed to the growth of both rates. In that case, acetic acid was more effective than propionic and butyric acids. At the highest dose of the acetic acid (COD of the waste water increased to 2100 mg $O_2 \cdot dm^{-3}$), both rates decreased.

It was also determined that higher amounts of the released and bound orthophosphate, in the presence of the particular acid, are not equivalent to higher efficiency of phosphorus removal. The evidence was the rates of orthophosphate release and uptake in the presence of acetic acid – in most cases higher than for the propionic and butyric acids. At the same time, the effectiveness of orthophosphate release in the reactor with acetic acid was not always higher than that observed in the presence of the propionic and butyric acids.

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KINETYKA UWALNIANIA I WIĄZANIA ORTOFOSFORANÓW W REAKTORZE TYPU SBR OCZYSZCZAJĄCYM ŚCIEKI MLECZARSKIE W OBECNOŚCI WYBRANYCH LOTNYCH KWASÓW TŁUSZCZOWYCH (LKT)

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S ł o w a k l u c z o w e: osad czynny, reaktory SBR, zewnętrzne źródło węgla, lotne kwasy tłuszczowe (LKT), uwalnianie i wiązanie ortofosforanów, kinetyka reakcji.

A b s t r a k t

Jednym ze sposobów podwyższania sprawności usuwania fosforu w komorach typu SBR jest doprowadzanie lotnych kwasów tłuszczowych (LKT). Efektywność działania kwasów zmienia się w zależności od czasu ich aplikacji.

Celem badań było określenie w warunkach dynamicznych wpływu kwasów octowego, propionowego i masłowego na kinetykę uwalniania i wiązania ortofosforanów, odpowiednio w fazie mieszania i napowietrzania. W skali laboratoryjnej przebadano przemiany ortofosforanów w komorze SBR oczyszczającej ścieki mleczarskie w obecności LKT.

Stwierdzono, że LKT wpływają na szybkość uwalniania i wiązania ortofosforanów. Zwiększanie ilości doprowadzanych kwasów propionowego i masłowego powodowało wzrost szybkości uwalniania i wiązania ortofosforanów w całym zakresie przebadanego stężenia (wzrost stężenia ChZT od 50 mg do 2100 mg $O_2 \cdot dm^{-3}$). Doprowadzanie kwasu octowego w ilościach powodujących wzrost stężenia od 50 do 1400 mg $O_2 \cdot dm^{-3}$ przyczyniało się do zwiększania obu szybkości. Wtedy też kwas octowy był bardziej efektywny niż kwasy propionowy i masłowy. Dla najwyższej dawki kwasu octowego (wzrost ChZT ścieków o 2100 mg $O_2 \cdot dm^{-3}$) zanotowano spadek obu szybkości.

Ustalono także, że większe ilości uwalnianych i wiązanych ortofosforanów w obecności danego kwasu, nie oznaczają wyższej sprawności procesu usuwania fosforu. Świadczą o tym szybkości uwalniania i wiązania ortofosforanów w obecności kwasu octowego – w większości przypadków wyższe niż dla kwasów propionowego i masłowego. W tym samym czasie sprawność usuwania ortofosforanów w reaktorze z kwasem octowym nie zawsze była wyższa od efektywności tego procesu w obecności kwasów propionowego i masłowego.

Introduction

The process of intracellular phosphorus uptake by the activated sludge, applicable in enhanced biological phosphate removal (EBPR) reactors, occurs in activated sludge tanks operating in anaerobic-aerobic (A/A) mode. The operational scheme is:

– in anaerobic conditions polyphosphate-accumulating bacteria (PABs) take up simple organic compounds (e.g. volatile fatty acids, VFAs) from waste water and synthesize poly-β-hydroxybutyric acid (PHB). The required energy is obtained from the hydrolysis of intracellular polyphosphate. Generated phosphates are released to the liquid;

– in aerobic conditions PHB becomes an endogenous source of energy and carbon for the growth of new cells. Surplus energy from PHB breakdown is retained in the cell due to the intracellular polyphosphate (PP) accumulation. As an effect, phosphates can be taken up from the interstitial water and accumulated as PP by the sludge (WENTZEL et al. 1991).

Orthophosphate release in anaerobic conditions is among others driven by the availability of easily-degradable organic compounds. Therefore, one of the methods to increase the efficiency of an EBPR activated-sludge system, including SBR, is to provide a so-called external carbon source (ARUN et al. 1989, SATOH et al. 1992, TAM 1992, RANDALL et al. 1997, JANCZUKOWICZ 2005). The method consists in extra feeding to the biologically-treated waste water with various substances comprising the source of carbon to phosphorus-removing organisms. Among those substances are methanol, sodium acetate, fermented sludge from municipal WWTP or the technical variety of VFAs. Data reported in the literature are unambiguous referring to acetate acid as the most effective in municipal waste water treatment (WENTZEL et al. 1991, TAM 1992). As for the specifically composed waste water, other acids prove more useful (RANDALL et al. 1997, RUSTRIAN et al. 1997). Attention is also paid to the fact that the acids' efficiency depends on the application time. Acids of low-efficiency in a single SBR cycle may turn out very efficient in the long run (RANDALL, LIU 2002, CHEN et al. 2004).

Still, there are no data in the literature on the relationship between the rates of phosphate release and uptake in the mix-phase and aeration phase and the type of organic substratum applied as external carbon source.

Study goal and scope

The goal of the study was to determine the effect of acetate, propionic and butyric acids on the kinetics of orthophosphate release and uptake in an SBR treating dairy wastewater.

The scope of the study was to determine

– the rate of orthophosphate release and uptake, depending on the dose of applied acids,

– the effect of the type of applied acid on the kinetics of phosphate release and uptake.

Methods

The experiment was run in four similar SBR reactors (R1, R2, R3, R4) with the unit active volume of 2.8 dm^3. The reactors were equipped with a thermo-

stat and covers restricting oxygen diffusion to the waste water during the mix phase. The contents, depending on the cycle phase, were mixed with magnetic stirrers (40 rpm) or aerated with compressed air (Figure 1). Oxygen concentration in the aeration phase was maintained above 2.5 mg $O_2 \cdot dm^{-3}$. The mix phase lasted 2.0 h, the aeration phase 5.0 h, and the settle phase 1.0 h.

Untreated waste water was dosed to the reactors at the start of the mix phase (three times a day) and the treated waste water was carried out after the sedimentation phase. The daily amount of the inflowing waste water was constant in all experiment series and equalled 25% of the reactor's active volume. Waste water temperature was maintained around 20°C.

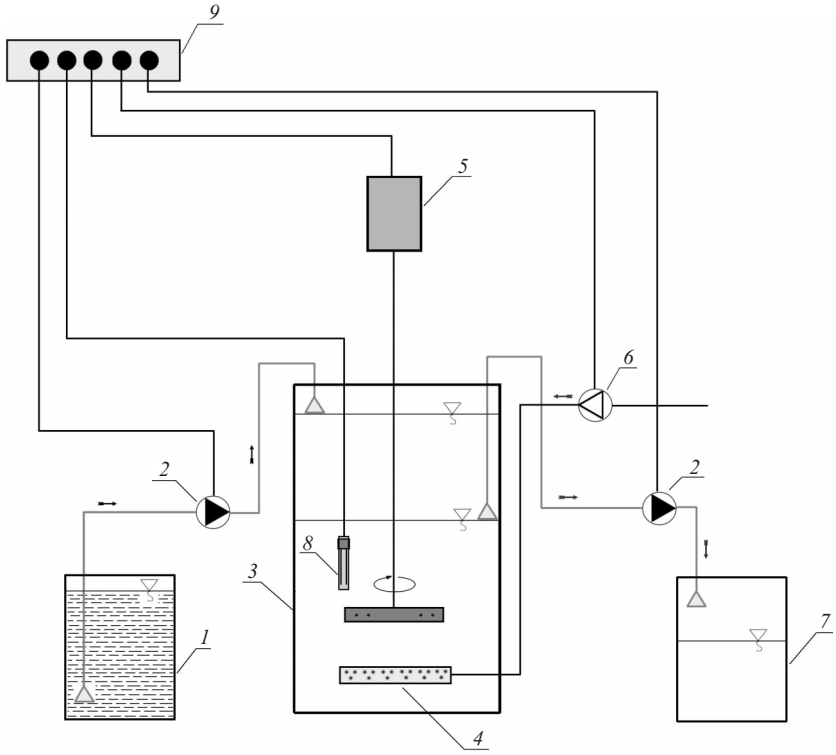

Fig. 1. Single experimental stand: *1* – container for untreated waste water, *2* – peristaltic pump, *3* – SBR, *4* – diffuser, *5* – stirrer, *6* – aerator, *7* – container for treated waste water, *8* – oxygen probe, redox potential, *9* – electronic control system

Synthetic waste water was used in the experiment, prepared from water and powdered milk (1 g milk per 1 dm^3 water) with the addition of the KH_2PO_4 solution.

Pollution indexes determined in the untreated waste water fed to the reactors were:

COD	1.050 mg $O_2 \cdot dm^{-3}$,
BOD_5	560 mg $O_2 \cdot dm^{-3}$,
Orthophosphate	46.5 mg $P_{PO_4} \cdot dm^{-3}$,
Total nitrogen	56 mg $N \cdot dm^{-3}$,
Ammonium nitrogen	0.84 mg $N_{NH_4} \cdot dm^{-3}$,
Nitrate nitrogen	3.6 mg $N_{NO_3} \cdot dm^{-3}$.

Waste water flowing to reactors 2, 3 and 4 was dosed with selected VFAs, i.e., acetate (R2), propionic (R3) and butyric (R4), in quantities corresponding with the experiment assumptions for the given series (1-6). R1 served as a reference reactor. Initial experiment conditions in the individual series are presented in Table 1.

Table 1

Initial conditions in the individual experiment series

Series	Reactor	COD increase in reactor inflow as a result of VFA addition (mg $O_2 \cdot dm^{-3}$)	MLSS concentration ($g \cdot dm^{-3}$)	Organic loading (g COD $\cdot g^{-1}$ d.w. $\cdot d^{-1}$)
1	R1	–	4.1	0.064
	R2,R3,R4	50	4.1	0.067
2	R1	–	4.0	0.066
	R2,R3,R4	150	4.0	0.075
3	R1	–	4.1	0.064
	R2,R3,R4	300	4.1	0.082
4	R1	–	4.1	0.064
	R2,R3,R4	700	4.1	0.107
5	R1	–	4.0	0.066
	R2,R3,R4	1.400	4.0	0.153
6	R1	–	4.1	0.064
	R2,R3,R4	2.100	4.1	0.192

After each experiment serie the kinetics of orthophosphate release and uptake was examined, with reference to the kind of the applied acid. Immediately after dosing the new portion of waste water and VFAs, analytical control of the orthophosphate concentration variations was initiated in the waste water during the mix and aeration phase. In the mix phase samples were taken for analyses in 15-min intervals. In aeration phase the interval was elongated to 30 min.

The reaction rate equation proposed by KLIMIUK (1998) was used to calculate reaction-rate constants for orthophosphate release and uptake. Reac-

tion rate equations of orthophosphate release and uptake were based on the hypothesis that in batch reactors orthophosphate increase in the waste water during the mix phase is the result of their release by PABs and of the hydrolysis of PP contained the waste water. Orthophosphate decrease occurs due to intracellular phosphorus uptake by PABs, biosynthesis processes, sorption, and chemical precipitation of orthophosphates in activated sludge (KLIMIUK 1998).

According to the above assumptions, the equation describing orthophosphate variations during release is

$$C_{PO_4} = (C_{Pmax} - C_{0,\,PO_4}) \cdot [1 - e^{-k_u \cdot t}] + C_{0,\,PO_4} \tag{1}$$

and the equation describing orthophosphate uptake is

$$C_{PO_4} = (C_{0,\,PO_4} - C_{k,\,PO_4})\, e^{-k_u \cdot t} + C_{k,\,PO_4} \tag{2}$$

where:

C_{PO_4} – orthophosphate concentration, mg $P_{PO_4} \cdot dm^{-3}$;
$C_{0,\,PO_4}$ – initial orthophosphate concentration, mg $P_{PO_4} \cdot dm^{-3}$;
C_{Pmax} – maximum orthophosphate concentration, mg $P_{PO_4} \cdot dm^{-3}$;
$C_{k,\,PO_4}$ – final orthophosphate concentration, mg $P_{PO_4} \cdot dm^{-3}$;
k_u – orthophosphate release reaction-rate, h^{-1};
k_w – orthophosphate uptake reaction-rate, h^{-1}.

The equation describing the simultaneous uptake and release processes and the resultant concentration variations over time is

$$C_{PO_4} = k \cdot t \cdot (C_{Pmax} - C_{0,\,PO_4}) \cdot e^{-k \cdot t} + (C_{0,\,PO_4} - C_{k,\,PO_4})\, e^{-k \cdot t} + C_{k,\,PO_4} \tag{3}$$

if

$$k_u = k_w = k$$

or,

$$C_{PO_4} = \frac{k_u}{k_u - k_w} \cdot (C_{Pmax} - C_{0,\,PO_4}) \cdot [e^{-k_w \cdot t} - e^{-k_u \cdot t}] + (C_{0,\,PO_4} - C_{k,\,PO_4})\, e^{-k_w \cdot t} + C_{k,\,PO_4} \tag{4}$$

if

$$k_u \neq k_w.$$

The reaction-rate constants (k_u, k_w) and C_{Pmax} and $C_{k,\,PO_4}$ were determined using the non-linear regression. The iterative method was applied in the programme. The obtained figures were used to compute orthophosphate release rate in anaerobic conditions (r_{uV}, r_{uX})and orthophosphate uptake rate in aerobic conditions (r_{wV}, r_{wX}). The figures with the subscript index V (r_{uV}, r_{wX}) and X (r_{uX}, r_{wX}) represent the reaction rates referring to the reactor's volume and the biomass contained in the reactor, respectively.

Results

The study was conducted to assess the effect of VFAs addition to dairy waste water on the kinetics of orthophosphate release in anaerobic conditions and orthophosphate uptake in aerobic conditions. Examined was the effect of the doses of acetic, propionic and butyric acids. The reaction-rate constants and the rates of orthophosphate release and uptake were computed on the grounds of the results presented in Table 2. Changes in the rate of orthophosphate release in anaerobic conditions and orthophosphate uptake in aerobic conditions, depending on the amount of the applied VFA, are shown in Figures 2 and 3.

The presented data reveal that the increasing input of the propionic and butyric acids raised the reaction rate of both orthophosphate release and uptake (Figures 2 and 3). For propionic acid, the orthophosphate release rate at the highest acid dose (COD increase by 2.100 mg $O_2 \cdot dm^{-3}$) is over 9 times higher than at the lowest dose (COD increase by 50 mg $O_2 \cdot dm^{-3}$). For butyric acid, the Figure is higher and exceeds the value of 12.5. Similar relations were observed regarding the orthophosphate uptake rates.

For acetic acid, the dose's increase in the range from 50 to 1.400 mg $O_2 \cdot \cdot dm^{-3}$ caused the reaction rate increase of both orthophosphate release and uptake (Figures 2 and 3). In the discussed range of doses, the rate of orthophosphate release at the highest concentration was over 6.6 times higher than at the lowest. As for the uptake rate, the value at the highest concentration was by almost 7.2 times higher than at the lowest concentration.

At the acetic acid's dose causing the COD increase by 2.100 mg $O_2 \cdot dm^{-3}$ a decrease was observed of both values, respectively by approximately 24.5% and 22%, compared to the figures measured for the value of 1.400 mg $O_2 \cdot dm^{-3}$. The reason was the apparent decline of the activated sludge's condition, an effect of its overloading with pollutants. As a consequence, the effluent quality deteriorated and orthophosphate removal efficiency diminished (Table 3).

Table 2

Kinetic constants and rate of phosphate release and uptake

Operations conditions	Parameters, units	Series 1-6	Series																
			1			2			3			4			5			6		
		R1	R2	R3	R4	R2	R3	R4	R2	R3	R4	R2	R3	R4	R2	R3	R4	R2	R3	R4
Anaerobic	kinetic constant k_u h^{-1}	0.78	0.74	0.80	0.67	0.99	0.82	0.87	0.77	0.85	0.78	0.90	0.97	0.91	1.01	1.12	0.97	0.68	1.54	1.45
	reaction rate:																			
	r_{uV}, mg $P_{PO_4} \cdot dm^{-3} \cdot h^{-1}$	11.86	11.34	11.66	9.04	13.51	12.98	10.68	18.55	16.78	12.63	42.43	35.09	25.42	75.42	59.65	47.02	56.92	111.12	113.24
	r_{uX} mg P_{PO_4} g^{-1} smo $\cdot dm^{-3} h^{-1}$	2.89	2.77	2.84	2.20	3.38	3.24	2.67	3.31	4.09	3.08	10.34	8.56	6.20	18.85	14.91	11.76	13.88	27.10	27.60
	$C_{Pmax} - C_{k,\,PO_4}$ mg $P_{PO_4} \cdot dm^{-3}$	15.21	15.32	14.57	13.49	13.65	15.83	12.28	24.09	19.74	16.19	47.14	36.18	27.93	74.67	53.25	48.47	83.71	72.16	78.03
Aerobic	kinetic constant k_w h^{-1}	0.78	0.74	0.80	0.67	0.99	0.82	0.87	0.77	0.85	0.78	0.90	0.97	0.91	1.01	1.12	0.97	0.68	1.54	1.45
	reaction rate:																			
	r_{wV}, mg $P_{PO_4} \cdot dm^{-3} \cdot h^{-1}$	13.80	13.45	13.33	10.63	18.12	14.65	12.95	25.46	18.70	14.65	48.37	39.94	31.05	99.06	79.08	52.29	77.24	130.21	131.51
	r_{wX} mg P_{PO_4} g^{-1} smo $\cdot dm^{-3} h^{-1}$	3.37	3.28	3.25	2.59	4.53	3.64	3.24	6.21	4.56	3.57	11.80	9.74	7.57	24.76	19.77	13.07	18.84	31.76	32.08
	$C_{Pmax} - C_{k,\,PO_4}$ mg $P_{PO_4} \cdot dm^{-3}$	17.69	18.17	16.66	15.86	18.30	17.87	14.89	33.06	22.00	18.78	53.73	41.17	34.12	98.08	70.61	53.91	113.59	84.55	90.70

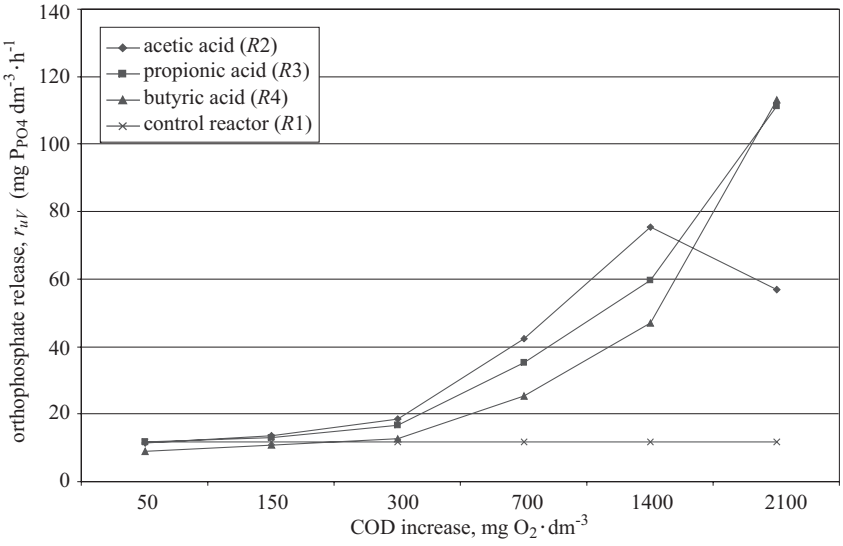

Fig. 2. Orthophosphate release rate vs. COD increase due to VFAs introduction to the waste water

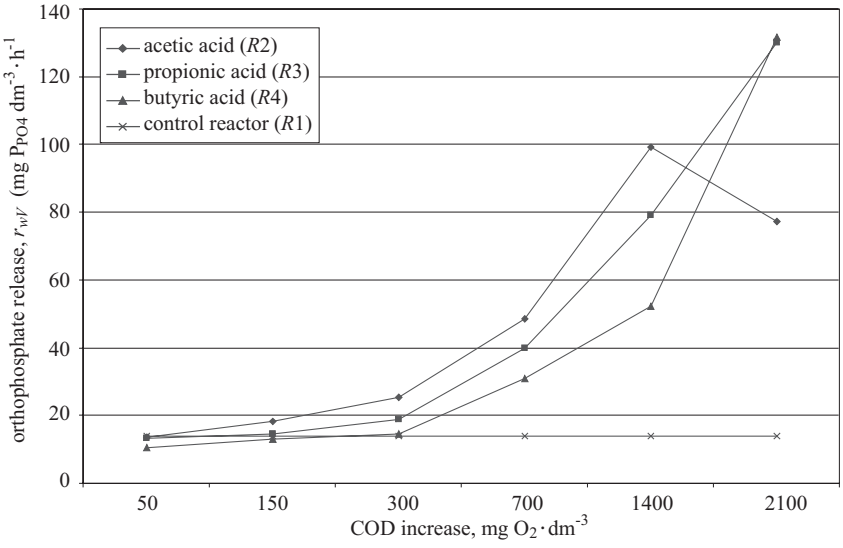

Fig. 3. Orthophosphate uptake rate vs. COD increase due to VFAs introduction to the waste water

Table 3

Effluent concentration (S_k) and phosphate removal efficiency (JANCZUKOWICZ 2005)

Reactor	Parameter	Series											
		1		2		3		4		5		6	
		mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
R 1 (control)	S_k, mg $P_{PO_4} \cdot dm^{-3}$	3.70	0.26	3.62	0.58	3.61	0.61	3.46	0.27	3.68	0.46	3.61	0.64
	efficiency	0.92	0.01	0.92	0.01	0.92	0.01	0.93	0.01	0.92	0.01	0.92	0.01
R2 (acetic)	S_k, mg $P_{PO_4} \cdot dm^{-3}$	5.34	1.01	3.88	0.57	3.42	0.39	2.96	0.22	2.20	0.23	6.79	0.94
	efficiency	0.89	0.02	0.92	0.01	0.93	0.01	0.94	0.00	0.95	0.01	0.85	0.02
R3 (propionic)	S_k, mg $P_{PO_4} \cdot dm^{-3}$	6.61	1.04	4.28	0.63	3.32	0.50	2.66	0.38	1.29	0.34	1.94	0.38
	efficiency	0.86	0.02	0.91	0.01	0.93	0.01	0.94	0.01	0.97	0.01	0.96	0.01
R4 (butyric)	S_k, mg $P_{PO_4} \cdot dm^{-3}$	5.30	0.66	3.76	0.14	3.39	0.36	3.09	0.19	2.34	0.41	1.92	0.43
	efficiency	0.89	0.01	0.92	0.00	0.93	0.01	0.93	0.00	0.95	0.01	0.96	0.01

In the concentration range from 50 to 1.400 mg $O_2 \cdot dm^{-3}$, the highest values of the rate of orthophosphate release and uptake were observed in the reactor fed with acetic acid. The lowest figures were determined in the reactors with propionic and butyric acids.

At the highest concentration (COD increase by 2.100 mg $O_2 \cdot dm^{-3}$), the rate of orthophosphate release in acetic acid presence was approximately 49% lower than in the presence of propionic and butyric acids, whereas the uptake rate was approximately 40% lower. The values of orthophosphate release and uptake for propionic and butyric acids dosed in this amount were comparable (111.12 and 113.14 mg $P_{PO4} \cdot dm^{-3} \cdot h^{-1}$, and 130.21 and 131.51 mg $P_{PO4} \cdot \cdot dm^{-3} \cdot h^{-1}$, respectively).

The orthophosphate release and uptake rates calculated for the lowest dose of propionic and butyric acids (series 1, COD increase by 50 mg $O_2 \cdot dm^{-3}$) were comparable to the values determined in the reactor fed only with the waste water (R1, Table 2). In the reactor with butyric acid both rates were approximately 23% lower than the figures determined for the other reactors. In series 2 – COD increase by 150 mg $O_2 \cdot dm^{-3}$ – the rates of orthophosphate release and uptake for acetic and propionic acids began to exceed the values determined in R1. In the reactor fed with butyric acid yet the COD increase by 300 mg $O_2 \cdot \cdot dm^{-3}$ increased the orthophosphate release and uptake rate to the level higher than in R1. In series 5 (COD increase by 1.400 mg $O_2 \cdot dm^{-3}$), orthophosphate release rates during the mix phase in the presence of acetic acid (R2), propionic acid (R3) and butyric acid (R4) were higher than in R1 by 6.4; 5 and 4 times, respectively. The corresponding rates of the orthophosphate uptake during the aeration phase were respectively higher than the value observed in R1 by 7.2; 5.7 and 3.8 times. In the last series, in the reactors with propionic and butyric acids the rates of release and uptake were more than 9 times higher than in the reactor with the waste water as the only carbon source. In the same series, in the reactor with acetic acid, despite the poor condition of the activated sludge, the rate of release and the rate of uptake of orthophosphate were higher than the corresponding figures in R1 (by 4.8 and 5.6 times, respectively).

Irrespective of the applied acid the rate of orthophosphate release was always higher than the uptake rate which comprised from 73 to 90% of the release rate. The same trend was observed in the reference reactor.

Concluding, the application of acetic, propionic and butyric acids in the quantities that increased the concentration of untreated waste water by 150 mg $O_2 \cdot dm^{-3}$ (acetic and propionic acid) or by 300 mg $O_2 \cdot dm^{-3}$ (butyric acid), had an effect on orthophosphate release rate in the mix phase and on the rate of orthophosphate uptake in the aeration phase. They were higher in the presence of these acids than in the reference reactor. Further increase of the quantity of propionic and butyric acids caused (in the examined range of

concentrations) an increase of the values of the discussed reaction rates. Similar tendency was characteristic for acetic acid, yet at the highest dose both reaction rates decreased.

Discussion

The experiment revealed that the rates of orthophosphate release and uptake (and consequently the amounts of the released and taken up orthophosphates) grow in parallel to the increasing amount of the dosed VFAs. Such regularity regards the propionic and butyric acids in the whole examined concentration-range and the first five examined concentrations in the case of acetic acid (COD from 50 mg to 1.400 mg $O_2 \cdot dm^{-3}$). Similar observations were made by RUSTRIAN et al. (1997), however, only with regard to the selected bacterial strains and some acids. This finding only partly overlaps with the results of the studies by KLIMIUK (1998). In the study of the effect of sodium acetate on the orthophosphate release and uptake kinetics in the municipal waste water she revealed that sodium acetate caused significant increase of orthophosphate release in the mix phase but simultaneously, decreased the uptake rate in the aeration phase. The reason was the high content of nitrates in the processed waste water and the use up of sodium acetate in the mix phase for denitrification.

For all acids, a regularity was observed that the rate of orthophosphate release was lower than the uptake rate in the oxic phase. In consequence, the amount of the taken up orthophosphates was higher than the released quantity. Moreover, it was observed that the more orthophosphates were released in the mix phase, the more was taken up in the aeration phase. This phenomenon regarded all examined acids. Similar regularities are described by CHEN et al. (2004).

However, the high values of orthophosphate release and uptake rate were not identical with the higher phosphorus (P) removal efficiency. For propionic acid, the higher release and uptake rates resulted in the higher P-removal efficiency as compared with the figures noted in the reactor fed with butyric acid. Simultaneously, the release and uptake rates higher for acetic acid than for propionic and butyric acids were not identical with the higher P-removal efficiency and the expected higher effluent quality. Likewise, the higher amounts of the orthophosphates released in the presence of acetic acid in the mix phase had no effect on the amount of orthophosphates taken up during the aeration phase and on the P concentration in the treated waste water.

Therefore, it may be concluded that the discussed kinetic parameters are mainly an illustration of the easiness of orthophosphate release and uptake in

the presence of acetic acid. It does not mean that the biomass of activated sludge in the presence of this acid as the carbon source removes orthophosphate more effectively.

Lack of the relationship between the amount of the released and taken up orthophosphate and the terminal efficiency of orthophosphate removal, has already been reported by RUSTRIAN et al. (1997). They studied the behaviour of the selected pure strains of *Acinetobacter* in the presence of acetic, propionic and butyric acids. The experiments showed that the amount of the orthophosphate taken up in the aeration phase was sovereign from the amount released in the mix phase. The quantity of removed orthophosphate did not depend on the applied amount of VFAs. Orthophosphate release in the anaerobic conditions was not always regulated by the amount of the taken up VFAs. All the investigated bacterial strains revealed the ability to accumulate phosphate, however, the amount of the removed phosphate was mostly correlated with the type of the strain. At the same time, each strain had a different efficiency of phosphate release and uptake, depending on the type of acid used to cultivate the bacteria (RUSTRIAN et al. 1997).

The result of the multi-month application of three different acids was a different composition of the biocenosis and the resultant dominance of the specific bacterial strains; i.e., those that adapted to the given substratum. However, it should not be ignored that except for a given VFA each reactor was fed with organic substrate that may have been transformed in the anaerobic phase into various volatile acids. Therefore, in each reactor, there was mainly the mixture of the acetic, propionic and butyric acids, however, in quite different proportions and variable over time. In the reactor with acetic acid the acid also dominated, followed in quantity by the propionic and butyric acids. In the reactor with propionic acid, at the beginning of the mix phase the propionic acid prevailed, followed by acetic and butyric acids. In the reactor with butyric acid, initially dominated the butyric acid. There is no certainty regarding the second by quantity acid in this reactor. Most possibly it was the acetic acid, followed by the propionic acid. It should not be also ignored that the applied acids were decomposed during the fermentation process and some butyric and propionic acids were transformed into acetic acid, with various reaction rate; the more acids were dosed, the more acetic acid was generated in the reactors with propionic and butyric acids. The reason is the fermentation occurring in anaerobic conditions simultaneously with the process of simple organic compounds uptake by PABs. The relationship described in this paper, and confirmed in the literature, is that the amount of orthophosphate released in anaerobic conditions and the organic carbon demand diminished in parallel to the increasing number of atoms in the acid molecule (ABU-GHARACH, RANDALL 1991).

Conclusions

The presented study allows concluding that,

– VFAs fed as external organic carbon source to the waste water treated in an SBR have effect on the kinetics of the release and uptake of orthophosphates in the mix and aeration phases, respectively.

– Increase of the applied amounts of propionic and butyric acids caused an increase of the orthophosphate release rate in the mix phase and the uptake rate during aeration. This tendency regarded the whole range of the examined concentrations (COD increase from 50 mg do 2.100 mg $O_2 \cdot dm^{-3}$).

– Acetic acid added to the treatment process in the amounts that increased COD concentration from 50 to 1.400 mg $O_2 \cdot dm^{-3}$ elevated the rate of orthophosphate release and uptake. Application of the acid's dose increasing COD by 2.100 mg $O_2 \cdot dm^{-3}$ diminished both reaction rates. The reason was the poor condition of the activated sludge biomass overloaded with the pollutants.

– In the concentration range from 50 mg to 1.400 mg $O_2 \cdot dm^{-3}$, i.e., when VFAs did not affect the condition of activated sludge, acetic acid caused the highest increase of the orthophosphates release and uptake rates. Propionic acid was more effective than butyric.

– Higher amounts of the released and taken up orthophosphates in the presence of the given acid are not identical to or do not explain the higher efficiency of P-removal. The evidence is that the rates of orthophosphate release and uptake determined for the biomass under acetic acid's influence were in most of the cases higher than for propionic and butyric acids. At the same time, the efficiency of the orthophosphate release in the reactor with acetic acid was not always higher than the efficiency noted for the propionic and butyric acids.

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References

ABU-GHARACH Z. H., RANDALL C. W. 1991. *The effect of organic compounds on biological phosphorus removal*. Wat. Sci. Technol., 23: 585-594.

ARUN V., MINO T., MATSUO T. 1989. *Metabolism of carboxylic acids located in and around the glycolytic pathway and the TCA cycle in the biological phosphorus removal process*. Wat. Sci. Technol., 21: 363-374.

CHEN Y., RANDALL A.A., MCCUE T. 2004. *The efficiency of enhanced biological phosphorus removal from real wastewater affected by different ratios of acetic to propionic acid.* Wat. Res., 38: 27-36.

JANCZUKOWICZ W. 2005. *Usuwanie fosforu ze ścieków mleczarskich w bioreaktorze sekwencyjnym w obecności wybranych lotnych kwasów tłuszczowych (LKT).* Wyd. UWM, Olsztyn, Rozpr. i Monogr., 107: 1-134.

KLIMIUK E. 1998. *Kinetyka przemian związków azotu i fosforu w osadzie czynnym w warunkach beztlenowo-tlenowych*. Wyd, ART, Olsztyn, Rozpr. i Monogr. 3.

SATOH H., MINO T., MATSUO T. 1992. *Uptake of organic substrates and accumulation of polyhydroxyalkanoates linked with glycolysis of intracellular carbohydrates under anaerobic conditions in the biological excess phosphate removal processes*. Wat. Sci. Technol., 26: 933-942.

RANDALL A.A., BENEFIELD L.D., HILL W.E., NICOL J.P. BOMAN G.K. JING S.R. 1997 . *The effect volatile fatty acids on enhanced biological phosphorus removal and population structure in anaerobic/aerobic sequencing batch reactor*. Wat. Sci. Technol., 35: 153-160

RANDALL A.A., LIU Y-H. 2002. *Polyhydroxyalkanoates from potentially a key aspect of aerobic phosphorus uptake in enhanced biological phosphorus removal*. Wat. Res., 36 (14): 3473-3478.

RUSTRIAN E., DELGENES J. P., MOLETTA R. 1997. *Phosphate release and uptake by pure cultures of Acinetobacter sp.: Effect of the volatile fatty acids concentration.* Curr. Microbiol., 34: 43-48.

TAM N.F.Y., WONG Y.S., LEUNG G. 1992. *Significance of external carbon source on simultaneous removal of nutrients from wastewater.* Wat. Sci. Technol., 26 (5-6): 1047-1055.

WENTZEL M.C., LÖTTER L.H., EKAMA G.A., LOEWENTHAL R.E., MARAIS G.V.R. 1991. *Evaluation of biochemical models for biological excess phosphorus removal*. Wat. Sci. Technol., 23: 567-576.

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THE SANITARY STATE OF SWIMMING SITES IN CHEŁMŻYŃSKIE LAKE

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K e y w o r d s: lake, water, pollution, indicator bacteria.

A b s t r a c t

This paper presents results from a study of the sanitary state of the northern section of Chełmżyńskie Lake. In order to identify the sanitary state of the lake, number of bacteria indicationg the degree of water contamination (TVC 22°C and TVC 37°C) and sanitary state (TC, FC, FS, *Clostridium perfringens* and *Salmonella*) were determined. According to our results, the surveyed lake water belongs to water quality class III (*Ministry of the Environmental Protection Directive from 11th* February, 2004; Dz.U. No. 32/2004, sec. 284) with respect to bacteria. Values of total coliform (TC) and fecal streptococcus (FS) indicators for water from all swimming sites of the Chełmżyńskie Lake did not exceed standards adopted for water quality at swimming sites (*Directive of the Ministry of Environmental Protection from 16 October*, 2002; Dz.U. No. 183/2002, sec. 1530). The permissible values of fecal coliform (FC) indicators were exceeded during the study period in water from two swimming sites (stations III and IX). Sanitary hazards associated with the presence of the *Salmonella* bacteria were found in water from two sites (stations IV and VII), which disqualifies these tested sites from suitability for swimming. According to the requirements specified by the U.S. Department of Interior Federal Water Pollution Control Administration (1968), Chełmżyńskie Lake conforms with standards for recreational water with respect to the TC indicator. However, we obtained slightly less favorable results with respect to the FC indicator. In this case, FC indicator levels in water from three research stations located in the Chełmżyńskie Lake (II, III, and IX) exceeded the permissible values.

STAN SANITARNY KĄPIELISK JEZIORA CHEŁMŻYŃSKIEGO

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S ł o w a k l u c z o w e: jezioro, bakterie, zanieczyszczenie, bakterie wskaźnikowe, woda.

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Abstrakt

Badano stan sanitarny płn. części Jez. Chełmżyńskiego. Oznaczono liczebność bakterii wskaźnikowych stopnia zanieczyszczenia wód jeziora (TVC 22°C oraz TVC 37°C), a także liczebność bakterii wskaźnikowych stanu sanitarnego (TC, FC, FS, *Clostridium perfringens* oraz *Salmonella*). Stwierdzono, że wodę badanego jeziora można zaliczyć pod względem bakteriologicznym do III klasy czystości (*Rozp. Min. Środ. z dnia 11 lutego 2004*; Dz.U. Nr 32/2004, poz. 284). Wskaźniki bakterii z grupy coli (TC) i paciorkowców kałowych (FS) w wodzie wszystkich badanych kąpielisk Jez. Chełmżyńskiego przyjmowały wartości dopuszczalne, zgodnie z wymogami, jakim powinna odpowiadać woda w kąpieliskach (*Rozp. Min. Zdr. z dnia 16 października 2002 r.*; Dz.U. Nr 183/2002, poz. 1530). W badanym okresie, w wodzie dwóch kąpielisk (stanowiska III i IX), zostały przekroczone wartości dopuszczalne wskaźnika bakterii z grupy coli typu kałowego (FC). W wodzie dwóch kąpielisk (stanowiska IV i VII) stwierdzono ryzyko sanitarne związane z obecnością pałeczek *Salmonella*, co dyskwalifikuje je pod względem przydatności do celów kąpieliskowych i rekreacyjnych. Według wytycznych U.S. Departament of Interior Federal Water Pollution Control Administration (1968), wody Jez. Chełmżyńskiego pod względem wskaźnika bakterii z grupy coli (TC) spełniają wymogi sanitarne stawiane wodom rekreacyjnym, natomiast pod względem wskaźnika bakterii z grupy coli typu fekalnego (FC) nie spełniają ww. wymogów, ponieważ na trzech stanowiskach badawczych (II, III, IX) wskaźnik FC przekroczył dopuszczalne normy.

Introduction

Chełmżyńskie Lake is a large water body suitable for recreation located in the southern section of the Chełmżyńskie Lake district (KONDRACKI 2000). Due to its scenic location and favorable morphological features, the lake is a desirable site for water sports. This has resulted in the dynamic growth of the tourist industry in recent years, especially in the southwestern section in direct proximity of the town of Chełmża, 15 thousand inhabitants, and its eastern suburbs. Due to the increased interest of the Chełmża and Toruń inhabitants in lake recreation, it is necessary to conduct continuous monitoring of the sanitary and bacteriological conditions of the lake. The purpose of this monitoring is to minimize the risk of human infections by pathogenic or potentially pathogenic microorganisms occurring in the water.

Invasive microorganisms, including human and animal pathogens, represent all basic taxonomic types, i.e. viruses, bacteria, fungi, and protozoa, and enter the lacustrine water from surface runoff from rain and melting snow, waterfowl, uncontrolled sewage discharge from summer houses and campgrounds, as well as from swimming humans and animals (ZUCKERMAN et al. 1997, MANSOUR, SIDKY 2003, VREDE et al. 2003, BETTAREL et al. 2004).

Bacteriological analyses used to determine water quality and sanitary condition focus on enumerating the total number of heterotrophic bacteria that are capable of growth at 22°C and 37°C. These abundances are used as indicators of the concentration of organic matter and invasive microorganisms (BOBROWSKI 2002). Additionally, the abundance of bacteria indicating the

sanitary condition was determined. These bacteria are typical representatives of saprophytic microflora inhabiting the alimentary canal of humans and warm-blooded animals (NIEWOLAK 1982, LIBUDZISZ, KOWAL 2000), and are excreted with feces and urine. These microorganisms indicate the fecal pollution of water, and the probability of occurrence of pathogenic bacteria in the aquatic environment. Among the typical representatives of microorganisms indicating sanitary condition are coliform bacteria (TC), fecal coliform bacteria and fecal streptococcus (FS) (NOBBLE et al. 2003), the *Clostridium perfringens* strains and *Salmonella* (NIEWOLAK 1982).

The purpose of this study was to determine the sanitary state of swimming sites located in the town's vicinity and to evaluate their suitability as swimming and recreational sites for people. The study includes enumeration of bacteria indicating the pollution level of the Chełmżyńskie lake water (heterotrophic bacteria capable of growth at 22°C and 37°C), bacteria indicating the sanitary state of the lake (i.e. TC, FC, FS, anoxic sulfate-reducing bacteria *Clostridium perfringens* and *Salmonella* bacteria), as well as determination of the water quality according to KORSH (1957).

Materials and Methods

Study area

Microbiological analyses were conducted in the northwestern section of Chełmżyńskie Lake, in direct proximity to the town of Chełmża. The lake, as noted above, is a relatively large ribbon lake, characterized by an irregular elongate shape stretching from northwest to southeast. Table 1 presents the most important morphometric and trophic data pertaining to the examined lake. The watershed of the lake is used for agricultural purposes and is largely woodless. The primary type of land use on the direct watershed is cultivation; arable land occupies 72.1% of the area, which in many cases reaches the shoreline. The town of Chełmża, whose runoff is discharged to the lake through a separate sewage system, is located on the northwestern section of the lake. The lake, as noted above, is used intensely for recreation. There are 5 resorts, 2 campgrounds, and 1 agrotourism farm located on the lakeshore. Summer housing development is also undergoing strong growth. Both the proximity of the town and the lake watershed type have negative impacts on the water quality of the lake. According to data published by the The Provincial Inspectorate of the Environmental Protection in Bydgoszcz, the water quality of Chełmżyńskie Lake, as described by standard indicators, belonged to water quality class III in 2000.

Table 1

Morphometric and trophic characteristics of Chełmżyńskie Lake (WIOŚ 2001)

Characteristic	Value
Area, ha	271.1
Maximal depth, m	27.1
Mean depth, m	6.1
Length of shore line, m	20 985
Total phosphorus, μg dm^{-3}	30.0 – 99.0
Total nitrogen, mg dm^{-3}	0.94 – 1.76
Chlorophyll *a,* μg dm^{-3}	26.4 – 56.9
Water transparency, m	1.2 – 1.9

Sampling stations

Sampling stations were designated on the three research transects (I-III, IV-VI, and VII-IX). Station I is a town beach, station III – an unguarded town beach, station II – the center of the transect I-III , station IV – a swimming site on "the park peninsula", station V – the transect center of IV-VI, station VII – a swimming site next to a summer house, station IX – a swimming site next to summer houses, and station VIII- the VII-IX transect center.

Sampling procedures

Water samples were collected monthly in sterile bottles from surface water (from a depth of ca. 15-20 cm) between May and October, 2004. The samples were transported to the laboratory in a cold container, whose interior was maintained at a temperature not exceeding +4°C. The time between sample collection and microbiological analyses did not exceed 6 hours.

Microbiological analyses

The sanitary and bacterial analyses included determination of:

– number of planktonic heterotrophic bacteria (cfu · cm^{-3}) cultured on broth agar for 72 hours at 22°C (TVC 22°C);

– number of planktonic heterotrophic bacteria (cfu · cm^{-3}) cultured on broth agar for 24 hours at 37°C (TVC 37°C);

– total number of bacteria, as estimated by a direct counting method on membranes, following ZIMMERMAN (1981).

– number of TC bacteria (MPN · 100 cm^{-3}) cultured on Eijkman culturing medium (BTL) for 48 h at 37°C;

– number of FC bacteria (MPN · 100 cm^{-3}) cultured on Eijkman culturing medium with brilliant yellow and green (BTL) for 24 h at 44.5°C;

– number of FS bacteria (cfu · 100 cm^{-3}) cultured on Slanetz and Bartley culturing medium (BTL) for 48 h at 37°C;

– number of anoxic sulfate-reducing bacteria *Clostridium perfringens* (cfu · 100 cm^{-3}) cultured on Wilson-Blair culturing medium (BTL) for 18 h at 37°C (preceded by sample pasteurization for 10 min at 80°C);

– *Salmonella* bacteria (presence in 1000 cm^3) grown up on peptone culturing medium and cultured on the selective culturing mediums SF and SS (BTL); colonies with Salmonella characteristics underwent diagnostic testing for *Salmonella* bacteria (TECRA International Pty Ltd.).

Prior to analyses, water samples were diluted 10-fold with sterile Ringer solution (PN-ISO 8199). The analyses were carried out according to Polish Standards (PN-ISO 6222, PN-75/C-04615/05, PN-77/C-04615/07, PN-82/C--046115/25, PN-EN 26461-2).

Results and Discussion

Surface waters are used by people for both industrial and recreational purposes. A specific quantity of water necessary for human existence and the functioning of the individual economic sectors constitutes the water requirements of a given area. These water requirements demand infrastructure development in the reservoir proximity and have a direct impact on the lake from both the land and water (CHOIŃSKI 1995). Unquestionably, the use of lacustrine water by the human population has an impact on the quality and pollution level of the lake water and is directly or indirectly related human

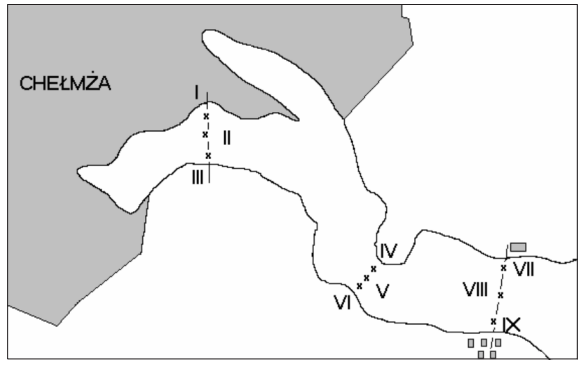

Fig. 1. Outline of studied Chełmżyńskie lake and sampling stations (I-IX)

health. Water may constitute a transmission medium for pathogenic microorganisms, which may infect humans as a result of contact with or intake of such water. Therefore, continuous sanitary and biological monitoring of not only drinking water, but also water in swimming pools, open swimming sites, and entire aquatic ecosystems is necessary (ZMYSŁOWSKA 2003).

Number of bacteria indicating the degree of pollution of the Chełmżyńskie Lake

Number of heterotrophic bacteria that are capable of growth at 22°C (TVC 22°C) and are indicators of water pollution with easily decomposable organic matter ranged from 0.10 to 68.80 · 102 cfu · cm^{-3} (Table 2). The broadest range of abundances was observed in the water of the swimming site next to a summer house (station VII), the narrowest in the water at the town beach (station I).

Table 2

Number of bacteria indicating the degree of water contamination (cfu · 10^2 · cm^{-3}) and water quality indicator according to Korsh (1957) in Chełmżyńskie Lake

Sampling station	Number of samples	TVC 22°C	TVC 37°C	Q
I	6	0.10 – 19.70* 7.58	0.10 – 3.50* 1.20	9-309* 110
II	6	2.10 – 33.50 14.49	0.45 – 4.49 2.39	15-76 73
III	6	0.20 – 36.00 16.65	0.40 – 16.00 3.84	9-128 64
IV	6	1.64 – 30.50 11.92	0.40 – 27.00 2.37	52-266 144
V	6	0.60 – 25.40 9.21	0.30 – 129 0.67	3-617 214
VI	6	1.00 – 68.00 2215	0.30 – 4.20 1.56	21-220 102
VII	6	0.30 – 68.80 31.18	0.30 – 18.50 4.28	96-352 370
VIII	6	1.50 – 27.57 8.38	0.40 – 1.40 1.22	41-453 187
IX	6	1.90 – 25.00 10.42	1.40 – 3.00 1.98	21-131 65

Explanations: TVC 22°C – number of heterotrophic bacteria capable of growth at 22°C; TVC 37°C – number of heterotrophic bacteria capable of growth at 37°C, * mean value and range; Q – water quality indicator; $0 < Q < 10$ highly polluted water; $10 < Q < 100$ – moderately polluted; $100 < Q < 1000$ – moderately pure; $Q > 1000$ – pure water

Number of heterotrophic bacteria in the Chełmżyńskie lake, which are capable of growth at 37°C (TVC 37°C) and are indicators of water pollution with invasive microflora, ranged from 0.10 to 27.00 · 102 cfu · cm^{-3} (Table 2). Water from the swimming site located on the park peninsula (station IV) was characterized by the greatest fluctuations in abundance of these microorganisms; whereas, the smallest fluctuations in abundance were observed in water from the second transect center (station V).

The highest number of planktonic heterotrophic bacteria, which are capable of growth at 22°C (cfu 22°C) and at 37°C (cfu 37°C), were found in water from the site located next to a summer house – station VII. The most probable reason for relatively high abundances of these two groups of microorganisms at station VII is the fact that meadows, which are used as pastures, are located in this section of the lake, as well as the presence of a private swimming site next to the summer house. A decline in water quality directly bordering meadows and pastures has been observed by numerous researchers (LEWANDOWSKA et al. 2000, GOŁAŚ et al. 2003, WILK, DONDERSKI 2005).

The lowest number of planktonic heterotrophic bacteria capable of growth at 22°C was observed in the water of the town beach, while the lowest abundance of bacteria growing at 37°C was recorded at station V.

Evaluation of water quality at Chełmżyńskie Lake swimming sites, based on the Korsh (1957) water quality indicator

Based on Korsh;s water quality indicator (Table 2), water from the surveyed swimming sites of Chełmżyńskie lake can be classified as moderately pure (stations I, IV, V, VI, VII, and VIII) and moderately polluted (stations II, III, and IX).

Number of bacteria indicating sanitary state

Table 3 presents data regarding numbers of bacteria indicating sanitary condition. According to these data, the abundance of TC bacteria ranged from 9.0 to 2400.0 cells in a 100 cm^3 sample. The maximum variation in their abundance was observed at station II, which was the mid-point of the first research transect, with minimal variations at station V (center of the second research transect).

The number of FC bacteria ranged from 4.0 to 1100.0 cells in a 100 cm^3 sample. The maximum variation in this indicator value was observed at station V, with the minimum variation occurring at the site next to the summer houses (station IX). Station II was characterized by the highest values of TC and FC, with station V – the lowest.

Table 3
Number of bacteria indicating the sanitary state of water of Chełmżyńskie Lake (*MPN – , **cfu in 100 cm^3 of sample)

Sampling station	Number of samples	TC*	FC**	FS**	*Clostridium perfringens*
I	6	93.0-240.0* 176.0	3.0-240.0* 75.8	1.0-260.0* 50.5	4.0-141.0*** 48.0
II	6	48.0-2400.0 748.0	23.0-1100.0 417.0	2.0-84.0 29.6	1.0-147.0 50.75
III	6	93.0-1100.0 477.4	48.0-1100.0 299.5	3.0-80.0 25.1	2.0-140.0 52.0
IV	6	15.0-930 63.3	9.0-93.0 39.0	3.0-30.0 14.2	4.0-101.0 56.3
V	6	15.0-93.0 39.0	9.0-48.0 18.7	3.0-21.0 8.9	3.0-107.0 43.5
VI	6	21.0-460.0 77.5	15.0-93.0 36.8	1.0-28.0 9.2	4.0-99.0 44.8
VII	6	48.0-1100.0 474.8	9.0-460.0 176.2	2.0-149.0 64.3	1.0-101.0 45.4
VIII	6	9.0-240.0 167.7	4.0-93.0 59.0	2.0-45.0 121	0.0-98.0 41.1
IX	6	9.0-1100.0 454.8	9.0-1100.0 312.8	2.0-62.0 25.2	4.0-144.0 74.2

Explanation: TC – number of total coliforms; FC – number of fecal coliforms ; FS – number of fecal streptococci, *Clostridium perfringens* – number of anoxic sulfate-reducing bacteria, *** – mean value and range

Number of fecal streptococci in the analyzed water ranged from 1.0 to 260.0 cells per 100 cm^3 of sample. The highest variability in bacterial abundance was observed in the town beach water (station I), while the lowest was observed at station V. The maximal abundance of fecal enterococcus was observed at station VII, with the minimal at station V.

The number of anoxic sulfate-reducing bacteria (*Clostridium perfringens*) ranged from 0 to 147.0 cells per 100 cm^3 of sample. The maximum variation was observed at station II, which was the mid-point of the first research transect, while the minimal was observed at the station located opposite the "park peninsula" (station VI). The maximal abundance of these microorganisms was observed at station IX, and the minimal at station VIII.

The water of two swimming sites presented a sanitary risk associated with the presence of *Salmonella* bacteria. These microorganisms were detected at stations II and VII.

It is evident from the data presented in Table 4 that pollution in the majority of analyzed surface water samples was of mixed – human and animal – (stations IV-IX) and human (stations I-III) origin.

Table 4

Indicator of fecal pollution in Chełmżyńskie Lake FC/FS

Range	Sampling stations								
	I	II	III	IV	V	VI	VII	VIII	IX
FC/FS < 0.7	16.7*	0.0	0.0	0.0	16.7	0.0	16.7	0.0	0.0
0.7 < FC/FS < 4	33.3	33.3	33.3	66.3	66.7	66.7	50	66.7	66.7
FC/FS > 4	50	66.7	66.7	33.3	16.7	33.3	33.3	33.3	33.3

Explanations: FC/FS < 0.7 – animal pollution; 0.7 < FC/FS < 4 – mixed (animal and human pollution); FC/FS > 4 – human pollution, * sample percent per range

According to results describing the abundances of bacteria indicating the sanitary condition, the mid-point of the second research transect (station V) had the cleanest water. Water from that station was characterized by the lowest values of TC, FC, and FS indicators as well as the lowest abundance of heterotrophic bacteria capable of growth at 37°C, which indicates that water pollution levels with invasive microflora are relatively low. In contrast, abundances of TC and FC bacteria and the presence of *Salmonella* bacteria suggest that the highest pollution levels were observed at station II, the mid-point of the first transect. This station was located in front of an interceptor sewer outlet, which discharges rainwater from the town into the lake. Eastward advection of the water due to prevailing westerly winds probably pushed pollution into the areas around station II and III, which was also characterized by relatively high values of TC and FC indicators.

Analysis of the FS indicator values indicates that water from station VII was found to be the most polluted. Water from that station contained *Salmonella* bacteria; furthermore, the highest mean values of heterotrophic bacteria capable of growth at 22°C were also observed at station VII. The latter indicator describes pollution with easily decomposable organic matter.

Relatively high values of sanitary indicators TC, FC and *Clostridium perfringens* were observed at the swimming site near summer houses on the southern shore of the lake (station IX). The pollution levels were affected by sewage discharge from the area and runoff of dung produced by animals grazing on pastures surrounding the summer house development.

Evaluation of water quality in Chełmżyńskie Lake based on legal regulations

According to the water classification system of the Directive of the Minister of the Environment from 11 February, 2004 (Dz.U. No. 32/2004, sec. 284), water of Chełmżyńskie Lake belongs to water quality class III (Table 5).

Table 5

Evaluation of water quality in Chełmżyńskie Lake according to the water classification system of the *Directive of the Minister of the Environment from 11 February, 2004* (Dz.U. No. 32/2004, sec. 284)

Range	Class of water quality	Sampling stations								
		I	II	III	IV	V	VI	VII	VIII	IX
total coliform (TC)										
< 50	I	0.0*	16.7	0.0	33.3	83.3	33.3	33.3	50.0	16.7
50 – 500	II	100.0	50.0	66.7	66.7	16.7	66.7	33.3	50.0	66.7
500 – 5000	III	0.0	33.3	33.3	0.0	0.0	0.0	33.3	0.0	16.7
5000 – 50 000	IV	0,0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
> 50 000	V	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
fecal coliform (FC)										
< 50	I	66.7	16.7	50.0	83.3	100.0	83.3	33.3	50.0	33.3
50 – 500	II	33.3	50.0	33.3	16.7	0.0	16.7	66.7	50.0	50.0
500 – 5000	III	0.0	33.3	16.7	0.0	0.0	0.0	0.0	0.0	16.7
5000 – 50 000	IV	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
> 50 000	V	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Explanations: * – sample percent per class

According to the data presented in Table 6, in 2004, values of the total coliform (TC) and fecal streptococcus (FS) indicators determined for water from all swimming sites in Chełmżyńskie lake did not exceed the permissible values adopted for water at swimming sites (Directive of the Ministry of Health from 16 October, 2002; Dz.U. No. 183/2002, sec. 1530).

Table 6

Bacteriological evaluation of water quality at swimming sites in Chełmżyńskie Lake according to *Directive of the Ministry of Environmental Protection from 16 October, 2002*; Dz. U. No. 183/2002, sec. 1530

Sampling stations	Bacteriological standards of water quality						
	total coliform (TC)		fecal coliform (FC)		fecal Streptococci (FS)		*Salmonella*
	< 500 *A*	< 10 000 *B*	< 100 *A*	< 1000 *B*	< 100 *A*	< 400 *B*	
I	100.0*	100.0*	83.3	100.0	83.3	100.0	absent
III	66.7	100.0	66.7	83.3	100.0	100.0	absent
IV	100.0	100.0	100.0	100.0	100.0	100.0	present
VI	100.0	100.0	100.0	100.0	100.0	100.0	absent
VII	66.7	100.0	50.0	100.0	66.7	100.0	present
IX	83.3	100.0	83.3	83.3	100.0	100.0	absent

Explanations: *A* – recommended values, *B* – acceptable values, * – sample percent per class

The permissible values of the fecal coliform (FC) indicator were exceeded in water from two swimming sites (stations III and IX). Sanitary hazards associated with the presence of the *Salmonella* bacteria were found in water from two sites (stations IV and VII), which disqualifies the tested sites from suitability for swimming.

Evaluation of Chełmżyńskie Lake water for recreational purposes according to the guidelines of the U.S. Department of Interior Federal Water Pollution Control Administration (1968)

The Polish legal system does not specify any requirements for water used for recreational purposes. Therefore, in order to describe the lake's suitability for these purposes the authors used guidelines provided by the U.S. Department of Interior Federal Water Pollution Control Administration (1968).

According to the data presented in Table 7, Chełmżyńskie Lake water conforms to the sanitary requirements of recreational waters based on the TC indicator, which in all samples did not exceed permissible values. However, the assessment of the FC indicator yielded slightly less favorable results. In this case, values of the FC indicator in the water from three research stations (II, III, and IX) exceeded the permissible standards.

Table 7

Evaluation of Chełmżyńskie Lake water for recreational purposes according to the guidelines of the U.S. Department of Interior Federal Water Pollution Control Administration (1968)

Sampling stations	Bacteriological standards			
	total coliform (TC)		fecal coliform (FC)	
	< 1000 *A*	< 5 000 *B*	< 200 *A*	< 1000 *B*
I	100.0*	100.0*	83.3	100.0
II	83.3	100.0	66.7	66.7
III	66.7	100.0	66.7	83.3
IV	100.0	100.0	100.0	100.0
V	100.0	100.0	100.0	100.0
VI	100.0	100.0	100.0	100.0
VII	66.7	100.0	50.0	100.0
VIII	100.0	100.0	100.0	100.0
IX	83.3	100.0	50.0	83.3

Explanations: see Table 6

Conclusions

1. Water of Chełmżyńskie Lake belongs to water quality class III (*Directive of the Minister of the Environment from 11 February*, 2004; Dz.U. No. 32/2004, sec. 284).

2. Values of total coliform (TC) and fecal streptococcus (FS) indicators for water from all swimming sites of the Chełmżyńskie Lake did not exceed standards adopted for water quality at swimming sites (*Directive of the Ministry of Environmental Protection from 16 October*, 2002; Dz.U. No. 183/2002, sec. 1530). The permissible values of the fecal coliform (FC) indicator were exceeded in water from two swimming sites (stations III and IX). Sanitary hazards associated with the presence of the *Salmonella* bacteria were found in water from two sites (stations IV and VII), which disqualifies the tested sites from suitability for swimming.

3. Chełmżyńskie Lake water conforms to the sanitary requirements of recreational waters (U.S. Department of Interior Federal Water Pollution Control Administration, 1968) based on the TC indicator, which in all samples did not exceed permissible values. However, the assessment of the FC indicator yielded slightly less favorable results. In this case, values of the FC indicator in the water from three research stations (II, III, and IX) exceeded the permissible standards.

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References

BETTAREL Y., SIME-NGANDO T., AMBLARDx 2CH., DOLAN J. 2004. *Viral activity in two contrasting lake ecosystems*. Appl. Environ. Microbiol., 70:2941-2951.

BOBROWSKI M. 2002. *Podstawy mikrobiologii sanitarnej*. Wyd. Ekonomia i Środowisko, Białystok.

CHOIŃSKI A. 1995. *Zarys limnologii fizycznej Polski*. Wyd. UAM, Poznań.

GOŁAŚ I., ZMYSŁOWSKA I., LEWANDOWSKA D., 2003. *Sanitary and bacteriological quality of water of western part of Kleberskie Lake*. AUNC, Limnol. Pap., 23: 235-246.

Komunikat o jakości wód jezior: Chełmżyńskie i Grażyna. 2001. Woj. Insp. Ochr. Środ. w Bydgoszczy.

KONDRACKI J. 2000. *Geografia regionalna Polski*. PWN, Warszawa.

KORSH L.E. 1957. *Ocenka effektivnostii meropriatij po sanitarnoj ochrane kanala imieni Moskvy*.Gig. i Sanit., 3: 19-25.

LEWANDOWSKA D., ZMYSŁOWSKA I., FILIPKOWSKA Z. 2000. *Sanitary and bacteriological Evaluation of the southern part of Legiński Lake for recreation Purpose*. Pol. J. Environ. Stud., 4: 341-353.

LIBUDZISZ Z., KOWAL K. 2000. *Mikrobiologia techniczna.* T. I. Wyd. Politechniki Łódzkiej.

MANSOUR S.A., SIDKY M.M. 2003. *Ecotoxicological Studies 6. The first comparative study between Lake Quarun and Wadi El-Rayan Wetland (Egypt), with respect to contamination of their major components*. Food Chem., 82:181-189.

Ministry of the Environmental Protection Directive from 11th February, 2004. Dz.U. No. 32/2004, sec. 284.

Ministry of Environmental Protection Directive from 16th October, 2002. Dz.U. No. 183/2002, sec. 1530.

NIEWOLAK S. 1982. *Drobnoustroje wskaźnikowe stanu sanitarnego.* Cz. II. *W: Biologia sanitarna*., Wyd. ART, Olsztyn, 38 ss.

NOBBLE R.T., MOORE D.F., LEECASTER M.K., MCGEE C.D., WEISBERG S.B. 2003. *Comparison of total coliform, fecal coliform and enterococcus bacterial indicator response for ocean recreational water quality testing*. Wat. Res., 37: 1637-1643.

VREDE K., STENSDOTTEER U., LINDSTRÖM E.S. 2003. *Viral and Bacterioplankton dynamics in two lakes with different humic contents*. Microb. Ecol., 46: 406-415.

Water Quality Criteria: Report of Committee on Water Quality Criteria. 1968. U.S. Department of Interior, Federal Water Pollution Control Administration. Washington, D.C.,1.

WILK I., DONDERSKI W. 2005. *Bacteriological – sanitary state of waters of north-west part of Chełmżyńskie Lake*. AUNC. Limnol. Pap., 25: 187-196.

ZMYSŁOWSKA I. 2003. *Intensive fish farming versus the microbiological aspects of environment contamination*. AUNC. Limnol. Pap., 23: 147-157.

ZUCKERMAN H., GOLD D., SHELEF G., ARMON R., 1997. *The presece of Giardia and Cryptosporodium in surface waters and effluens in Israel*. Wat. Sci. Tech., 35: 381-384.

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THE BACTERIOLOGICAL AND SANITARY STATE OF SEWAGE IN AN ON-SITE WILLOW WASTEWATER TREATMENT FACILITY

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K e y w o r d s: heterotrophic bacteria, coliform bacteria, fecal coliform bacteria, fecal streptococci, hydrobotanical sewage treatment plants.

A b s t r a c t

The numbers of heterotrophic bacteria, capable of growing at 22°C and 37°C, as well as the following indicator bacteria used to determine the sanitary and bacteriological conditions, were determined in an on-site willow wastewater treatment facility in the Czarne Błoto, Zławieś Wielka district: coliforms bacteria (TC), fecal coliforms bacteria (FC), and fecal streptococci (FS). The study demonstrated that the number of heterotrophic bacteria, capable of growing at 22°C and 37°C, as well as indicator bacteria was greatly reduced after wastewater passed through all stages of treatment in the soil – willow filter. The greatest reduction in the bacterial abundance was observed between the raw sewage that flows into the soil-willow filter and the treated sewage (even in winter). Reduction of coliform and fecal coliform bacteria was the greatest following all stages of sewage treatment (on average 95.2% and 94.6%). The reduction of fecal streptococcus equaled 90.4% on average. The lowest reduction in abundance (on average 82.2%) was observed for heterotrophic bacteria capable of growth at 22°C.

STAN SANITARNO-BAKTERIOLOGICZNY ŚCIEKÓW W PRZYDOMOWEJ OCZYSZCZALNI WIERZBOWEJ

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S ł o w a k l u c z o w e: bakterie heterotroficzne, bakterie z grupy coli, bakterie z grupy coli typu kałowego, paciorkowce kałowe, hydrobiologiczna oczyszczalnia ścieków.

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Abstrakt

Badano liczebność bakterii heterotroficznych zdolnych do wzrostu w temp. 22°C i 37°C oraz bakterii wskaźnikowych stanu sanitarno-bakteriologicznego: bakterii z grupy coli (TC), bakterii z grupy coli typu kałowego (FC) oraz paciorkowców kałowych (FS) w przydomowej oczyszczalni ścieków z układem wierzbowym, w miejscowości Czarne Błoto, gmina Zławieś Wielka. Wykazano znaczne zmniejszenie liczebności bakterii heterotroficznych zdolnych do wzrostu w temp. 22°C i 37°C oraz bakterii wskaźnikowych po przejściu ścieków przez wszystkie etapy oczyszczania na filtrze gruntowo-wierzbowym. Największy spadek liczebności badanych bakterii stwierdzono między ściekiem surowym dopływającym do filtra gruntowo-wierzbowego a ściekiem oczyszczonym (również w okresie zimowym). Po przejściu ścieków przez wszystkie etapy oczyszczania stwierdzono największą redukcję bakterii z grupy coli i bakterii z grupy coli typu kałowego (średnio 95,2% i 94,6%). Redukcja paciorkowców kałowych wynosiła średnio 90,4%. Najmniejszą redukcję liczebności (średnio 82,2%) stwierdzono wśród bakterii heterotroficznych zdolnych do wzrostu w temp. 22°C.

Introduction

The problem of rural wastewater utilization has been on the increase since the beginning of the 1970s, when water systems began undergoing an uncontrollable development without simultaneous introduction of sewage management solutions. During the last two decades, the number of farms connected to the water system has increased four-fold. Currently, over 80% of villages are connected to the water supply system. The most common sewage treatment systems in rural households are holding or septic tanks, which are often improperly located and constructed. However, public interest in the application of natural methods of sewage treatment has been rising for the past few years. Treatment facilities with emergent macrophytes are called botanical, aquatic plant, root-zone, or constructed wetland treatment systems (OZIMEK, RENMAN 1996). The botanical treatment systems utilize the natural ability of aquatic macrophytes to intercept chemical compounds that are present in wastewater; their high resistance to elevated pollution content is also very advantageous. The removal of pollution in hydrophyte treatment systems is a result of the process of sorption of biochemical pollution, reduction and oxidation reactions, and the biological activity of aquatic micro-organisms and plants (BŁAŻEJEWSKI 1997). The most common hydrophytes are macrophytes that are rooted on the bottom and have the vegetative and generative stems growing above the water surface, for example, the common reed (*Phragmites australis*), osier willow (*Salix viminalis*), common cattail, and rush (*Juncus* sp.) (OZIMEK, RENMAN 1996). Currently, the willow is considered the most effective plant in the removal of heavy metals and other toxic compounds from soils.

The purpose of this study was to determine the level of reduction of indicator bacteria in an on-site willow wastewater treatment facility.

Materials and Methods

Object of the study

The study was conducted in the area of an on-site willow wastewater treatment facility in the village of Czarne Błoto, Zławieś Wielka district. This district is located in the central part of the Kujawsko-Pomorskie voivodship in the Toruń province (second level of local administration in Poland). The district spreads along the right bank of the Vistula River from the city limits of Toruń to that of Bydgoszcz and occupies an area of 177.5 km^2; it has 10 000 residents. Agricultural production dominates in the district. Among the main enterprises in the district are farms, agriculture and food industry firms as well as agriculture related services.

Characteristics of the on-site willow wastewater treatment facility

The on-site willow treatment facility has treated domestic sewage in the village of Czarne Błoto since 2003. It is a no-outlet sewage treatment system with a capacity of 60 m^3. The septic tank is made of medium-density polyethylene and it has a solid body with no seams or welds. Such construction ensures that the tank is fully watertight. The tank was placed on a 30 cm layer of dry concrete and then was gradually filled with water and simultaneously covered with sand and dry concrete. Dry concrete was applied in sections where groundwater might come into contact with the tank.

Construction of the willow system

A 12 x 5 x 1 m pit was lined with sand and 0.8-1.2 mm foil. The bottom was then covered with fine gravel in order to ensure efficient flow of graywater. Perforated tubes were attached widthwise to the T-connector of the pipe, which supplies the wastewater. This wastewater inlet was covered with fine gravel, which connected the entire system with the gravel base at the bottom. This arrangement facilitates the leaching of graywater throughout the entire system. Finally, a pipe that collects and channels out the possible excess wastewater to a control tank was installed. The remaining sections of the pit were filled with soil matrix. The area of the excavation was planted with 10 osier willows (*Salix viminilis*), which are fed with graywater.

Sample collection

Microbiological tests were conducted from July to December of 2004, and included all developmental stages of the osier willow. Sewage samples were collected sequentially from three sampling stations in 250 ml sterile bottles. The samples were transported to the laboratory in an ice-filled container. The time between sample collection and the beginning of the microbiological analyses did not exceed 4 hours. The samples were collected in an inspection chamber (sewage flowing into the willow filter) from the filter (sewage during treatment), and from an inspection chamber located downstream of the willow filter (treated sewage).

Microbiological studies

Microbiological tests contained number of heterotrophic bacteria capable of growing at 22°C (TVC 22°) and 37°C (TVC 37°), coliform bacteria (TC), fecal coliform bacteria (TC), and fecal streptococci (FS). Heterotrophic bacteria capable of growing at 22°C (TVC 22°) and 37°C (TVC 37°C) were determined using a pour-plate technique according to the Polish Standard (PN-ISO 6222) CFU. Total coliforms and fecal coliforms were enumerated with a multiple-tube fermentation technique according to the Polish Standard (PN-75/C-04615/05, PN-77/C-04615/07) MPN. The number of fecal streptococci was determined with a membrane filter technique on BTL Slanetz-Bartley medium according to the Polish Standard (PN-82/C-04615/25).

Results

The number of heterotrophic bacteria capable of growth at 22°C (TVC 22°C) and 37°C (TVC 37°C) varied with the type of sewage and studied month (Table 1, Figure 1). The highest number of heterotrophic bacteria was found in wastewater flowing into the willow filter, while the lowest was found in the effluent after all stages of the treatment process. For all sewage samples, the highest bacterial count was found in the months of July and September, the lowest in December. Reduction in the number of heterotrophic bacteria that are capable of growth at 22°C equaled 76.1 – 86%; whereas, the number of the same bacteria capable of growth at 37°C was observed to be reduced by 80.6 – – 95.5% (Figure 2).

Table 1
The number of heterotrophic bacteria, capable of growing at 22°C (TVC 22°C) and 37°C (TVC 37°C) (CFU · 1 cm^{-3} sewage)

The type of sewage	Bacteria	Date of sampling					
		02.07.04	09.08.04	01.09.04	13.10.04	04.11.04	02.12.04
Sewage flowing into the willow filter	TVC 22°C TVC 37°C	1485 945	16 750 2400	9300 2750	4500 2200	2345 1530	1230 605
Sewage during treatment	TVC 22°C TVC 37°C	1420 755	12 800 1900	2600 980	1800 690	1260 240	200 32
Treated sewage	TVC 22°C TVC 37°C	355 144	2345 465	1450 365	780 350	480 150	190 27

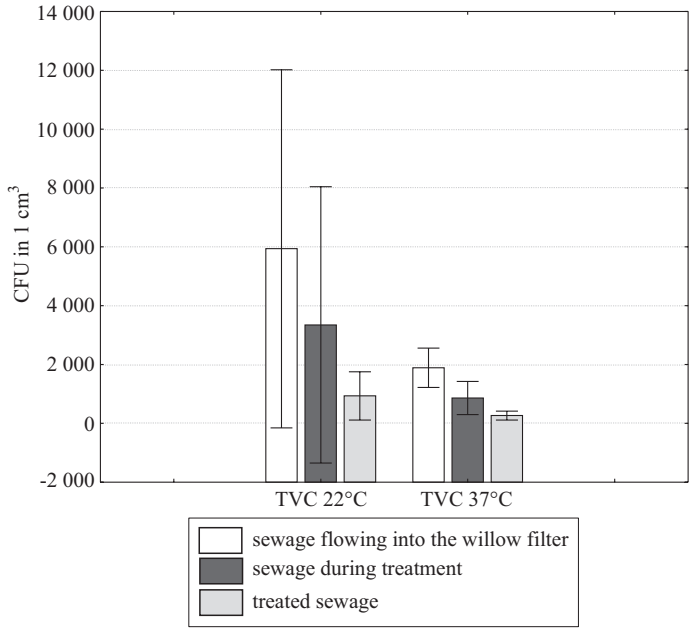

Fig. 1. Average numbers of heterotrophic bacteria, capable of growing at 22°C (TVC 22°C) and 37°C (TVC 37°C). Vertical bars represents SD

The number (MPN) of total coliforms in the sewage varied in the studied months and was related to the type of sewage (Table 2, Figure 3). The highest concentration of TC was found in the sewage flowing into the willow filter in July (45 000 cells 100 cm^{-3} of sewage), and the lowest in the treated sewage in the months of August and September (250 cells 100 cm^{-3} of sewage). The reduction of the number of coliform bacteria after passing through all treatment stages equaled 90-99% (Figure 4).

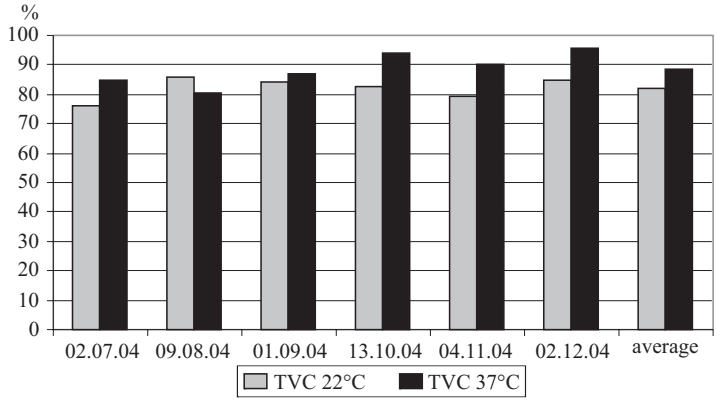

Fig. 2. Reduction in number of heterotrophic bacteria, capable of growing at 22°C (TVC 22°C) and at 37°C (TVC 37°C) after passing through all treatment stages

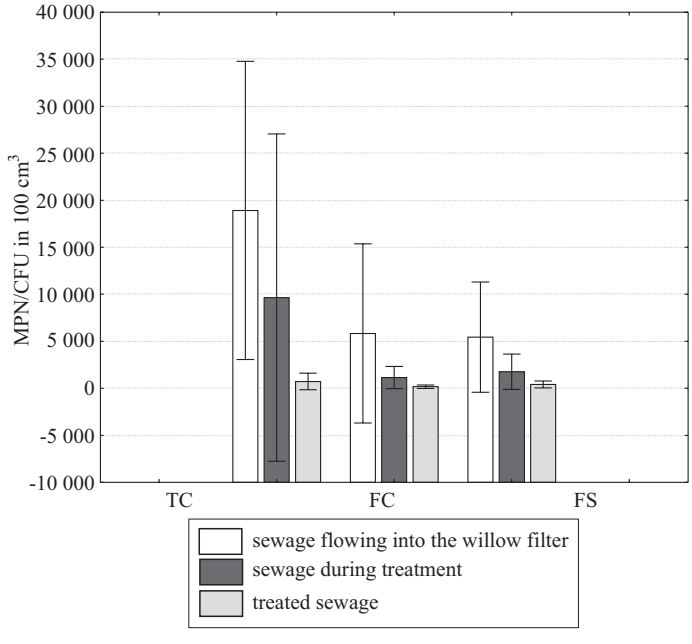

Fig. 3. Average MPN of coliform (TC), fecal coliform (FC) and CFU fecal streptococci (FS). Vertical bars represents SD

Table 2

The number of coliform bacteria (TC), fecal coliform (FC), fecal streptococci (FS), MPN/CFU in 100 cm^3 sewage

The type of sewage	Bacteria	Date of sampling					
		02.07.04	09.08.04	01.09.04	13.10.04	04.11.04	02.12.04
Sewage flowing into the willow filter	TC	45 000	25 000	25 000	4500	4500	9500
	FC	25 00	2500	250	2500	250	4500
	FS	16 550	1120	3700	2500	7245	1550
Sewage during treatment	TC	45 000	450	2500	4500	950	4500
	FC	2500	42	250	2500	93	1500
	FS	5250	850	1750	2200	555	0
Treated sewage	TC	2500	250	450	250	450	450
	FC	450	0	23	250	15	250
	FS	1050	158	460	522	282	0

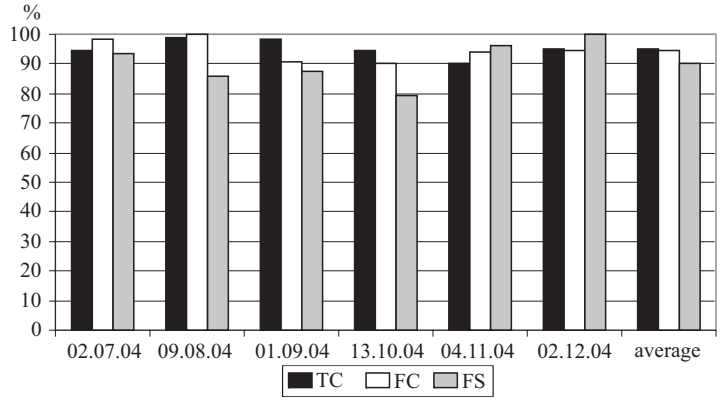

Fig. 4 Reduction in number (MPN)of total coliform (TC), fecal coliform (FC) and (CFU) fecal streptococci (FS) after passing through all treatment stages

Similar changes in numbers were observed for FC bacteria. The highest concentration of FC was found in the sewage flowing into the treatment system, and the lowest in the treated sewage in the months of August and September (0 and 15 cells 100 cm^{-3} of sewage respectively) – Table 2, Figure 3. The reduction in FC bacterial concentration after passing through all treatment stages equaled 90-100% (Figure 4).

The FS bacterial count varied across months of the survey and was related to the sewage type (Table 2, Figure 3). The highest concentrations of FS were found in the sewage flowing into the willow filter in July (16 550 cells 100 cm^{-3}), and the lowest in December when no FS occurrences were observed. Reduction of fecal streptococcus during the willow system treatment was also high, and ranged from 79.1 to 100% (Figure 4).

Discussion

Application of a hydrophyte sewage treatment system is an economic and environmentally friendly, alternative way of treating wastewater. Hydrophytes effectively remove pollutants and pathogenic organisms, which is very important in small, home sewage treatment facilities. Our assessment of the effectiveness of hydrophyte treatment systems in pathogenic organism removal consisted exclusively of analyses of indicator organisms, coliforms, *Escherichia coli*, and fecal streptococci.

Heterotrophic bacteria participate in the remineralization of organic substances, and as a result contribute to transfer of matter and energy in nature (PALUCH 1973, DONDERSKI 1983). Bacteria capable of growth at 22°C indicate the level of water pollution with organic matter that is easily decomposed. Whereas, the occurrence of heterotrophic bacteria capable of growth at 37°C may indicate the presence of pathogenic microflora (ZMYSŁOWSKA 2002, LALKE-PORCZYK et al. 2006). The results of this study demonstrate that the concentration of CFU at 22°C ranged from 1.9 to $167.5 \cdot 10^2$ cells cm^{-3} of sewage. LALKE-PORCZYK et al. (2006), when analyzing the indicator bacteria in a sand-reed filter of a wastewater treatment system in Wielka Nieszawka, observed much higher concentrations of these bacteria in sewage ($1.0 \cdot 10^4 - 6.2 \cdot 10^6$ cells cm^{-3}). Lower concentrations of heterotrophic bacteria capable of growth at 22°C in the willow system may reflect the lower content of organic substances utilized by these bacteria and, simultaneously, the presence of various pollutants that impede heterotrophic bacterial growth. Furthermore, the on-site treatment system in Czarne Błoto is smaller than the one in Wielka Nieszawa, and handles smaller quantities of wastewater. Similarly, the concentration of heterotrophic bacteria capable of growth at 37°C was substantially lower in the Czarne Błoto treatment facility ($2.7 - 275 \cdot 10^1$ cells cm^{-3} of sewage) than in the Wielka Nieszawa facility, where, according to LALKE-PORCZYK'S et al. (2006), the concentrations ranged from $2.0 \cdot 10^3$ to $8.4 \cdot 10^5$ cells cm^{-3}. This study demonstrated that the greatest number of bacteria that are capable of growth at 22°C and 37°C occurred in influent sewage, and the lowest in the treated sewage. According to OBARSKA-PEMPOWIAK (2002), a decrease in bacterial concentration along the filter could be related to a reduction in the quantity of organic compounds, which provide food for these organisms. However, JUCHERSKI and WALCZKOWSKI (2000) pointed out that during the process of wastewater filtration, micro-organisms from the sewage may also be eliminated. DECAMP and WARREN (2001) maintain that the reduction in bacterial concentration may be caused by mechanical damage to micro-organism cells that occurs during filtration.

The average reduction in concentration of heterotrophic bacteria capable of growth at 22°C and 37°C equaled 85.5%. WALCZAK and DONDERSKI (2004), when analyzing the impact of the Bydgoszcz wastewater treatment plant "Kapuściska" on the Brda River, found that the concentration of these bacteria was reduced by only 66.5%. Similarly, LALKE-PORCZYK et al. (2006) observed a lower reduction (60%) in bacteria capable of growth at 22°C; a reduction in bacteria capable of growth at 37°C was lowest (30%).

IGNASZAK (2005, unpub. data), in studing the wastewater pollution indicator BOD_5 in the on-site sewage treatment system in Czarne Błoto concluded that the sewage flowing into the willow filter had the highest values of BOD_5. The BOD_5 values were observed to decrease as the sewage passed through the willow filter. The authors of the present study found a similar correlation. The concentration of bacteria was the highest in the influent sewage and decreased as the sewage flowed through the filter. LALKE-PORCZYK et al. (2006) observed the same phenomenon when analyzing the heterotrophic bacterial count in a sand and reed bed treatment system.

The bacteriological sanitary indicators include bacteria that are present in the digestive tract of humans and warm-blooded animals, and are excreted with stool. Their presence in water signifies the risk of pathogenic bacterial infection. They include: coliforms, fecal coliforms, and fecal streptococci.

Based on the results of the study, we conclude that the reduction of indicator bacteria in the willow system was high (on average: 95.2% for TC, 94.6% for FC, and 90.4% for FS). In contrast, LALKE-POLCZYK et al. (2006), when analyzing bacterial concentrations in a sand and reed bed system, observed that only the coliform count was considerably reduced (on average 98.7%). FC and FS bacterial concentrations were reduced by only 51.2% and 10.6%, respectively. Analyzing the effectiveness of the wastewater treatment plant "Kapuściska", WALCZAK and DONDERSKI (2004) observed a lower reduction (51.5%) of TC concentration and a higher reduction (61.9%) of FS concentration.

A high level of reduction in indicator bacteria in the on-site willow sewage treatment facility may be a result of the effectiveness of this system. Willow is the most effective plant used in hydrophyte sewage treatment systems. Willow roots intercept over 80% of pollution. Results of microbiological analyses conducted by FILIPKOWSKA et al. (2004) confirmed the high efficiency (51 – 99.9%) of soil and plant filters with a vertical flow of wastewater (also in winter: 70 – 85%).

Conclusions

1. The number of heterotrophic bacteria capable of growth at 22°C (TVC 22°C) and 37°C (TVC 37°C) was highest in the sewage flowing into the soil and willow filter, and the lowest, in the treated sewage.

2. The number (MPN) of TC, FC, and FS were related to the type of sewage. The greatest numbers of these bacteria were observed to be present in the influent sewage and the lowest in the effluent.

3. The highest level of reduction of TC, FC, and FS concentrations was observed between the sewage flowing into the soil-willow filter and the effluent: from 79.1% to 100%.

4. After all stages of sewage treatment, reduction of coliform and fecal coliform bacteria (on average 95.2% and 94.6%) was found to be the greatest.

5. The number of fecal streptococci after all treatment stages was reduced by 90.4% on average.

References

Błażejewski R. 1997. *Botanical sewage treatment systems.* II Int. Sci. and Techn. Conf., Poznań.

Decamp O., Warren A. 2001. *Abundance, biomass and viability of bacteria in wastewaters: impact of treatment in horizontal subsurface flow constructed wetlands*. Wat. Res., 35 (14): 3496-3501.

Donderski W. 1983. *Heterotrophic aerobic bacteria in lakes of different trophy.* Nicolaus Copernicus University Press, Toruń.

Filipkowska Z., Pesta J., Korzeniowska E., Gotkowsa-Płachta A. 2004. *Sanitary and microbiological aspects of sewage treatment in soil and plant filters.* Post. Mikrobiol. 43, Supl. 1, 441.

Jucherski A., Walczkowski A. 2000. *Domestic sewage treatment systems in on-site soil and plant facilities located in mountainous regions.* Warszawa.

Lalke-Porczyk E., Swiontek-Brzezinska M, Donderski W. 2006. *Changes in the numbers of bacteria sanitarny in a sand-reed filter of a wastewater treatment system*. XX Zjazd Hydobiologów, Toruń.

Obarska-Pempkowiak H. 2002. *Hydrophyte sewage treatment plants*. Publication of the Technical University, Gdańsk.

Ozimek T., Renman G. 1996. *The role of helophytes in botanical sewage treatment systems.* II Int. Sci. and Techn. Conf., Academy of Agriculture, Poznań.

Paluch J. 1973. *Microbiology of water*. PWN, Warszawa.

Oznaczanie bakterii grupy coli metodą fermentacyjną probówkową. PN-75/C-04615/05.

Oznaczanie bakterii grupy coli typu kałowego, fekalnego metodą fermentacyjną probówkową. PN-77/C-04615/07.

Oznaczanie paciorkowców kałowych metodą filtrów membranowych i metodą probówkową. PN-82/C-04615/25.

Oznaczanie ilościowe mikroorganizmów zdolnych do wzrostu. Określenie ogólnej liczby kolonii metodą posiewu na agarze odżywczym. PN-EN ISO 6222.

Walczak M., Donderski W. 2004. *Assessment of the impact of wastewater treatment plants, "Kapuściska" and "Fordon" (opened in 2000) on the Wisła and Brda Rivers based on microbiological parameters.* Post. Mikrobiol., 43, Supl. 1, 468.

Zmysłowska I. 2002. *General and environmental of microbiology*. Univ. of Warmia and Mazury in Olsztyn.

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ELIMINATION OF INDICATORS (TC, FC, FS) AND *ENTEROBACTERIACEAE* FAMILY BACTERIA DURING THE SEWAGE TREATMENT PROCESS

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K e y w o r d s: microorganisms removal, fecal indicators, antibiotic resistance, wastewater treatment.

A b s t r a c t

The occurrence and removal of sanitary indicator bacteria (TC, FC, FS) and bacteria of *Enterobacteriaceae* family during the standard sewage treatment process were investigated. In addition, we determined the antibiotic-resistance of *Enterobacteriaceae* bacteria in raw and treated sewage. The result of the conducted study demonstrated that bacteria of *Enterobacteriaceae* family were most numerous among the surveyed microorganisms, and fecal streptococci was the least numerous bacteria group. The survey of antibiotic resistance of *Enterobacteriaceae* family demonstrated that these bacteria are the least sensitive to streptomycin and erythromycin. The increase in the proportion of bacterial strains resistance to tested antibiotics in the subsequent months of the study was correlated with the upward seasonal trend in human population morbidity, and in turn the usage antibiotic therapy.

ELIMINACJA BAKTERII WSKAŹNIKOWYCH (TC, FC, FS) ORAZ BAKTERII Z RODZINY *ENTEROBACTERIACEAE* PODCZAS PROCESU OCZYSZCZANIA ŚCIEKÓW

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A b s t r a k t

Badano występowanie i stopień redukcji bakterii będących wskaźnikami sanitarnymi (TC, FC, FS) oraz bakterii z rodziny *Enterobacteriaceae* podczas standardowego procesu oczyszczania ścieków. Ponadto oznaczano także antybiotykooporność bakterii z rodziny *Enterobacteriaceae* wyizolowanych ze ścieku surowego i oczyszczonego. Stwierdzono, że najliczniejszą grupę stanowiły bakterie z rodziny *Enterobacteriaceae,* a najmniej liczną – paciorkowce kałowe. W badaniach nad antybiotykoopornością bakterii należących do rodziny *Enterobacteriaceae* wykazano, że bakterie te są najmniej wrażliwe na streptomycynę i erytromycynę. Obserwowano również wzrost proporcji szczepów opornych na badane antybiotyki w kolejnych miesiącach badań, co było skorelowane z sezonowym wzrostem zachorowań populacji ludzkiej i zwiększonym stosowaniem antybiotykoterapii.

Introduction

Rivers are the primary source of water for consumption and industrial purposes. River water usability depends on its quality, which in turn is related to the amount and quality of the inflowing waters including sewage (BARNESS et al. 1981).

The purpose of sewage treatment plants is to prevent degradation of rivers by removal of nutrients, toxic substances, and BZT5. However, in few cases are treatment plants designed for the purpose of sewage microflora elimination (KOIVUNEN et al. 2003). Despite the fact that domestic sewage contains large quantities of fecal microorganisms, including pathogens, reduction of their populations has never been the priority of sewage treatment (GEORGE et al. 2002). Furthermore, the quantities of fecal microorganisms contained in raw sewage are enormous. It is estimated that in raw domestic sewage, the abundance of total coliforms (TC) reaches from 10^7 to 10^9 cells 100 ml^{-1} and fecal coliforms (FC) – 10^6-10^8 cells 100 ml^{-1} (ROSE et al. 1996). As a result, considering even a significant reduction in abundance, the amount of bacteria entering the rivers with the treated sewage varies from a few hundred to a more thousand cells in 100 ml. In addition to bacteria indicating sanitary condition, pathogenic bacteria enter the natural environment; water containing pathogens cannot be reused for consumption or recreation purposes.

Another serious hazard for people and animals using such water arises from the fact that pathogenic bacteria that leak out of the treatment plant are microorganisms that have acquired antibiotic-resistance. The sewage flowing into the treatment plant also contains waste produced by carriers, or ill and infected people who underwent antibiotic treatment (GONI-URIZA et al. 2000). Additionally, the amount of antibiotic-resistant bacteria may increase during the treatment process as a result of transfer of antibiotic-resistance genes (GUARDABASSI et al. 2002).

The purposed of this study was to survey the abundance reduction of sanitary condition indicator bacteria (TC, FC, and FS) and *Enterobacteriaceae* family bacteria during the standard sewage treatment process, and to determine the antibiotic-resistance of these bacteria in raw and treated sewage.

Materials and Methods

Object of the study

During the study, the author surveyed raw and treated sewage collected from Municipal Sewage Treatment Plant in Toruń in November, December, January, February, and March of 2004/2005. The above plant utilizes mechanical and biological (aeration tanks with active sludge) processes to treat domestic and industrial sewage from the city of Toruń. The plant's processing capacity equals 90 000 m^3 per day and the sewage retention time is approx. 24 hours. Currently the sewage treatment plant treats about 60 000 m^3 of sewage per day. The treated sewage is disposed in the Vistula river.

Microbiological analyses

Enumerating total coliform (TC) and fecal coliform (FC) abundances was conducted with the Most Probable Number (MPN) method using the three-tube system and Eijkman (MERCK) liquid culture medium. In the case of the TC bacteria, the incubation lasted for 48 hours at 37°C, while the FC bacteria were incubated for 48 hours at 44°C. Enumeration of fecal streptococci (FS) was carried out according to the EN ISO 7899-2: 2002 standard. The abundance of *Enterobacteriaceae* bacteria was determined by the spread plates method on McConkey's agar (MERCK). Pink and red bacterial colonies that grew on this agar base belonged to the *Enterobacteriaceae* family. The quantity of bacterial colonies (CFU) was estimated using the dilution ratio of the original bacteria sample.

The *Enterobacteriaceae* strains were isolated from the cultures used for bacterial enumeration. One hundred strains were inoculated from each type of analyzed sample (raw and treated sewage) and transferred on semisolid Nutrient Agar (MERCK) in tubes. After 5 days of incubation at 30°C, the strains were stored at 4°C to preserve them for further analysis.

Determination of antibiotic resistance of the isolated *Enterobacteriaceae* bacteria was conducted with the disc diffusion method. In order to test the

antibiotic sensitivity, the surveyed strains were pre-incubated for 72 hours in a nutrient agar slant. Bacteria cultured on agar slants were then suspended in 5 ml of sterile physiological salt solution. The obtained suspensions of individual bacterial strains were brought to the same optical density (0.15), which was determined by a Marcel Pro spectrometer at the wavelength $\lambda = 560$ nm. Bacteria were then inoculated from the suspension on Mueller Hinton 2 agar (MERCK).

After the inoculation, discs saturated with relevant antibiotics (Becton Dickinson BBL™) were dispensed on the culture medium surface. Four different antibiotics (streptomycin, erythromycin, oxytetracycline, and doxycycline) were tested during the study. In order to ensure the diffusion of antibiotics to the base, inoculated plates were kept at 4°C for 1 hour. Following this period, they were transferred to the incubator and cultured for 48 hours at 30°C. Bacterial strain susceptibility or resistance to individual antibiotics was determined by measuring the diameter of the bacterial inhibition zone around the antibiotic discs, and comparing these results with data provided by the disc manufacturer.

Results

The results of the study on numerous and reduction rate of investigated bacterial groups are presented in Table 1 and Figure 1. The results of the conducted study demonstrated that bacteria of the *Enterobacteriaceae* family were most numerous among the surveyed microorganisms. Their average abundance in raw sewage equaled $118.20 \cdot 10^3$ cells cm^{-3}, and in treated sewage, $4.51 \cdot 10^3$ cells ml^{-1}. The abundance reduction rate of the *Enterobacteriaceae* family during the sewage treatment process ranged from 90.00% to 98.33% with a mean value of 96.18%.

The second most numerous group present in the surveyed sewage were coliform bacteria (TC). On average, during the entire research process, their abundance reached $100.00 \cdot 10^3$ cells cm^{-3} in raw sewage and $2.72 \cdot 10^3$ cells cm^{-3} in the treated sewage. During sewage treatment, coliform bacterial reduction rate ranged from 96.25% to 98.31% (mean value: 97.28 %).

During the entire research program, the average abundance of fecal coliform (FC) equaled $34.20 \cdot 10^3$ cells cm^{-3} in the raw sewage and $0.93 \cdot 10^3$ cells cm^{-3} in the treated sewage. The reduction rate of these bacteria ranged from 95.31% to 98.40% with a mean value of 97.28%.

Fecal streptococci (FS) was the least numerous bacterial group. Reduction of FS population size during the sewage treatment process was the least effective, with reduction rates ranging from 70.84% to 95.38% (mean value: 85.97).

Table 1

Abundance of bacteria of surveyed groups in raw and treated sewage (cells · 10^3 cm^{-3})

Data of sampling	Total coliform bacteria (TC)			Fecal coliform bacteria (FC)			Fecal streptococci (FS)			Bacteria from *Enterobacteriaceae* family		
	raw sewage	treated sewage	reduction rate (%)	raw sewage	treated sewage	reduction rate (%)	raw sewage	treated sewage	reduction rate (%)	raw sewage	treated sewage	reduction rate (%)
November	200.00	7.50	96.25	75.00	1.60	97.86	2.00	0.15	92.50	220.00	11.00	95.00
December	65.00	1.10	98.31	25.00	0.40	98.40	0.30	0.04	86.67	64.50	6.44	90.00
January	95.00	2.40	97.47	30.00	0.95	96.83	0.24	0.07	70.84	112.50	2.00	98.22
February	75.00	1.50	98.00	25.00	0.95	96.20	0.65	0.03	95.38	104.00	1.60	98.46
March	65.00	1.10	98.31	16.00	0.75	95.31	0.45	0.07	84.45	90.00	1.50	98.33
Average	100.00	2.72	97.28	34.20	0.93	97.28	0.73	0.07	85.97	118.200	4.51	96.18

Table 2

Antibiotic-resistance of bacteria from *Enterobacteriaceae* family (bacteria in %)

Data of sampling	Streptomycin						Erythromycin						Doxycycline						Oxytetracycline					
	raw sewage			treated sewage			raw sewage			treated sewage			raw sewage			treated sewage			raw sewage			treated sewage		
	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I
November	0	85	15	0	83	17	0	85	15	0	100	0	15	30	55	28	22	50	15	35	50	28	33	39
December	0	92	8	0	88	12	0	96	4	0	100	0	8	44	48	20	20	60	4	52	44	20	24	56
January	0	92	8	4	84	12	0	100	0	0	100	0	8	36	56	28	28	44	16	24	60	20	24	56
February	0	84	16	0	80	20	0	100	0	0	100	0	8	56	36	36	24	40	0	56	44	20	32	48
March	0	100	0	0	100	0	0	100	0	0	100	0	8	64	28	16	44	40	12	48	40	28	48	24
Average	0	90.6	9.4	0.8	87.0	12.2	0	96.2	3.8	0	100	0	9.4	46.0	44.6	25.6	27.6	46.8	9.4	43.0	48.4	23.2	32.2	44.6

S – sensitive, R – resistant, I – intermediate

The survey of antibiotic resistance of the *Enterobacteriaceae* family demonstrated that these bacteria are the least sensitive to streptomycin and erythromycin (Table 2, Figure 2). During the study, no bacterial strains were found to be sensitive to erythromycin in both raw and treated sewage. Furthermore, the percentage of strains resistant to erythromycin increased during treatment; for example, in the samples of raw sewage collected in November, 85%

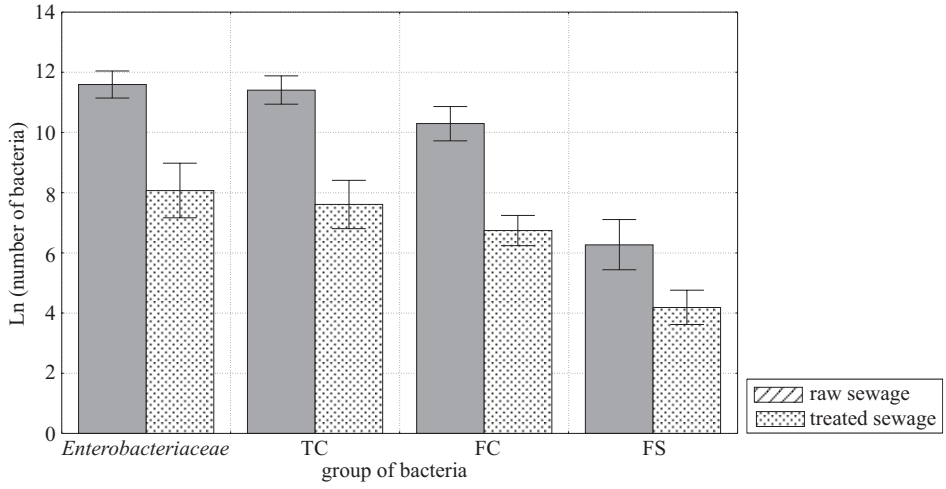

Fig. 1. Elimination of examinated bacteria during the sewage treatment process. Vertical bars represents SD

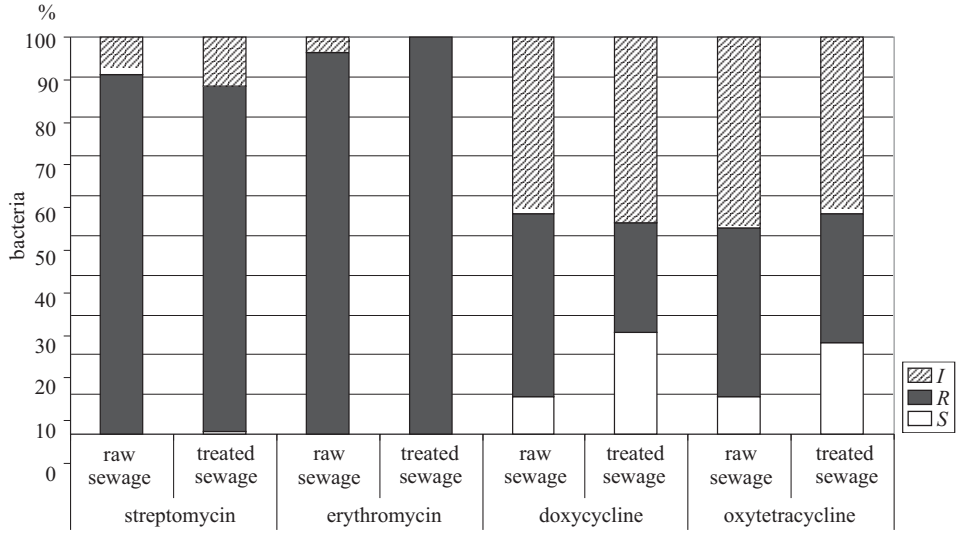

Fig. 2. Antibiotic resistance of *Enterobacteriaceae* bacteria isolated from tested sewage (mean values)

of strains were resistant and for the treated sewage this value reached 100%. An analogous increase in resistance to erythromycin was noted for the December samples.

In the case of streptomycin, no sensitive strains were found, while the average rate of resistant strains was 90.6%. Among the strains isolated from the treated sewage, only 4% of strains from January samples were sensitive to streptomycin (average rate for the entire research period – 0.8%), while 87% were resistant to this antibiotic.

The largest number of strains were found to be sensitive to doxycycline (mean value for raw sewage – 9.4%; for treated sewage – 25.6%) and oxytetracycline (mean value for raw sewage – 9.4%; for treated sewage – 23.2%). The strains resistant to these antibiotics constituted for the raw sewage an average of 46% for doxycycline and 43% for oxytetracycline, while for the treated sewage these values equaled 27.6% and 32.2%, respectively.

During the sewage treatment process, the proportion of strains resistant to doxycycline and oxytetracycline did not increase.

In comparing the percentage rates of strains resistant to erythromycin, doxycycline, and oxytetracycline in raw sewage in subsequent months of the study, a gradual increase in their numbers is visible (Table 2). In November, 85% of strains were found to be resistant to erythromycin; whereas in March, 100% of strains were resistant. In the case of doxycycline, these numbers equaled 30% for November and 64% for March, while for oxytetracycline, 35% for November and 56% for February. The increase in the proportion of bacterial strains resistant to these antibiotics in the subsequent months of the study is correlated with the upward seasonal trend in population morbidity, and in turn the usage antibiotic therapy.

Discussion

With the advancement of civilization, water as an element of the natural environment has been undergoing gradual degradation. This degradation is a result of the inflow of increasing amounts of various pollutants, usually in the form of sewage into the water environment. Pathogenic microorganisms, which commonly occur in farm and domestic sewage, constitute a serious problem. These microorganisms include primarily representatives of *Enterobacteriaceae* family, which in addition to being pathogenic are often resistant to antibiotics (GUARDABASSI et al. 1998).

Results obtained from this study regarding the population sizes of the TC, FC, FS and *Enterobacteriaceae* bacterial groups in raw sewage are in accordance with the results of earlier research (KOIVINEN et al. 2003, GEORGE et al.

2002, DIONISIO et al. 2000), and confirm that the treatment process utilizing active sludge technology does not eliminate all intestinal bacteria.

The TC bacterial reduction rate for the surveyed sewage treatment plant ranged from 96.25 to 98.31%, with an average value for the entire research period of 97.28%. The obtained reduction rate is consistent with KOIVUNEN'S (2003) results, whose rate ranged from 96.2 to 99.6%. Similarly, the abundances of bacteria from the FC group, which were at a level of $34.20 \cdot 10^3$ cells cm^{-3} in raw sewage and $0.93 \cdot 10^3$ cells cm^{-3} in treated sewage, confirm the earlier results of GEORGE et al. (2002). This author found that the abundance of FC bacteria in raw sewage ranges from $104 - 10^5$ cells cm^{-3}. The FC abundance obtained during this study equaled $34.20 \cdot 10^3$ cells cm^{-3}, ii is the same like $3.4 \cdot 10^4$. The mean reduction rate of the FC abundance in treated sewage equaled 97.28% and is similar to the values (94.3 – 99.8%) obtained by KOIVUNEN et al. (2003).

The abundance of fecal streptococci (FS) was much lower in both the raw and treated sewage than previously discussed bacterial groups (TC and FC), but the reduction rate during the treatment process was also lower. The FS reduction rate reached 85.97%. However, according to the commonly accepted standards, the reduction rate of intestinal bacteria during the treatment process should range from 90 to 99% (TEITGE et al. 1986, KAYSER et al. 1987). In light of KOINVUNEN;S (2003) results, the fecal streptococci elimination rate (85.97%), determined in this study, is not particularly low. It is also noteworthy that during the treatment process the characteristic chains of fecal streptococci undergo at least partial fragmentation into single cells. These cells, in the form of micrococcus, are poorly absorbable by flocks of active sludge and may not undergo sedimentation in the secondary sedimentation tank.

The survey of *Enterobacteriaceae* bacterial abundance demonstrated that bacteria of this family are present in the treated sewage. This fact provides evidence that these microorganisms, including their pathogenic forms, penetrate to the natural environment.

The sewage treatment processes have a high impact on abundance of *Enterobacteriaceae* microorganisms and other bacterial groups. The mean abundance of the bacteria from this family in the treated sewage equaled $4.51 \cdot 10^3$ cells cm^{-3}, while in the raw sewage, $118.20 \cdot 10^3$ cells cm^{-3}. These numbers demonstrate that the reduction rate of the treatment process was considerable; on average, it reached 96.18%.

According to PIKE and CURDS (1971), the considerable decrease in microorganism abundance is a result of the active sludge properties. Bacterial cells that flow with the sewage to the aeration tank adhere to sedimentation flocks and, as a result of this process, their abundance in the treated sewage

decreases. Moreover, the reduction of these bacteria during treatment is enhanced by microorganisms that are present in the active sludge, primarily protozoa and, to a lesser degree, bacteriophages, and *Bdellovibrio* (PIKE, CURDS 1971).

The analysis of the bacteria of *Enterobacteriaceae* family that were collected from the technological line of the Municipal Sewage Treatment Plant in Toruń demonstrated that these bacteria were, for the most part, resistant to all surveyed antibiotics.

The data regarding the antibiotic resistance of the strains to erythromycin (macrolide antibiotics) demonstrate their significant resistance to this antibiotic. Among all tested bacterial strains, 96.2% of bacteria in the raw sewage and 100% of bacteria in the treated sewage were resistant to erythromycin.

Significant resistance to this antibiotic was also demonstrated by SCHWARTZ et al. (2003), who found that all analyzed bacterial strains collected in raw sewage of *Enterobacteriaceae* family were resistant to erythromycin. The lower effectiveness of this antibiotic towards *Enterobacteriaceae* bacteria, which was observed in this study, is probably a result of a natural property of erythromycin, which is responsible for poor performance against gram-negative enteric bacteria (PODLEWSKI, CHWALIBOGOWSKA-PODLEWSKA 1999).

The results obtained from this study demonstrate that on average 90.6% and 87.1% of bacterial strains collected in raw and treated sewage, respectively, were resistant to streptomycin. According to PODLEWSKI and CHWALIBOGOWSKA-PODLEWSKA (1999), the disadvantage of streptomycin is the rapid occurrence of strains resistant to this antibiotic. For example, *Mycobacterium tuberculosis* became resistant to this antibiotic after several days of application. The rapid occurrence of antibiotic-resistant bacteria could have had a decisive impact on the results obtained during this study.

The conducted analyses proved that *Enterobacteriaceae* bacteria were least resistant to tetracyclines (doxycycline and oxytetracycline). According to the obtained data, 46% and 27.6% of bacterial strains collected from raw and treated sewage, respectively, were resistant to doxycycline. In the case of oxytetracycline, the resistance of isolated bacterial strains equaled 43 and 32.3% for raw and treated sewage, respectively.

However, despite the fact that the analyzed strains were most sensitive to tetracyclines, the author of this study observed that a large number of *Enterobacteriaceae* bacteria were resistant even to doxycycline. REINTHALER et al. (2003) obtained similar results. They observed a significant increase in *Enterobacteriaceae* bacterial resistance to tetracyclines, especially among *Escherichia coli* (57% of analyzed strains) collected from sewage.

The data analysis conducted for this study identified differences in antibiotic resistance of the surveyed bacterial strains in individual stages of the

treatment process in the Municipal Sewage Treatment Plant in Toruń. Results demonstrated that the treatment process caused a gradual reduction in the proportion of strains resistant to streptomycin, doxycycline, and oxytetracycline. In the case of streptomycin, these changes were small, but were more pronounced for doxycycline and oxytetracycline.

It is hard to identify the factor responsible for these results. These results were most probably due to properties of the active sludge, whose flocks could have caused a reduction in their abundance in the treated sewage through bonding the cells of the enteric bacteria. However, the impact of physicochemical factors, which change in the course of the technological process of sewage treatment, on the bacteria antibiotic resistance should not be unquestionably ruled out.

After the data analysis, it was concluded that in the case of the bacteria of *Enterobacteriaceae* family, the sewage treatment process may also have contributed to their antibiotic resistance. This was true in the case of erythromycin; the highest abundance of strains resistant to this antibiotic was found in the treated sewage. The observed increase in resistance during the treatment process may have been caused by the transfer of resistance genes in the form of R plasmids, transposons, introns or gene cassettes by three mechanisms: conjugation, transduction, and transformation (SCHMIDT et al. 2001).

The occurrence of antibiotic-resistance bacteria of *Enterobacteriaceae* family in sewage is a commonly reported fact (GONI-URRIZA et al. 2000), and has also been confirmed by the results of this study. The presence of these bacteria is a result of constantly increasing and improper application of antibiotics, primarily in medicine but also in veterinary medicine, agriculture, and households. Furthermore, the observed increase in the number of strains resistant to tested antibiotics in the months (November – March) of soaring morbidity demonstrates an often unnecessary and improper usage of antibiotics in health care. This increase constitutes a serious problem because this group of bacteria comprises pathogens, which acquire resistance to antibiotics used in medicine.

There is also a risk of transfer of microorganisms, some of which are pathogenic and have acquired antibiotic resistance, to the natural water environment with the sewage.

Coclusion

This paper presented data of abundance reduction of sanitary indicator bacteria (TC, FC, FS) and *Enterobacteriaceae* family bacteria in raw and treated sewage of Municipal Sewage Treatment Plant in Torun (Poland).

1. The average abundance of bacteria from *Enterobacteriaceae* family in raw sewage equaled $118.20 \cdot 10^3$ cells cm^{-3}, and in treated sewage, $4.51 \cdot 10^3$ cells cm^{-3}. Fecal streptococci (FS) was the least numerous group. The average abundance of this bacteria was $0.73 \cdot 10^3$ in raw, and $0.07 \cdot 10^3$ cells cm^{-3} in treated sewage.

2. The analysis of the bacteria from *Enterobacteriaceae* family that were collected, demonstrated that these bacteria were, for the most par, resistant to all surveyed antibiotics.

3. The increase in the proportion of bacteria strains resistant of tested antibiotics in the subsequent month (November – March) was correlated with the upward seasonal trend in population morbidity, and in turn the usage antibiotics therapy.

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References

BARNESS D., BLISS P.J., GOULD B.W., VALLENTINE H.R. 1981. *Water and wastewater engineering systems.* Pitman Press, London, 180-221.

DIONISIO L.C., RHEINHEIMER G., BORREGOS J.J. 2000. *Microbiological pollution of Ria Formosa (South of Portugal).* Marine Poll. Bull., 40 (2): 186-93.

GEORGE I., CROP P.H., SERVAIS P. 2002. *Fecal coliform removal in wastewater treatment plants studied by plate counts and enzymatic methods.* Wat. Res., 36 (10): 2607-2617.

GONI-URRIZA M., CAPDEPUY M., ARPIN C., RAYMOND N., CAUMETTE P., QUENTIN C. 2000. *Impact of an urban effluent on antibiotic resistance of riverine Enterobacteriaceae and Aeromonas spp.* Appl. Environ. Microbiol., 66 (1): 125-132.

GUARDABASSI L., PETERSEN A., OLSEN J.E., DALSGAARD A. 1998. *Antibiotic resistance in Acinetobacter spp. Isolated from sewers receiving waste effluent from a hospital and a pharmaceutical plant.* Appl. Environ. Microbiol., 64 (9): 3499-3502.

GUARDABASSI L., DANILA M.A., WONG L.F., DALSGAARD A. 2002. *The effects of tertiary wastewater treatment on the prevalence of antimicrobial resistant bacteria.* Wat. Res., 36 (8): 1955-1964.

KAYSER R., BOLL R., MÜLLER H.E. 1987. *Quantitative determinations of the elimination of salmonellae by biological treatment of wastewater.* Zentralbl Bakteriol Mikrobiol Hyg B, 184: 195-205 (in German).

KOIVUNEN J., SIITONEN A., HEINONEN-TANSKI H. 2003 *Elimination of enteric bacteria in biological--chemical wastewater treatment and tertiary filtration units.* Wat. Res., 37 (3): 690-698.

PIKE E.B., CURDS C.R. 1971. *Microbial ecology of the activated sludge process. Microbial aspect of pollution.* Acad. Press, London and New York, 123-147 pp.

PODLEWSKI J.K., CHWALIBOGOWSKA-PODLEWSKA A. 1999. *Drugs of contemporary therapy.* XIV ed. Split Trading, Warsaw, 672-754 pp. (in Polish).

REINTHALER F.F., POSCH J., FEIERL G., WUST G., HAAS D., RUCKENBAUER G., MASCHER F., MARTH E. 2003. *Antibiotic resistance of E. coli in sewage and sludge.* Wat. Res., 37 (8): 1685-1690.

ROSE J.B., DICKSON L.J., FARRAH S.R., CARNAHAN R.P. 1996. *Removal of pathogenic and indicator microorganisms by full-scale water reclamation facility.* Wat. Res., 30 (11): 2785-2797.

SCHMIDT A.S., BRUUN M.S., DALSGAARD J., LARSEN J.L. 2001. *Incidence, distribution and spread of tetracycline resistance determinations and integron-associated antibiotic resistance genes among Motile Aeromonads from a fish farming enviroment.* Appl. Environ. Microbiol., 67 (12): 5675-5682.

SCHWARTZ T., KOHNEN W., JANSEN B., OBST U. 2003. *Detection of antibiotic-resistant bacteria and their resistance genes in waste water, surface water and drinking water biofilms.* FEMS Microbiol. Ecol., 43 (3): 325-335.

TEITGE E., GERHARDT G.G., GUNDERMANN K.O. 1986. *Quantitative studies of salmonella at two sewage purification plants in Schleswig-Holstein.* Zentralbl Bakteriol Mikrobiol Hyg B, 180: 120-130 (in German).

Water quality. Detection and enumeration of intenstial enterococci. Part 2: *Membrane filtration method.* 2000. European Comitee for Standarization, EN ISO 7899-2, Bruxelles, Belgium.

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APPLICATION OF TWO SAMPLING METHODS TO ASSESSMENT OF BACTERIOLOGICAL CONTAMINATION OF ATMOSPHERIC AIR IN OLSZTYN

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K e y w o r d s: atmospheric air, bacteria, sedimentation method, impaction method.

A b s t r a c t

Atmospheric air samples for bacteriological assays were collected by the sedimentation and impaction methods. The samples were used for quantification of psychrotrophic and mesophilous bacteria in atmospheric air in the centre of Olsztyn and in Kortowo, a suburb of the town. It was found that counts of microorganisms were much higher when air was sampled by the sedimentation method rather than the impaction one. Besides, it was demonstrated that the determined groups of bacteria were more numerous in summer (July, August) than in the other months. Higher bacteriological contamination was determined at the three sampling sites in the centre of Olszyn than at the control site in a forest by Kortowskie Lake, in Kortowo.

ZASTOSOWANIE DWÓCH METOD BADAŃ W OCENIE BAKTERIOLOGICZNEGO ZANIECZYSZCZENIA POWIETRZA ATMOSFERYCZNEGO OLSZTYNA

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S ł o w a k l u c z o w e: powietrze atmosferyczne, bakterie, metoda sedymentacyjna, metoda zderzeniowa.

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Abstrakt

W próbkach powietrza atmosferycznego – pobranych do badań bakteriologicznych metodami sedymentacyjną i zderzeniową w centrum miasta Olsztyna i dzielnicy Kortowo – oznaczono ilościowo bakterie psychrotrofowe i mezofilne. Stwierdzono kilka razy większą liczebność badanych grup bakterii w próbkach powietrza pobranych metodą sedymentacyjną. Ponadto wykazano, że liczebność oznaczanych grup bakterii była wyższa latem (w lipcu, sierpniu) niż w pozostałych miesiącach badań. Stwierdzono także większe zanieczyszczenie bakteriologiczne powietrza na trzech stanowiskach w centrum Olsztyna niż na stanowisku kontrolnym w lesie, przy Jeziorze Kortowskim, w dzielnicy Kortowo.

Introduction

Excessive exploitation of natural resources and their processing, along with increasing areas of urbanised lands (DI GIORGIO et al. 1996), economic activity of people and higher numbers of employees working in towns – all these factors add to progressing degradation of natural environment (BIESZCZAD, SOBOTA 1993). The atmosphere receives emissions of large amounts of harmful pollutants, such as ashes, organic compounds, non-organic substances and microorganisms (BOVALLIUS et al. 1978, MANCINELLI, SHULLS 1978, KRZYSZTOFIK 1992, LIGHTHART 1997). Although atmospheric air is not a hospitable living environment for microorganisms, mainly because of the influence, pathogenic microorganisms (viruses, bacteria and fungi) as well parasites which enter the outside environment, often in the form of aerosols, demonstrate different survival rates in air. Pathogenic microorganisms in air can cause infections and illnesses of humans and animals, particularly ailments of the respiratory system (TERZIEVA et al. 1996, JUOZAITIS et al. 1998, MARKS et al. 2001). Carried by air currents, such pathogenic agents can travel over large distances.

Survival of aerosols in air depends on their size and composition (SHAFFER, LIGHTHART 1997, RAGA et al. 2001) and on certain environmental factors such as temperature, atmospheric pressure, relative humidity of air, intensity of solar radiation, time of the day and season of the year and the location of a given area (DI GIORGIO et al. 1996, ZMYSŁOWSKA, JACKOWSKA 2003). All those environmental factors affect the qualitative and quantitative composition of microflora present in atmospheric air.

The purpose of the study was to evaluate the degree of contamination of atmospheric air with psychrotrophic and mesophilous bacteria, in the centre of Olsztyn (three sites) and in a forest near Kortowskie Lake (one site). The air was sampled by the sedimentation and impaction methods.

Material and Methods

Sampling. Air samples for bacteriological counts were collected at four sampling sites, in three series, from April to August 2002 at monthly intervals and in October 2002 and February 2003. Two methods were applied: the sedimentation method in compliance with the recommendations of the Polish Norms (PN-89 Z-04111/01, PN-89 Z-04111/02, PN-89 Z-04008/08) and the impaction method using a MAS 100 Eco™ microbiological air sampler manufactured by Merck (ZMYSŁOWSKA, JACKOWSKA 2005).

Sampling sites. Three sites were set up in the centre of Olsztyn (Figure 1). In addition, one sample was established in a suburban area of Olsztyn called Kortowo. The sites were designated as site 1 (near the Dukat department store) site 2 (a square in front of the Court of Law), site 3 (the crossroads at the Town Hall), site 4 (control; in the forest by Kortowskie Lake in Kortowo, part of Olsztyn). The three sites in the centre differed from the control site in Kortowo in that they were situated in a built-up area, much heavier traffic and a high number of pedestrians.

Fig. 1. Location of the sampling sites in the centre of Olsztyn

Bacteriological assays. The quantitative assays of bacteria in the atmospheric air of Olsztyn comprised determinations of the following groups of bacteria:

– total count of psychrotrophic bacteria on nutrient agar, incubated at 22°C for 72 hours, including the proportion of pigment bacteria. In order to create good conditions for bacteria to produce pigment, incubation was conducted under daylight. After the incubation, colour (pigment) and colourless colonies were counted separately.

– total count of mesophilous bacteria on nutrient agar incubated at 37°C for 24 hours.

All determinations were done in three parallel replications using both methods of air sampling. The results obtained with the sedimentation method were converted into colony forming units in 1 m^3 (cfu · m^{-3}) using Omelianski's formula as modified by Gogoberidze (PN-89 Z-04111/02). The results obtained with the impaction method were converted into cfu · m^{-3} using the formula reported by KRZYSZTOFIK (1992).

Meteorological measurements. While collecting air samples for bacteriological assays, meteorological data was recorded, including temperature, atmospheric pressure, relative air humidity, wind speed and direction and sunlight.

Statistical analysis. The results of quantitative bacteriological assays of atmospheric air underwent statistical interpretation. In order to reveal significant differences between counts of psychrotrophic and mesophilous bacteria versus the sites located in the town centre and in Kortowo, a recreational area of Olsztyn, or the date of air sampling, the Kruskal-Wallis non-parametric ANOVA test was applied (STANISZ 1998). The level of significance at $p \leq 0.05$ was assumed for all statistical tests.

Results

The results of the quantitative bacteriological assays of the atmospheric air in Olsztyn sampled by the sedimentation method showed that the total count of psychrotrophic bacteria ranged between 40 cfu · m^{-3} in February at site 4 to about 8 500 cfu · m^{-3} in May at site 3 and in July at site 4 (Figure 2 a). In air collected with the impaction method the count of psychrotrophic bacteria varied from 0 cfu · m^{-3} in April and February at site 4 to 12 500 cfu · m^{-3} in August at site 1 (Figure 2b).

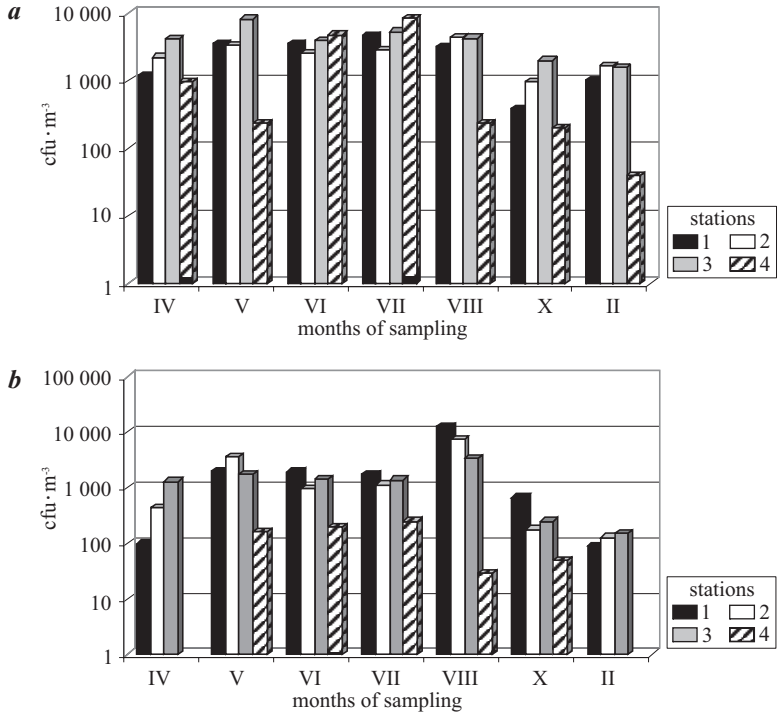

Fig. 2. Counts of psychrotrophic bacteria in 1 m^3 of air at the sites located in the town centre (1, 2, 3) and in Kortowo, a suburb of Olsztyn (4), determined with: *a* – sedimentation method, *b* – impaction method

Of all psychrotrophic bacteria revealed during our assays, the pigment forms made up from 40% in February to 100% in October (Figure 3a) when the sedimentation method had been applied, or from 25% in August to 100% in October following the application of the impaction method (Figure 3b).

The total count of mesophilous bacteria in the analysed atmospheric air collected by the sedimentation method ranged between 40 cfu · m^{-3} in August at site 4 to about 3 000 cfu · m^{-3} in July at site 1 (Figure 4a). In the air sampled by the impaction method, the total count of these bacteria ranged from 0 cfu · m^{-3} in April and August at site 1 to about 800 cfu · m^{-3} in May at site 2 and in July at site 1 (Figure 4b).

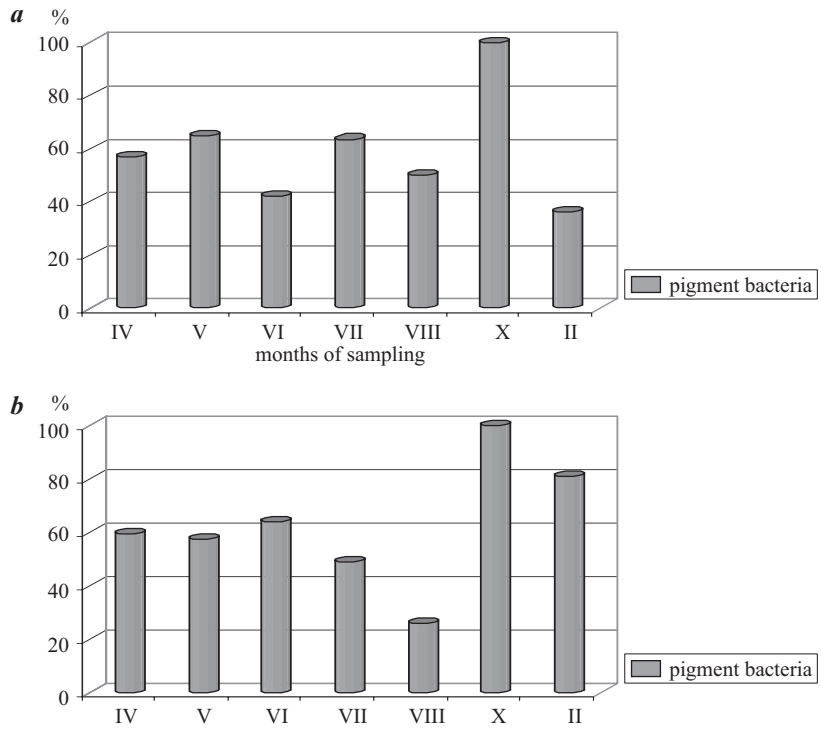

Fig. 3. Percentage of pigment forms in the total count of psychrotrophic bacteria in 1 m^3 of atmospheric air at the sites in the town centre (1, 2, 3) and in Kortowo, a suburb of Olsztyn (4), determined by: *a* – the sedimentation method, *b* – the impaction method

In general, higher counts of both psychrotrophic and mesophilous bacteria were determined at sites 1, 2 and 3, all located in the centre of the town, than at site 4, the control one, which lay in the outskirts of Olsztyn (Figures 2, 4).

The highest average counts of psychrotrophic bacteria, ca 6 000 cfu $\cdot$ m^{-3} (Figure 5a) and mesophilous ones, ca 2 000 cfu $\cdot$ m^{-3} (Figure 5b), were determined in July in 1 m^3 of air collected by the sedimentation method. In air samples gathered by the impaction method, the highest counts of psychrotrophic bacteria were obtained in August (ca 6 000 cfu $\cdot$ m^{-3}), whereas the maximum number of mesophilous bacteria appeared in July (ca 400 cfu $\cdot$ m^{-3}). The lowest counts of those bacteria occurred in April, October and February, irrespective of which determination method was applied (Figure 6a,b).

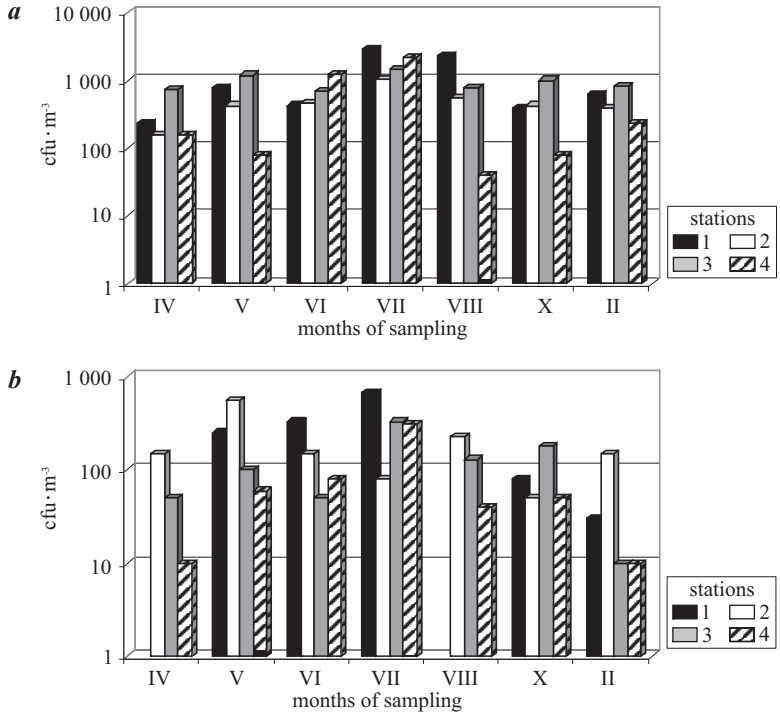

Fig. 4. Counts of mesophilous bacteria in 1 m^3 of atmospheric air at the sites in the town centre (1, 2, 3) and in Kortowo, a suburb of Olsztyn (4), determined by: *a* – the sedimentation method, *b* – the impaction method

The statistical analysis, with an aid of the Kruskal-Wallis non-parametric ANOVA test, showed that the month of air sample collection had a significant influence on counts of psychrotrophic bacteria determined by the sedimentation method (Figure 7). In contrast, particular dates of sample collection showed no significant effect on counts of psychrotrophic bacteria in air sampled by the impaction method or counts of mesophilous bacteria determined via either of the methods. Besides, no statistically significant effect of particular sampling sites on psychrotrophic and mesophilous bacterial counts (both the sedimentation and impaction methods) was proved. Consequently, these results were excluded from the present report.

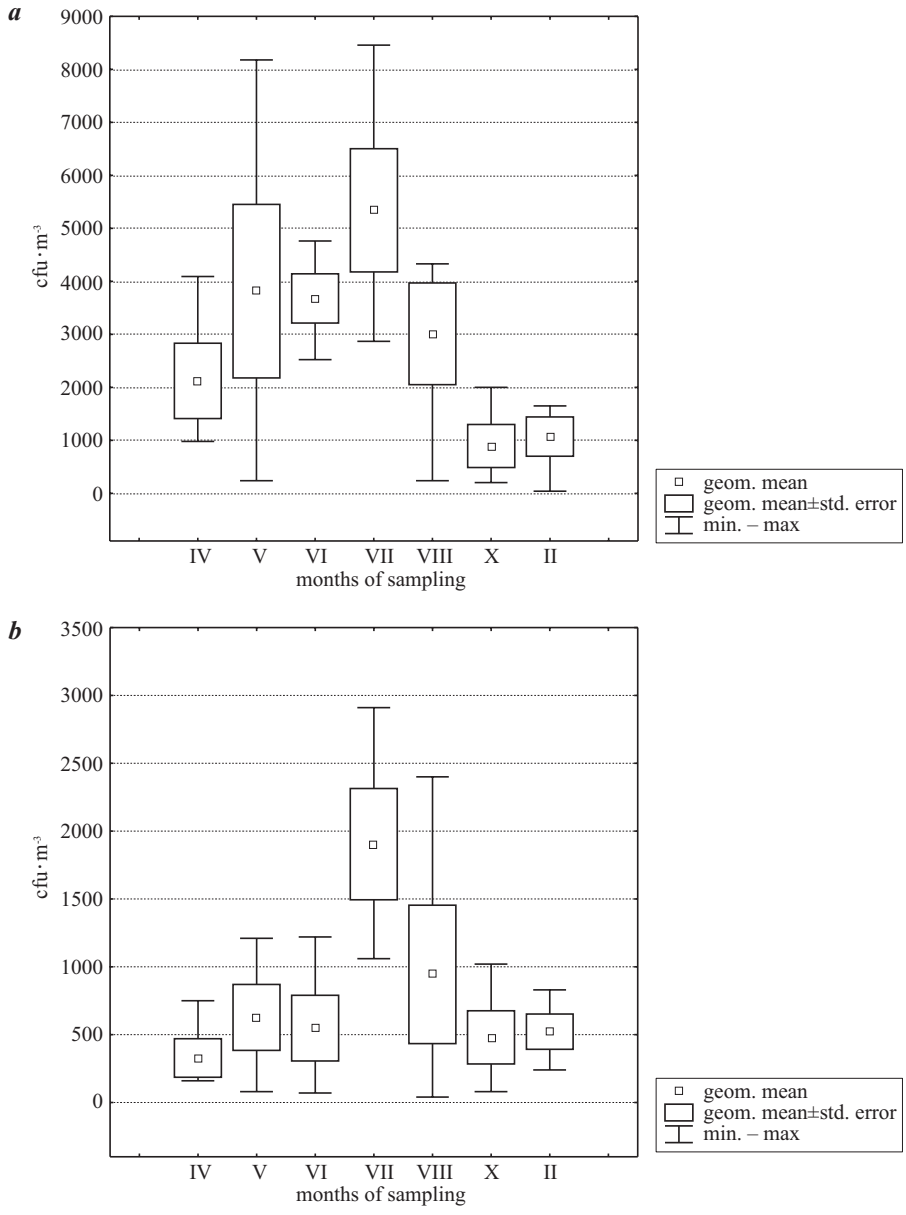

Fig. 5. Means and ranges of counts of: *a* – psychrotrophic, *b* – mesophilous bacteria in 1 m^3 of atmospheric air in Olsztyn sampled by the sedimentation method in particular months of the study

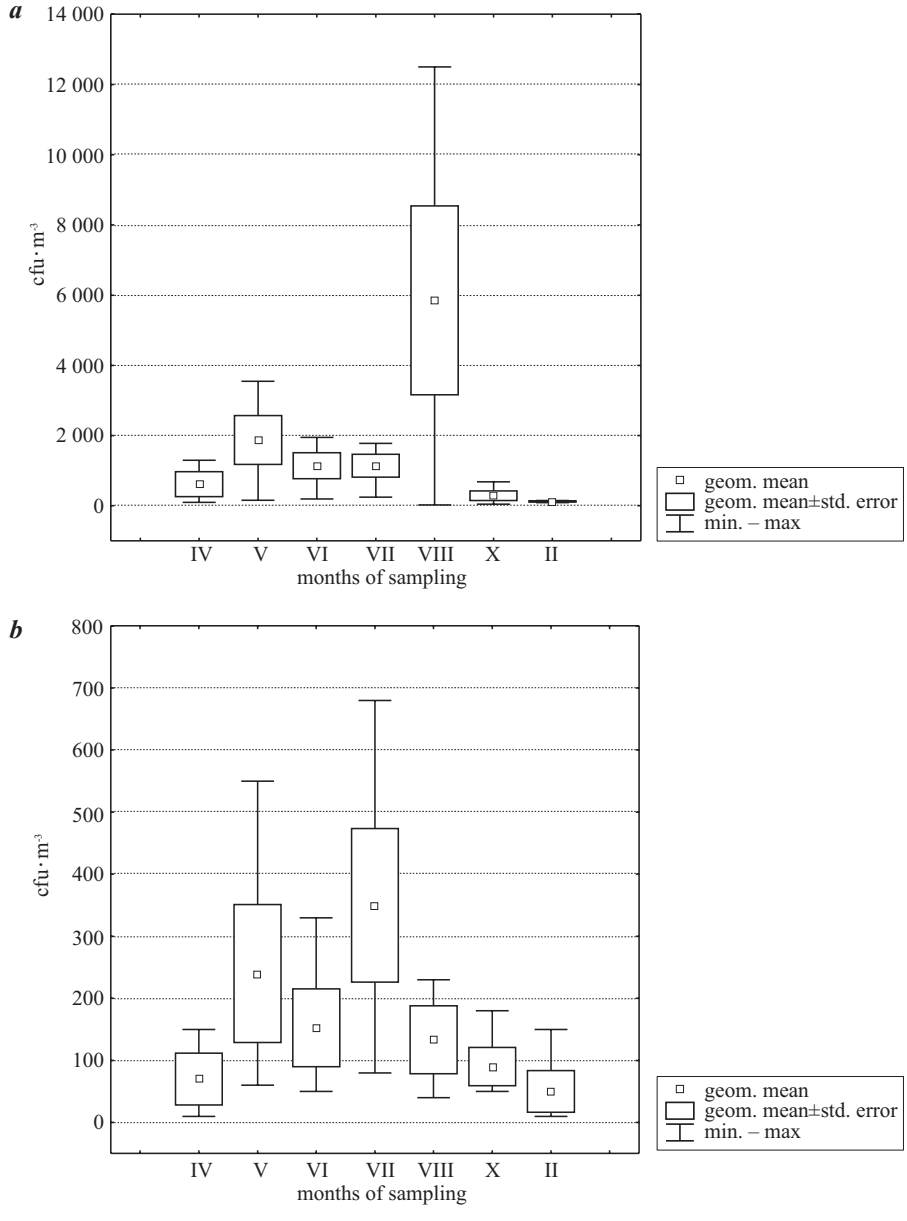

Fig. 6. Means and ranges of counts of: *a* – psychrotrophic, *b* – mesophilous bacteria in 1 m^3 of atmospheric air in Olsztyn sampled by the impaction method in particular months of the study

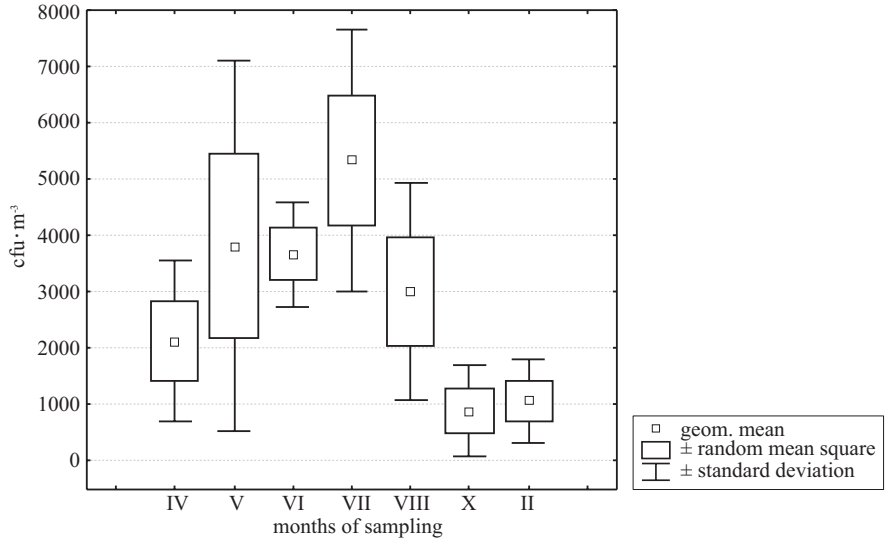

Fig. 7. Results of the statistical analysis with the Kruskal-Wallis ANOVA test at $p \leq 0.05$. Evaluation of the effect of a month of air sampling on psychrotrophic bacteria, determined with the sedimentation method

Table 1 shows the results obtained from the measurements of the meteorological parameters, which were taken at a time of air sampling.

Discussion of Results

The bacteriological assays of atmospheric air in Olsztyn, collected simultaneously by two methods: the sedimentation and the impaction ones, showed certain variation in the counts of psychrotrophic and mesophilous bacteria, which was caused by the use of two completely different methods of air sampling. It can be concluded that results of quantitative bacteriological assays obtained with an aid of the sedimentation and impaction methods are not comparable. When using the impaction method, an air stream of certain capacity is sucked into an apparatus for a certain period of time (KRZYSZTOFIK 1992). In the sedimentation method, the microorganisms which fall onto a known surface of a bacteriological medium during a certain time period are counted (BOŻKO et al. 1961, NOWAK et al. 1998). Thus, it can be assumed that exposed media on Petri plates contain a larger proportion of thick cells than impact plates in an aspirator. It should also be noticed that higher relative air humidity favours formation of larger agglomerates of microbes, thus accelerating their sedimentation (KRUCZALAK et al. 2002). Although the results of bacteriological assays obtained via the sedimentation or the impaction method

Table 1

Meteorological measurements

Month of air sampling	Site	Time of sampling	Temperature (°C)	Relative air humidity (%)	Atmospheric pressure (hPa)	Wind direction and speed (m s^{-1})	Sunlight	Precipitation
IV	1 2 3 4	17^{30} 8^{15} 900 1200	8 8 8.5 8.5	77 71 71 68	1016	1.0-3.0 NE	complete cloud cover	no precipitation
V	1 2 3 4	720 755 815 1200	10 8 11 17	70 69 70 79	1018	2.7-3.8 SW	sunny	thunderstorm a day before
VI	1 2 3 4	710 750 830 1240	24 24 24 23.5	43 47 43.5 35	1018	moderately strong SW	partly cloudy	no precipitation
VII	1 2 3 4	725 805 840 1215	18 17 18 22	57 60 58 47	1004	moderately strong SW	varied cloud cover	
VIII	1 2 3 4	725 800 835 1215	20 21 21 25	60 70 68.5 70	1011	moderately strong N	sunny	
X	1 2 3 4	720 755 835 1235	11.5 5 6 8	61 71.5 72.5 68.5	1011	strong S	complete cloud cover	
II	1 2 3 4	735 812 850 1215	-3 -2 1 3	59 63 68.5 67	1028	1.0-3.0 NE	complete cloud cover	

cannot be compared, they may be used for comparison with results cited by other authors, who applied corresponding methodology. Besides, either of the methods can be selected for a research project depending on the assumed focus.

Apart from colourless bacteria, the air contained between 40 and 100% pigment cells. The 100% ratio of pigment bacteria to the total population of psychrotrophic bacteria determined in October using both air sampling methods can be ascribed to the elevated survival rate of these microorganisms in the time period preceding the sample collection procedure, owing to the air temperature (8°C) and high relative air humidity (68%). Pigments present in bacterial cells protect them from the effect produced by visible sunlight (SHAFFER, LIGHTHART 1997), therefore the survival rate of pigment bacteria is higher than that of colourless forms exposed to sunrays (SCHLEGEL 2001).

With the data obtained during our study, we can conclude that the counts of bacteria in the town centre obtained via the impaction method were several-fold lower than those yielded with the use of the sedimentation method (except samples collected in August). A similar relationship was confirmed by KRUCZALAK et al. (2002), who completed bacteriological assays of air in the shoreline zone of the Gulf of Gdańsk. Our own studies showed that the highest counts of mesophilous bacteria in 1 cubic meter of atmospheric air appeared in July (2000 cfu $\cdot$ m^{-3} in air sampled by the sedimentation method and 400 cfu $\cdot$ m^{-3} in air sampled by the impaction method). Increased concentrations of bacteria determined in the summer months and their less numerous occurrence in the other months have been reported by other researchers (BOŻKO et al. 1961, BOVALLIUS et al. 1978, DI GIORGIO 1996).

The evaluation of the bacteriological purity of air regarding counts of mesophilous bacteria, as compared with the Polish Norms (PN-89 Z-04111/02), shows that on one occasion, in July, the air sampled by the sedimentation method was moderately contaminated, whereas all the samples collected by the impaction method can be described as uncontaminated with mesophilous bacteria.

The statistical analysis of the data revealed that the sampling date had a significant effect on counts of psychrotrophic bacteria. It could be hypothesised that this effect was due to the meteorological conditions which occurred on a month when air samples were collected.

Conclusions

1. Bacteriological assays of the atmospheric air in Olsztyn sampled from April 2002 to February 2003 by the sedimentation and impaction methods yielded different counts.

2. Higher bacteriological contamination was determined in summer (July, August) than in the other months of the study, in air samples collected by either of the methods.

3. In general, counts of psychrotrophic and mesophilous bacteria in air sampled in the town centre, with either of the sampling methods, were higher than those obtained at the control site near the forest by Kortowskie Lake.

4. The bacteriological purity of atmospheric air in Olsztyn (from April 2002 to February 2003) depended on the season of the year (month) and location (site) of sampling.

5. The atmospheric air in Olsztyn during the time period covered by our study, evaluated in terms of bacteriological purity according to the Polish Norms ([PN-89 Z-04111/02), can be classified as moderately contaminated (the sedimentation method) or uncontaminated (the impaction method).

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References

BIESZCZAD S., SOBOTA J. 1993. *Zagrożenia, ochrona i kształtowanie środowiska przyrodniczo-rolniczego*, Wyd. AR Wrocław.

BOVALLIUS A., BUCHT B., ROFFEY R., ANÄS P. 1978. *Three-year investigation of the natural airborne bacterial flora at four localities in Sweden.* Appl. Environ. Microbiol., 35: 847-852.

BOŻKO L., CYPRYK-OSSOWSKA K., KRZYSZTOFIK B. 1961. *Mikroflora powietrza zakładów gastronomicznych i powietrza atmosferycznego miasta Warszawy.* Acta Microbiol. Pol., 10: 307-322.

DI GIORGIO C., KREMPFF A., GUIRAUD H., BINDER P., TIRET C., DUMENIL G. 1996. *Atmospheric pollution by airborne microorganisms in the city of Marseilles.* Atmos. Environ., 30: 155-160.

JUOZAITIS A., LUGAUSKAS A., SVEISTYTE L., ULEVICIUS V., WILLEKE K., GRINSHPUN S. A., REPONEN T. 1998. *Assessment of microorganism transfer from the Baltic Sea waters to the atmosphere.* J. Aerosol Sci., 29: S547-S548.

KRUCZALAK K., OLAŃCZUK-NEYMAN K., MARKS R. 2002. *Airborne microorganisms fluctuations over the Gulf of Gdańsk Coastal Zone (Southern Baltic),* Pol. J. Environ. Stud., 11: 531-536.

KRZYSZTOFIK B. 1992. *Mikrobiologia powietrza*. Wyd. Politechniki Warszawskiej.

LIGHTHART B. 1997. *The ecology of bacteria in the alfresco atmosphere*. FEMS Microbiology Ecology, 23: 263-274.

MANCINELLI R., SHULLS W.A. 1978. *Airborne bacteria in an urban environment.* Appl. Environ. Microbiol., 35: 1095-1101.

MARKS R., KRUCZALAK K., JANKOWSKA K., MICHALSKA M. 2001. *Bacteria and fungi in air over the Gulf of Gdańsk and the Baltic Sea.* Aerosol Sci., 32: 237 -250.

NOWAK A., PRZYBULEWSKA K., TARNOWSKA A. 1998. *Badania rozkładu dobowego zanieczyszczenia mikrobiologicznego powietrza na terenie Szczecina.* Chem. Inż. Ekol., 5 (1-2): 52-65.

Ochrona czystości powietrza. Badania mikrobiologiczne. Postanowienia ogólne i zakres normy. 1989. Polski Komitet Normalizacji, Miar i Jakości, PN-89 Z-04111/0.1

Ochrona czystości powietrza. Badania mikrobiologiczne. Oznaczanie liczby bakterii w powietrzu atmosferycznym (imisja) przy pobieraniu próbek metodą aspiracyjną i sedymentacyjną. 1989. Polski Komitet Normalizacji, Miar i Jakości, PN-89 Z-04111/02..

Ochrona czystości powietrza. Pobieranie próbek. Pobieranie próbek powietrza atmosferycznego (imisja) do badań mikrobiologicznych metodą aspiracyjną i sedymentacyjną. 1989. Polski Komitet Normalizacji, Miar i Jakości, PN-89 Z-04008/08.

RAGA G.B., BAUMGARDNER D., CASTRO T., MARTÏNEZ-ARROYO A., NAVARRO-GONZÄLEZ R. 2001. *Mexico city air quality: a qualitative review of gas and aerosol measurements (1960-2000),* Atmos. Environ., 35: 4041-4058.

SHAFFER B.T., LIGHTHART B. 1997. *Survey of culturable airborne bacteria at four diverse locations in Oregon: urban, rural, forest and coastal.* Springer-Verlag, New York, 34: 167-177.

SCHLEGEL H. G. 2001. *Mikrobiologia ogólna.* PWN, Warszawa.

STANISZ A. 1998. *Przystępny kurs statystyki w oparciu o program STATISTICA PL na przykładach z medycyny*. StatSoft Polska Sp. z o.o., Kraków, 263-292.

TERZIEVA S., DONNELLY J., ULEVICIUS V., GRINSHPUN S.A., WILLEKE K., STELMA G.N., BRENNER K.,P. 1996. *Comparison of methods for detection and enumeration of airborne microorganisms collected by liquid impingement.* Appl. Environ. Microbiol., 62: 2264-2272.

ZMYSŁOWSKA I., JACKOWSKA B. 2003. *Microbiological contamination of the air in the centre and recreational areas of the city of Olsztyn.* Pol. J. Natur. Sc., 15 (3): 689-700.

ZMYSŁOWSKA I., JACKOWSKA B. 2005. *The occurrence of the airborne bacteria genus Staphylococcus in the city of Olsztyn.* Arch. Environ. Protect., 31 (1): 13-24.

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APPLICATION OF NANOFILTRATION FOR DEMINERALIZATION AND DEACIDIFICATION OF TWAROG ACID WHEY

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K e y w o r d s: twarog, acid whey, demineralisation, deacidification, nanofiltration.
Abbreviations: NF – nanofiltration, DF – diafiltration, NEU – neutralisation.

A b s t r a c t

Valourization of acid whey is one of the greatest problem of the dairy industry. The utilization of acid whey is expensive because of its high biological oxygen demand (BOD), high lactic acid and minerals contents and low solids content. Increasing trend in fresh cheeses production forces to develop acid whey utilization technology in order to recover valuable whey components and obtain a better economic return.

In this connection the aim of the study was to recover and purify the valuable acid whey components by application of nanofiltration (NF). NF membrane processing was investigated as a method of demineralizing and deacidifying twarog cheese acid whey as a function of pH depending on location of acid whey neutralisation. The performance of NF membranes was characterized by in terms of ash and lactic acid retention characteristics. This study was focused on improving the acid whey demineralization and deacidification rates and finding the optimal place for neutralisation (NEU).

The most advantageous localization of neutralisation showed after nanofiltration. The use of nanofiltration has been applied to acid whey to concentrate its solids content around three fold. The results showed that retention depends on acid whey pH. Acid whey was demineralised to a degree of 40% by NF and 60% by diafiltration (DF). The obtained deacidification levels were 30% for NF and 44% for DF. Comparison of obtained ash and lactic acid reductions showed that acid whey should be neutralised after NF. The produced purified acid whey concentrate can be used as an ingredient of food products such ice cream, yoghurt or sweet syrup.

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ZASTOSOWANIE NANOFILTRACJI DO DEMINERALIZACJI I ODKWASZANIA SERWATKI KWASOWEJ

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S ł o w a k l u c z o w e: twaróg, serwatka kwasowa, demineralizacja, odkwaszanie, nanofiltracja.

A b s t r a k t

Jednym z poważniejszych problemów przemysłu mleczarskiego jest zwiększenie wartości serwatki kwasowej. Jej utylizacja wymaga dużych nakładów z powodu wysokiego biochemicznego zapotrzebowania na tlen (BZT), wysokiej zawartości kwasu mlekowego i związków mineralnych oraz niskiej zawartości składników suchej substancji.

Celem badań była próba odzysku i oczyszczenia wartościowych składników serwatki kwasowej za pomocą nanofiltracji (NF) oraz był wybór najkorzystniejszego miejsca neutralizacji w procesie jej odsalania i odkwaszania. Wydajność membran NF określano na podstawie ich charakterystyki retencyjnej, tj. popiołu i kwasu mlekowego.

Najkorzystniejszym wariantem okazało się umiejscowienie neutralizacji po procesie nanofiltracji. Wykazano, że retencja zależy od pH serwatki. Stopień demineralizacji serwatki kwasowej metodą NF wyniósł 40%, natomiast po zastosowaniu zabiegu diafiltracji (DF) – 60%. Stopień odkwaszenia w przypadku NF wyniósł 30%, w przypadku DF 44%. Z porównania redukcji zawartości związków mineralnych oraz kwasu mlekowego wynika, że serwatka kwasowa powinna być neutralizowana po NF. Otrzymany oczyszczony koncentrat serwatki kwasowej może być stosowany w produkcji takich produktów spożywczych, jak lody, jogurty oraz syropy słodzące.

Introduction

Acid whey is a by-product of fresh cheese [cottage cheese and twarog production] and casein production. Twarog is fresh un-ripened cheese widely produced in Eastern Europe countries by milk acidification by starter cultures and cutting, heating, draining and self-pressing of obtained coagulum. The remaining whey is a diluted mixture contains 50-60% of the solids present in the original whole milk including proteins, lactose, minerals and water-soluble vitamins. Especially whey proteins are valuable components with excellent nutritional and functional properties (DE WIT 1988).

Acid whey is produced in large quantities world-wide: 1 ton of cheese results in the production of 8 tons of liquid whey (MARSHALL 1982). Volume of acid whey grows in the consequence of increasing tendency of fresh cheeses production. About 0.67 mld[3] of acid whey was produced in 2001 in Poland, what presents around 35% of total Polish whey production (including sweet whey). Only 3% of this amount was industrially processed for food purposes. As much as 90% of acid whey is used as a feed in unprocessed form.

This situation is brought about by difficulties of acid whey disposal treatment due to high BOD (Biochemical Oxygen Demand), corrosive nature, high lactic acid and low solids contents (MINH NGUYEN et al. 2003). Moreover whey is often thought of as having an undesirable flavour that is unpleasant to consumers, which limits its use in bland or delicately flavoured foods (WHETSTINE et al. 2003). For this reasons acid whey incorporation into other food products is limited. Thus its utilized to animal feed however frequently is released into the wastewater treatment process or even just fed into the canal.

The necessity of recovering acid whey components, high cost of its disposal and need to reduce environmental pollution have prompted considerable efforts to developing acid whey industrial processing technology. The high lactic acid and ash contents cause lower value of acid whey, limit the range of application of for example acid whey powders and make its processing very difficult (e.g. evaporation and/or drying).

Thus lowering minerals content seems to be essential for use of acid whey in human food products, such as infant foods and low-sodium foods (MCDONOUGH et al. 1974). Acid whey demineralization increases its value as a component of food (VAN DER HORST et al. 1995). Demineralization of whey can be done either by nanofiltration (NF), electrodialysis or by ion exchange resins. NF is membrane pressure-driven separation process based on the principle of concentration-fractionation treatment of solutions without phase change. NF molecular weight cut-off is situated between reverse osmosis and ultrafiltration. This process allows separation of organics and monovalent salts in the molecular weight range 300 – 1000 Da (ERIKSSON 1988). NF membranes have a high permeability for monovalent salts (as NaCl, KCl) and organic compounds with low molecular weight. However, it has a very low permeability for organic compounds of molecular weight higher than 300 Da as lactose and proteins (RAUTENBACH, GRÖSCHL 1990). As a result of its unique ability to simultaneous demineralization, deacidification and concentration NF has found wide field of application (PEDERSEN 1990). The two main applications of nanofiltration in the food industry are partial water desalination and concentration/demineralization of whey. According to van der HORST (1995) 20 000 m^2 of NF membranes have already been installed worldwide for the demineralization of salted, acid and sweet cheese whey. Applying NF technique to whey processing crucial is maximum removal of minerals and minimal lactose loss (KELLY, KELLY 1995, VAN DER HORST 1995). Whey processing by using membrane filtration technology enables increasing the value of whey products, improving the utilization of valuable whey components and helps reduce of costs of its utilization and evaporation (KELLY et al. 1991).

The fundamentals of NF are discussed, e.g. by RAUTENBACH and GRÖSCHL (1990). Reports on application of NF to other types of acid whey were already

presented (MORR 1990, KELLY, KELLY 1995, BARRANTES, MORR 1997, MINH NGUYEN et al. 2003). However until now no information is available on NF membranes applied to twarog acid whey processing. The aims of the study were to recover and purify acid whey solids components by demineralization and deacidification by NF and find the optimal localization of neutralisation process in the technological process.

Materials and Methods

Acid whey pretreatment

Experiment organization is shown on Figure 1. Acid whey samples after production of twarog were centrifuged for casein fines and fat removal and pasteurized.

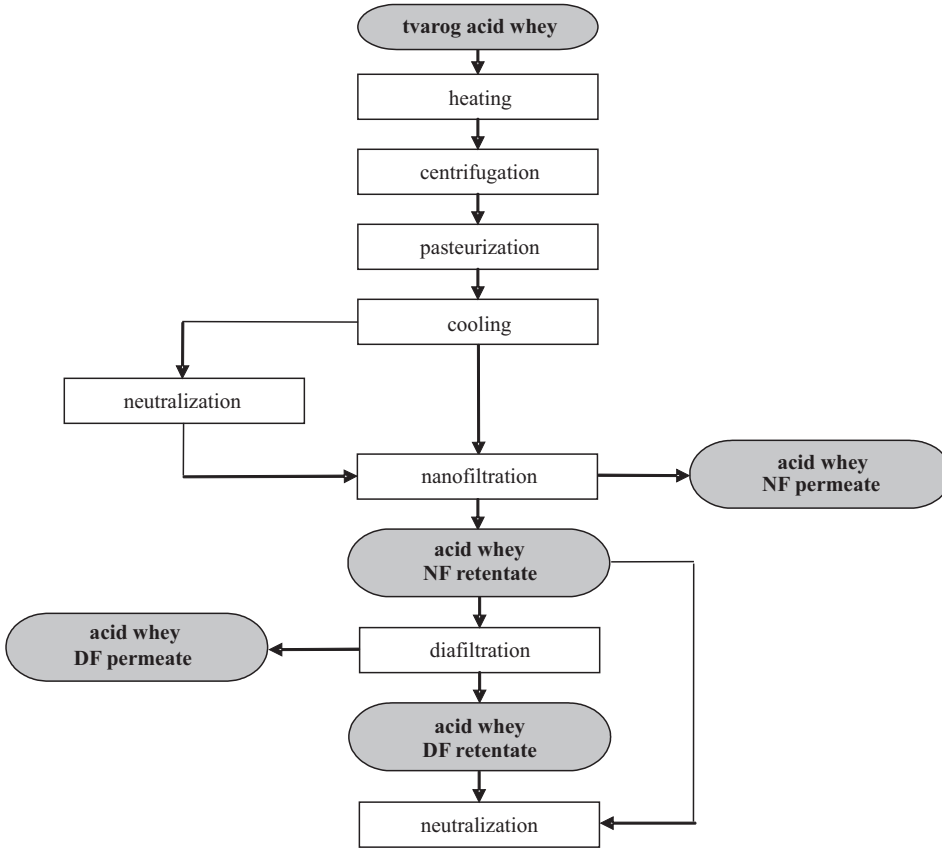

Fig. 1. Experiment organization

Acid whey neutralisation

The initial study using conventional evaporation/drying technology showed the necessity of acid whey neutralisation due to not acceptable acidic taste of the concentrate and precipitation of proteins and minerals during evaporation and drying. Acid whey samples were neutralised to sweet whey pH level by addition of NaOH of analytical grade (30% NaOH solution) which was chosen as neutraliser on the basis of preliminary work due to the highest ability of lactic acid binding.

Three variants of neutralisation were studied:

– (1) before NF,
– (2) after NF and,
– (3) after DF.

Acid whey nanofiltration and diafiltration

Twarog acid whey after pre-treatment was concentrated by using pilot--scale unit equipped with a spiral-wound module fitted with a polyamide thin film composite membrane with MWCO 200-300 Da and an effective area of 8 m^2. The plant was configured in a batch recirculation mode.

The work parameters determined were ash and lactic acid reduction (%), solutes retention factor r (%), operative temperature and pressure, permeate flux ($L\ m^{-2}\ h^{-1}$) and concentration factor of the process stream during each trial. During the experiments acid whey was concentrated about threefold to obtain about 17-18% total solids content in retentate.

The initial inlet and outlet pressures were adjusted to respectively 23-26 and 20-23 bar and controlled by regulation valves. The temperature was kept constant in all experiments at 20-22°C during the whole process by using double-pipe heat exchanger.

Performance of NF membrane was characterised by the following parameters:

– Ash and lactic acid reduction value (%) was equivalent to lowering the ash in total solids content from acid whey to retentate.

– Solute retention factor r (dry matter, lactose, ash and lactic acid):

$$r(\%) = (1 - \frac{C_p}{C_r})\ 100,$$

where:

C_p – solute concentration in permeate (%),
C_r – solute concentration in retentate (%);

– Volume concentration reduction (*VCR*):

$$VCR = \frac{V_F}{V_R},$$

where:
V_F – volume of feed (L),
V_R – volume of retentate (L).

The system was cleaned and rinsed thoroughly prior to commencement according to producer procedure.

Diafiltration (DF) process relied on addition of de-ionized water to NF retentate in amount equal to volume of drained off permeate and repeating NF.

The following analyses were performed in duplicates on feed, retentate and permeate: total solids (by drying in an oven at 102°C for 2 h), protein (not for permeate, by Kjeldahl method), ash (by incineration in the muffle furnace at 500°C for 4 h), lactic acid (by iodometric titration) and lactose (by Bertrand method) contents, pH and conductivity (by using conductmeter of the type HANNA HI9033). All of the chemical reagents used for assays were of analytical grade.

Results

Influence of neutralisation on acid whey properties

Table 1 shows the physicochemical properties of acid whey used in the study and the influence of neutralisation (NEU) on acid whey properties. The pH of sweet whey (6.2-6.6) was adopted as the target neutralisation level.

Neutralisation led into lactic acid content reduction of about 70% as a result of lactic acid bonding by neutraliser due to formation of sodium lactate not lactic acid removal. In consequence NEU caused 10-15% ash content

Table 1

Physicochemical properties of non- and neutralized acid whey

Feed	Parameters						
	pH	total solids (%)	protein (%)	lactose (%)	ash (%)	lactic acid (%)	conductivity (mS)
Acid whey	4.54	5.57	0.65	3.79	0.72	0.43	7.0
Neutralized acid whey	6.58	5.43	0.63	3.71	0.82	0.13	8.4

Means of duplicate determinations

increase in acid whey, thus conversed whey acidic into salty taste. Addition of neutraliser also resulted in increase of sample's electrolytic conductivity presumably due to increase of sodium ions content. This clearly indicates the necessity of application of NF for acid whey both demineralisation and deacidification.

Comparison of properties of produced acid whey retentates and permeates

The obtained results showed that applied variant of acid whey NEU had significant influence on composition both retentate and permeate which are put in Tables 2 and 3.

Table 2

Physicochemical properties of acid whey NF/DF retentates

Acid whey treatment	Parameters						
	pH	total solids (%)	protein (%)	lactose (%)	ash (%)	lactic acid (%)	conductivity (mS)
NF	4.66	17.39	2.26	11.87	1.22	1	7.4
NEU-NF	6.40	13.90	2.08	9.73	1.50	0.34	9.0
NF-NEU	6.43	15.30	2.27	11.98	1.29	0.32	7.9
NF-DF	4.75	15.78	2.65	13.01	0.70	0.69	5.3
NF-DF-NEU	6.56	17.48	2.74	13.31	0.99	0.19	6.8

Means of duplicate determinations

Table 3

Physicochemical properties of acid whey NF/DF permeates

Acid whey treatment	Parameters					
	pH	total solids (%)	lactose (%)	ash (%)	lactic acid (%)	conductivity (mS)
NF	4.57	1	0.12	0.44	0.17	4.3
NEU-NF	5.17	0.72	0.086	0.38	0.06	6.3
NF-DF	4.79	0.57	0.13	0.21	0.12	2.3

Means of duplicate determinations

The main observations can be made after comparison of NEU variants namely before and after NF. Acid whey retentate neutralised after NF was characterized by lower ash content (1.29%) than this one from acid whey neutralised before NF (1.50%) in spite of similar pH value (about 6.4). This

content is insignificantly higher than for retentate of acid whey (1.22%). In accordance with the expectations retentate after DF was characterized by the lowest ash content (0.7%) which increased to 0.99% after NEU. These values have met the confirmation in composition of permeates (Table 3). The higher ash content in permeate is accompanied lower ash content in retentate.

The level of mineral permeation by NF membrane was estimated by decrease in conductivity. The ash contents in retentates and permeates correspond with their conductivity. The obvious tendency was decrease in conductivity directly proportional to the decrease of the ash content and inversely.

Acid whey retentate neutralised after DF was marked by the lowest lactic acid content.

Produced permeates differed by total solids, ash and lactic acid contents. The lowest solids content was written down for those from neutralised acid whey. This confirmed lower ash and lactic acid reductions for variant NEU--NF. The highest solids content including ash and lactic acid contents was found in permeate of non-neutralised acid whey.

Influence of treatment on acid whey demineralization

Ash content reduction values depending on treatment are shown in Table 4. The comparison of these values clearly indicated that acid whey NEU before NF lowers the ash reduction level.

Table 4

Ash reduction

Ash reduction (%)	Treatment	Ash in TS content (%)	
		initial acid whey	retentate NF or DF
40.17	NF	11.50	6.88
18.47*	NEU-NF	12.02	9.80
33.44	NF-NEU	12.23	8.14 after NEU
66.49 after DF 56.56 after NEU	NF-DF-NEU	13.10	4.39 5.69 after NEU

* NEU caused increase of ash content in acid whey by 15% (from 12.02 %TS to 13.87 %TS).

Above 40% demineralisation degree was obtained after 3xconcentration of acid whey pH 4.66 (ash reduction from 11.5 %TS to 6.88% TS). Next NF of neutralised acid whey (NEU-NF, pH 6.58) resulted in merely 18% ash reduction (reduction from 12.02% TS to 9.8% TS). As a result of performing acid whey NEU after NF higher demineralization was obtained (above 33%). The

DF process increased ash reduction to 66%. However NEU of retentate produced in this way lowered demineralization level to around 56%.

Influence of treatment on acid whey deacidification

The conducted studies showed the ability of NF membrane to deacidify whey (Table 5). Nanofiltration deacidified of non-neutralised acid whey by almost 29%. Much greater lactic acid reductions were obtained for neutralised acid whey (above 71% for NEU either before and after NF). However in case of NEU before NF above 88% of this total lactic acid reduction is due to NEU as a result of lactic acid bonding and formation of salt sodium lactate. It caused undesirable increase of ash content in the final acid whey retentate. The more advantageous variant was with NEU after NF. Then above 63% of total lactic acid reduction was due to NF as a result of lactic acid removal. This high lactic acid removal was reflected in lower ash content in this way produced retentates and less usage of neutraliser. The latter benefit appears significant especially for industrial scale processing of acid whey from the economical point of view.

Table 5

Lactic acid reduction

Lactic acid reduction (%)	Treatment	Lactic acid in TS content (%)	
		initial acid whey	retentate NF or DF
28.95	NF	7.22	5.13
71.14*	NEU-NF	8.04	2.32
71.52**	NF-NEU	7.97	2.27 after NEU
44.89 after DF 85.50 after NEU***	NF-DF-NEU	7.93	4.37 after DF 1.15 after NEU

* Above 88% of total lactic acid reduction is due to NEU as a result of lactic acid bonding.
** Above 63% of total lactic acid reduction is due to NF as a result of lactic acid removal.
*** Above 52% of total lactic acid reduction is due to NF and DF as a result of lactic acid removal.

Diafiltration of acid whey increased the deacidification level to almost 45%. Neutralisation of DF retentate increased lactic acid reduction to 85%.

In this study for each treatment retention factors r_F was calculated which are presented in Table 6. The obtained values of retention factors confirmed that localization of acid whey NEU process had significant influence on minerals permeation by NF membrane. Higher retention of ash was obtained for acid whey neutralised before NF (76%) than after NF (65%). This compari-

son could also explain lower ash content in acid whey retentate neutralised after NF.

Table 6

Comparison of retention factors r_F depending on treatment

Component	Treatment		
	NF	NEU-NF	NF-DF
Total solids	94.29	94.87	96.41
Ash	65.70	76.11	70.16
Lactic acid	82.06	88.65	82.02
Lactose	98.98	99.31	98.92

Discussion

The obtained results confirmed that NF is effective method of ash and lactic acid removal also from twarog acid whey (BARRANTES, MORR 1997, HORTON 1996). Demineralisation level for nanofiltered acid whey (40%) is similar to work of VAN DER HORST (1995), Nguyen and co-workers (2003) – about 42% higher to KELLY and KELLY (1995) – 32%. These differences can be attributed to different membrane material molecular weight cut-off, type of feed pre-treatment, process conditions and level of whey concentration (TSURU et al. 1991).

The obtained results showed that pH value of acid whey had significant influence on demineralization level. Better demineralization level (33%) for acid whey samples neutralised after NF than before NF (18%) resulted from better minerals permeation at lower acid whey pH. Similar observation was made by AHN KYU-HONG and co-workers (1999) who suggested that higher ash retention at high pH can be caused by calcium phosphate precipitation and bonding it to the proteins. ALKHATIM and co-workers (1998) stated that pH affected mainly monovalent ions retention (sodium and potassium). So larger ions such as magnesium and calcium are too large to pass through the membrane and they enrich nutritional value of the final products (MINH NGUYEN et al. 2003). Kelly also observed higher demineralization effectiveness by NF at lower pH and explained it by presumable undergoing of some minerals from colloidal to ionic form as a result of linking with acid which easier permeated into permeate fraction (KELLY and KELLY 1995). Other researcher suggested that increase of mineral retention together with feed pH increase could be caused by changes in properties of NF membrane (BERG et al. 1997) and electrostatic repulsion of ions from charged NF membrane.

DF process of non-neutralised acid whey increased demineralization level to 66%. This demineralization level is comparable with the combined process of evaporation/electrodialysis (VAN DER HORST et al. 1995). KELLY and KELLY (1995) received 41% ash reduction for DF as a result of fourfold concentration (18-20% TS in retentate) of acid casein whey.

This high lactic acid removal by NF (29%) and DF (45%) resulted in less usage of neutraliser for acid whey deacidification and thereby lower ash content in the final product. KELLY and KELLY (1995) obtained 42% deacidification level of cottage cheese acid whey (1995) and BARRANTES and MORR (1997) 31% after of cottage cheese acid whey threefold concentration. Also CHERYAN (1998) showed that organic acids could easier penetrate NF membrane at lower pH because at higher pH they formed salts which were rejected by NF membrane.

However DF increased lactic acid reduction, in Kelly's study even to 67% (KELLY, KELLY 1995), its the main disadvantages were appreciable loss of lactose and other whey total solids components which increase pollution load of NF permeate (BARRANTES, MORR 1997). The acid whey NF and DF permeates were composed mainly of minerals, acids and lactose traces (VAN DER HORST et al. 1995, ALKHATIM et al. 1998). In this connection there is possibility to lower pollution load by reverse osmosis processing of NF permeate what could obtain soft, conditioned water for cleaning-in-place (CIP) system cleaning or boiler water (JELEN 1991).

Conclusions

Neutralisation of acid whey enabled its proper evaporation and drying without components precipitation. However this process only converted whey acidic taste to salty. Disadvantage of high ash content in whey after NEU was minimized by NF. Nanofiltration technique showed to be effective method of simultaneous demineralization, deacidification and concentration of acid whey. Application of NF [concentrating to 18% total solids] caused 40% demineralization of acid whey, and DF increased it to 66%. Resulted obtained showed higher minerals rejection at lower pH values. Best demineralization was obtained for non-neutralised acid whey at pH 4.6-4.8.

The deacidification levels (29% for NF and 45% for DF) influenced on lower neutraliser consumption for NF retentates and in consequence lower ash content in the final product – neutralised NF retentate. Thus acid whey NEU process should be evidently performed after whey filtration.

Application of NF into acid whey processing could help with recover of whey components, reduce the pollution treatment cost and redesign cheese

production as more environmentally friendly and "less waste" process. Acid whey fractionation by NF imitated it to sweet whey and transformed the troublesome by-product into high value product with wide range of application.

NF permeate because of some lactose and minerals content should not be directed to sewage treatment plant but processed, for example, by reverse osmosis technique to recover water for production of boiler or washing/rinsing water and reduce sewage volume.

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References

AHN KYU-HONG, KYUNG-GUEN SONG, HO-YOUNG CHA, ICK-TAE YEOM 1999. *Removal of ions in nickel electroplating rinse water using low-pressure nanofiltration.* Desalination, 122, 77-84.

ALKHATIM H.S., ALCAINA M.I., SORIANO E., IBORRA M.I., LORA J., ARNAL J. 1998. *Treatment of whey effluents from dairy industries by nanofiltration membranes*, Desalination, 119: 177-184.

BARRANTES L.D., MORR C.V. 1997. *Partial deacidification and demineralization of cottage cheese whey by nanofiltration.* J. Food Sc., 62: 338-341.

BERG P., HAGMEYER G., GIMBEL R. 1997. *Removal of pesticides and other micropollutants by nanofiltration.* Desalination, 113: 205-208.

CHERYAN M. 1998, *Ultrafiltration and microfiltration handbook.* Technomic Publ. Company, Inc.

ERIKSSON P. 1988, *Nanofiltration extends the range of membrane filtration.* Environ. Prog., 7, 58-62.

HORST VAN DER H.C. 1995. *Fouling of organic membranes during ultrafiltration and reverse osmosis of dairy liquids.* Int. Dairy Fed. Spec. Iss., 9504: 36-52.

HORST VAN DER H.C., TIMMER J.M.K., ROBERTSON T., LEENDERS J. 1995. *Use of nanofiltration for concentration and demineralization in the dairy industry: model for mass transport,.* J. Membr. Sci., 104: 205.

HORTON B. 1996. *Wheys of recovery.* Dairy Ind. Int., 5: 39-42.

JELEN P. 1991. *Pressure-driven membrane processes: principles and definitions.* IDF Spec. Iss., 9201: 7-14.

KELLY P.M., HORTON B.S., BURLING H. 1991. *Partial demineralization of whey by nanofiltration.* IDF Spec. Iss., 9201: 130-140.

KELLY J., KELLY P. 1995. *Desalination of acid casein whey by nanofiltration.* Int. Dairy J., 5: 291-303.

MARSHALL K.R. 1982. *Industrial isolation of milk proteins: whey protein.* In: *Developments in dairy chemistry.* I. *Proteins.* Appl. Sci. Publ., London, 339-373 pp.

MCDONOUGH F.E., HARGROVE R.E., MATTINGLY W.A., POSATI L.P., ALFORD J.A. 1974. *Composition and properties of whey protein concentrates from ultrafiltration.* J. Dairy Sci., 57: 1438-1443.

NGUYEN M., REYNOLDS N., VIGNESWARAN S. 2003. *By-product recovery from cottage cheese production by nanofiltration.* J. Cleaner Prod., 11: 803-807.

MORR C.V. 1990. *Whey protein functionality: current status and the need for improved quality and functionality.* Proc. of the Dairy Prod. Technical Conf., Chicago, USA, p. 69.

PEDERSEN R. 1990. *Development of membrane processes in milk processing.* APV Pasilac AS.
RAUTENBACH R., GRÖSCHL A. 1990. *Separation potential of nanofiltration membranes.* Desalination, 77: 73.
TSURU T., URAIRI M., NAKAO S., KIMURA S. 1991. *Negative rejection of anions in the loose reverse osmosis separation of mono- and divalent ion mixtures.* Desalination, 81: 219.
WHETSTINE C.J., PARKER J.D., DRAKE M.A. LARICK D.K. 2003. *Determining flavor and flavor variability in commercially produced liquid Cheddar whey.* J. Dairy Sci., 86: 439-448.
WIT DE J.N. 1988. *Functional properties of whey proteins.* A review, NIZO, Ede, Netherlands, 1988.

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CHARACTERISTICS OF ADHESIVE PROPERTIES OF *LACTOBACILLUS* STRAINS SYNTHESISING BIOSURFACTANTS*

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K e y w o r d s: adhesion, Caco-2 cell line, *Lactobacillus*, biosurfactants.

A b s t r a c t

This study provides an assessment of the adhesive properties of *Lactobacillus spp.* strains which synthesise biosurfactants to a mono monolayer of intestinal epithelium *in vitro*, represented by the Caco-2 cell line.

It also examines the effect of impregnating a polystyrene surface with a solution of biosurfactants synthesised by the *Lactobacillus casei rhamnosus* CCM 1825 strain on the adhesion of *Klebsiella pneumoniae* 2.

All tested *Lactobacillus* strains showed adhesion to Caco-2 cells. The highest adhesion was observed for the *Lactobacillus fermenti* 126 strain: 3000 cells to 1000 Caco-2 cells or 420.000 cells to 1 cm^2 of the intestinal epithelium, while the lowest was for the *Lactobacillus casei rhamnosus* CCM 1825, whose 2400 and 310.000 cells adhered to 1000 cells or 1 cm^2 of Caco-2 tissue, were determined respectively.

A 50% reduction in the population of *Klebsiella pneumoniae* 2 cells adhering to the surface previously impregnated with a solution of biosurfactants synthesised by *Lactobacillus casei rhamnosus* CCM 1825, after the 3-hour contact with the tested surface was also observed.

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CHARAKTERYSTYKA WŁAŚCIWOŚCI ADHEZYJNYCH SZCZEPÓW *LACTOBACILLUS* SYNTETYZUJĄCYCH BIOSURFAKTANTY

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S ł o w a k l u c z o w e: adhezja, linia komórkowa Caco-2, *Lactobacillus*, biosurfaktanty.

A b s t r a k t

Oceniano właściwości przylegania szczepów z rodzaju *Lactobacillus* syntetyzujących biosurfaktanty do monowarstwy komórek nabłonka jelitowego *in vitro* reprezentowanej przez linię komórkową Caco-2. Testowano także wpływ impregnacji powierzchni polisterynowej roztworem biosurfaktantów syntetyzowanych przez szczep *Lactobacillus casei rhamnosus* CCM 1825 na przyleganie bakterii *Klebsiella pneumoniae* 2.

Wszystkie testowane szczepy *Lactobacillus* przylegały do komórek Caco-2. Największą adhezję wykazano w przypadku szczepu *Lactobacillus fermenti* 126 (3000 komórek *L. fermenti* przylegało do 1000 komórek Caco-2 lub 420 000 komórek do 1 cm^2 tkanki nabłonka jelitowego), a najmniejszą w przypadku *Lactobacillus casei rhamnosus* CCM 1825 (2400 oraz 310 000 komórek *L. casei rhamnosus* przylegało odpowiednio do 1000 komórek lub 1 cm^2 tkanki Caco-2).

Ponadto wykazano 50% redukcję populacji komórek *Klebsiella pneumoniae* 2 przylegających do powierzchni wcześniej impregnowanej roztworem biosurfaktantów syntetyzowanych przez *Lactobacillus casei rhamnosus* CCM 1825, po ich 3 h kontakcie z testowaną powierzchnią.

Introduction

Metabolites of some lactic fermentation bacteria include biosurfactants, i.e. surface active agents (SERVIN 2004). They are increasingly frequently applied in medicine as components of therapeutic agents, playing a key role in preventing and controlling infections caused by various pathogens (RODRIGUES 2006).

Biosurfactants synthesised by some *Lactobacillus* strains show extraordinary adhesive properties and, for this reason (among others), they can be applied as impregnates for medical equipment surfaces (VELRAEDS 2000, SINGH, CAMEOTRA 2004). It should be emphasised that only the biosurfactants produced by *Lactobacillus* show the ability to bind the substances comprising extracellular stroma and mucins – the mucus which covers human digestive tract.

Due to this ability, lactic fermentation bacteria can colonise the human digestive and urogenital systems. This, in turn, makes it much more difficult

for pathogenic microflora to adhere to intestinal and urogenital epithelium cells in humans (VELRAEDS et al. 1997, VELRAEDS 2000).

A significant feature of *Lactobacillus spp.* strains showing probiotic properties is their high affinity to receptors on the surface of intestinal cells, which favours them in their competition with pathogenic microflora of the digestive system (BERNET 1994, KALLIOMAKI 2001, CAMPO 2005).

An important criterion of the probiotic properties of lactic fermentation bacteria is their adhesiveness to the intestinal epithelium cells, cultured *in vitro* and represented by the Caco-2 cell line (TUOMOLA, SALMINEM 1998, FORESTIER 2001).

The aim of the study was to assess the adhesive properties of selected *Lactobacillus spp.* strains which synthesise biosurfactants to a monolayer of intestinal epithelium cells *in vitro*, represented by the Caco-2 cell line, and determine the effect of impregnating a polystyrene surface with a solution of biosurfactants synthesised by a selected strain of *Lactobacillus* sp. on the adhesion of pathogens.

Materials and Methods

Studying the adhesive abilities of selected biosurfactant synthesising *Lactobacillus* sp. strains

Selected biosurfactant synthesising strains of *Lactobacillus* sp. were cultured in MRS (Merc) bullion for 18 h at 37°C. After 18 hours, the culture was centrifuged, suspended in a buffered solution of physiological saline (pH 7.2) and diluted to the population density of $1 \cdot 106$ cfu ml^{-1}. Thus the biomass obtained was suspended in an Eagle bullion, modified by Dulbeco (DMEM, Sigma) without the addition of an antibiotic, filled to 1 ml.

A culture of Caco-2 epithelium cells was established at the optimum initial density of $1 \cdot 10^4$ Caco-2 cells cm^{-2}. The Caco-2 cells (ATTC HTB 37) were kept in six-well plates (Nunc) in Eagle;s medium, modified by Dulbecco (DMEM, Sigma), with an addition of 20% fetal bovine serum bovine (FBS, Gibco BRL), 1% of non-exogenous amino acids 100x (NEAA, Sigma) and 50 mg l^{-1} gentamicin (Gibco BRL) for 14 days, i.e. until a monolayer had been produced, in an atmosphere consisting of 95% air and 5% CO_2. The culture medium on the plates was changed daily. The number of Caco-2 cells on the membranes was calculated in a Neunbauer;s chamber.

In order to examine the adhesion of biosurfactant synthesising bacteria to Caco-2 epithelium cells, their monolayer was washed twice with a solution of physiological saline and, subsequently, the suspensions of bacteria in the Eagle

medium (modified by Dulbeco (DMEM, Sigma without antibiotics) were put onto the prepared surface and incubated for 1 hour at 37°C. After the incubation, the medium was removed from the wells and the cell monolayer was washed twice with physiological saline to remove the non-adhering bacteria. In order to isolate the Caco-2 cells with the adjacent bacteria, 1 ml of trypsin-EDTA was put onto the monolayer and incubated for 5 min at room temperature. The cell suspension was centrifuged (600 g for 10 min) and the isolated biomass was lysed for 5-10 min in 1 ml of 1% solution of Triton X (Sigma). The resulting deposit was suspended in 1 ml of physiological saline.

When determining the number of bacteria which adhered to the surface, decimal dilutions were performed, followed by inoculation by the plate method on the MRS agar medium.

Determination of adhesion of *Klebsiella pneumoniae* 2 cells to native polystyrene surface and to such surface impregnated with a solution of biosurfactants from *Lactobacillus casei rhamnosus* CCM 1825

Klebsiella pneumoniae 2 cells were cultured in McConkey's bullion at 37°C in aerobic conditions for 18 hours. The culture was then centrifuged (10.000 · g, 5 min, 10°C) and the biomass was suspended in 0.9% NaCl and diluted to a population density of $3 \cdot 10^6$ cfu ml^{-1}.

To test the *Klebsiella pneumoniae* 2, 4 Eppendorf tubes with a bacteria density of $3 \cdot 10^6$ cfu ml^{-1} were used. The fifth tube was used for the control of bacteria population. In order to determine their population in 1 ml, the surface inoculation method was employed.

Adhesion was tested by introducing 2 ml of suspension of the tested strain of *Klebsiella pneumoniae* 2 at the stationary growth phase and the density of $3 \cdot 10^6$ cfu ml^{-1} to a well of a sterile polystyrene plate. The plate was then incubated at 37°C for 1, 3 and 5 hours. The sterile polystyrene plate (Serstedt) was impregnated with a solution of biosurfactants at a surface tension of 48.1 mN m^{-1} and was isolated from *Lactobacillus casei rhamnosus* CCM 1825 by putting 2 ml of the solution in sterile wells of a polystyrene plate. The plates were then incubated for 18 hours at 4°C. After this period, the wells were washed twice with 2 ml of sterile distilled water. Subsequent stages of the experiment consisted in introducing 2 ml of an 18-hour suspension of bacterial cells with a density of $3 \cdot 10^6$ cfu ml^{-1}. The prepared polystyrene plates were kept for 4 hours at 4°C. In order to reduce the possibility of external infections, the plates were covered with parafilm.

After the appropriate incubation time, the wells were washed twice with

2 ml of sterile PBS buffer solution in order to remove the non-adhering bacterial cells. In the next stage, the adhered cells of the tested bacteria were counted. Next, 2 ml of the dyied solution of 4',6-diamidino-2-phenylindole (DAPI, Sigma) was placed in each well, which area was 9.6 cm^2, for 15 min. Then, after the wells had been washed twice with 2 ml of sterile distilled water, the bacteria adhering to the tested surfaces were counted. The observations were conducted under a fluorescence microscope (OLYMPUS, BX 51, magnification x 200), at a wavelength of 350 nm, and the adhering bacteria were counted in twenty randomly chosen fields of vision. The area of one such field of vision was 0.16 mm^2.

Results and Discussion

Adhesive abilities of selected biosurfactants producing strains of *Lactobacillus*

The adhesion of biosurfactant producing *Lactobacillus*: *Lactobacillus acidophilus* PG 8/4, *Lactobacillus casei rhamnosus* CCM 1825, *Lactobacillus fermenti* 126 were examined. The degree of adhesion of the tested bacteria was determined from the number of the cells adhering to 1000 cells or 1 cm^2 of Caco-2 cell monolayer, representing epithelium in vitro.

The average comparative scale used to determine the adhesive properties of bacteria describes them as good when adhering 1010 to 3000 cells adhere to 1000 intestinal epithelium cells (e.g. Caco-2 cells) monolayer. Average properties are shown by strains when 1000 epithelial cells have from 210 to 1000 bacterial cells adhering to them; weak adhesive properties are shown when the number of adhering cells ranges from 0 to 200.

The study showed that 3.000, 2.800 and 2.400 of *Lactobacillus fermenti* 126, *Lactobacillus acidophilus* PG 8/4 and *Lactobacillus caei rhamnosus* CCM 1825, respectively, adhered to 1.000 cells of Caco-2, representing intestinal epithelium *in vitro* (Table 1, Figure 1).

When adhesion to 1 cm^2 of Caco-2 tissue was examined, the following numbers of bacteria adhered to it: 420.000, 360.000 and 310.000 for: *Lactobacillus fermenti* 126, *Lactobacillus acidophilus* PG 8/4 and *Lactobacillus caei rhamnosus* CCM 1825, respectively (Table 1, Figure 1).

When the results were related to the average comparative scale, which describes adhesive properties of bacteria, it was shown that the biosurfactant producing strains of *Lactobacillus* have good adhesive properties, which can mean that the main criterion of probioticity is fulfilled.

Good adhesiveness of probiotic strains is commonly linked to the inoculum

Table 1
Adhesion abilities comparison of *Lactobacillus* strains synthesizing biosurfactants towards to cells intestine epithelium monolayer in vitro, represented by Caco-2 cell line

Tested strain of bacteria	Applied bacterial culture density of genus *Lactobacillus*	Applied cell culture density of Caco-2 cm^{-2}	Average count of *Lactobacillus* bacterias, which revealed adhesion abilities to 1000 Caco-2 cells (10^3)	Average count of *Lactobacillus* bacterias, which revealed adhesion abilities to 1 cm^2 Caco-2 tissue (10^3)
Lactobacillus acidophilus PG 8/4	$1 \cdot 10^6$	$1 \cdot 10^4$	2.8	360
Lactobacillus casei rhamnosus CCM 1825	$1 \cdot 10^6$	$1 \cdot 10^4$	2.4	310
Lactobacillus fermenti 126	$1 \cdot 10^6$	$1 \cdot 10^4$	3.0	420

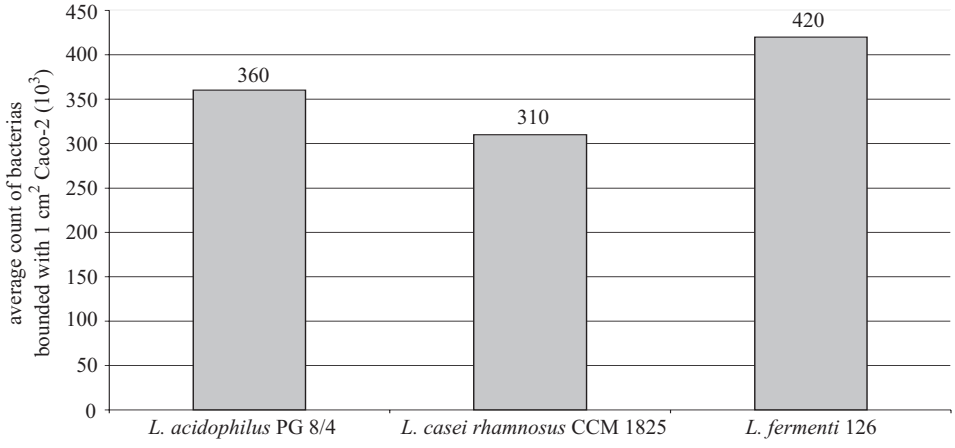

Fig. 1. Comparision of adhesion abilities of selected bacteria;s strain of genus *Lactobacillus* synthesizing biosurfactants to cell intestine epithelium monolayer *in vitro* represented by Caco-2 cell line, expressed by average bacterial cell population bounded with 1 cm^2 Caco-2 tissue

dose for the bacteria, introduced to Caco-2 culture (SAREM-DARMERDJI 1995, LEE 2000, MATIJASIC 2006).

However, a study conducted by Tuomola et al., showed that the rule is not always in force. They tested the ability of probiotic strains to adhere to Caco-2 epithelial cells, cultured in vitro, and found that the bacterial inoculum (diluted to an absorbance corresponding to 0.5) contained a lower number of probiotic bacteria of more adherent strains (TUOMOLA, SALMINEM 1998).

When trying to interpret the differences, the authors suggested that the high optic density was not associated with the bacteria count, but was caused by surfactant components produced by the bacteria which made the bacterial suspension cloudier (TUOMOLA, SALMINEM 1998).

The results obtained in a study conducted by Rodrigues indicate that the good properties of biosurfactants from the strains of lactic bacteria manifest themselves not only in their extraordinary adhesive properties, but also in those which are usually attributed to antibiotics (RODRIGUES 2004).

In the study conducted by Rodrigues et al., the authors showed the existence of the antibiotic effect of biosurfactants produced by probiotic bacteria of lactic fermentation: *Lactococcus lactis* 53 and *Streptococcus thermophilus* A towards strains of pathogenic bacteria and fungi isolated from voice prostheses (RODRIGUES 2004).

In the experiment, biosurfactants of various concentrations were examined: 5, 10, 25, 50 and 100 mg, isolated from *Lactococcus lactis* 53 and 3, 5, 10, 50 and 100 mg, isolated from *Streptococcus thermophilus* A; all were tested on pathogenic bacteria and fungi isolated from voice prostheses.

Rodrigues et al. found the biosurfactants to inhibit the growth of *Staphylococcus epidermidis* GB 9/6, *Streptococcus salivarius* GB 24/9 and *Staphylococcus aureus* GB 2/1, as well as *Rothia dentocariosa* GBJ 52/2B, *Candida albicans* and *Candida tropicalis* GB 9/9 (RODRIGUES 2004).

These results indicate that the biosurfactants synthesised by strains of lactic fermentation bacteria present in food can contribute to reducing the pathogenic microflora, e.g. in the digestive system, by effectively preventing pathogens from adhering to intestinal epithelium cells or by their antibiotic-like action.

Examining the effect of impregnating polystyrene surface with a solution of biosurfactants isolated from the *Lactobacillus casei rhamnosus* CCM 1825 strain on the adhesion of *Klebsiella pneumoniae* 2 cells

The research objective was to study the adhesion of pathogenic *Klebsiella pneumoniae* 2 to polystyrene surface impregnated with a solution of biosurfactants (surface tension 48.1 mN m^{-1}), synthesised by a selected strain of *Lactobacillus*. The evaluation was based on the differences in the count of cells adhering to 1 cm^2 of the native polystyrene surface and to that impregnated with the biosurfactants.

An analysis of the experiment results shows that impregnating polystyrene with a biosurfactant inhibited the adhesion of *Klebsiella pneumoniae* 2 cells to

the polystyrene surface. In the experiment described above, the observed adhesion was 1875, 1875 and 3750 cfu cm^{-2}; the number of adhered cells was reduced by 25, 50 and 25% after, respectively, 1, 3 and 5-hours of contact with the surface impregnated with the biosurfactant solution (Table 2, Figure 2).

Table 2
Evaluation of biosurfactants solution isolated from *Lactobacillus casei rhamnosus* CCM 1825 strain influence on *Klebsiella pneumoniae* 2 adhesion to polystyrene surface in relation to contact duration

Strain / Time	Bacterial cell population bounded with polystyrene surface (cfu cm^{-2})		
	1 h	3 h	5 h
Klebsiella pneumoniae 2	2500	3750	5000
Strain / Time	bacterial cell population bounded with polystyrene surface impregnated with biosurfactants solution (cfu cm^{-2})		
	1 h	3 h	5 h
Klebsiella pneumoniae 2	1875	1875	3750
Reduction of *Klebsiella pneumoniae* 2 bacterial cell population (%)	25	50	25

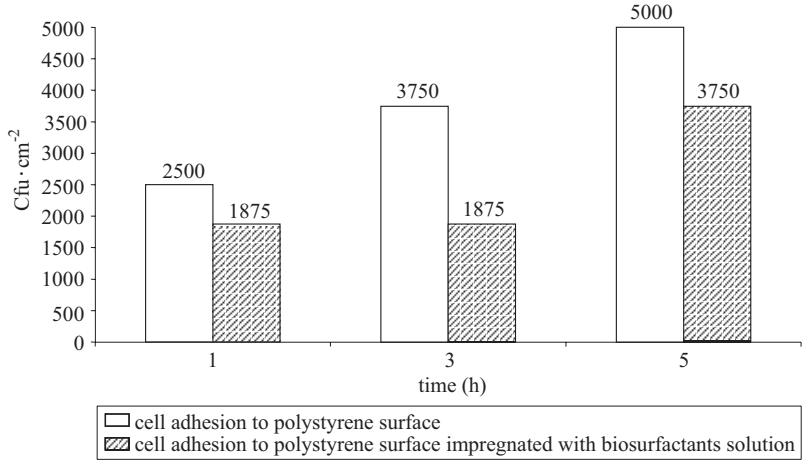

Fig. 2. Influence of polystyrene surface impregnation with biosurfactants solution of *Lactobacillus casei rhamnosus* CCM 1825 on *Klebsiella pneumoniae* 2 bacterial cell adhesion

Results presented in scientific reports confirm the inhibiting effect of biosurfactants on the adhesion of pathogenic bacteria to various bases.

Reid has shown that *Lactobacillus acidophilus* NCFM and *Lactobacillus fermentum* RC-14 synthesise biosurfactants which significantly inhibit the adhesion of the pathogenic *Enterococcus faecalis* 1131 strain to a polystyrene base (REID 2000).

The studies conducted by Velraeds et al. have confirmed that the biosurfactants synthesised by lactic fermentation bacteria inhibit the adhesion of pathogenic bacteria both in models and in natural conditions (the urogenital and digestive systems) and give the competitive advantage to the *Lactobacillus spp.* strains in conquering ecological niches (VELRAEDS et al. 2000).

Research into the inhibiting effect of biosurfactants isolated from selected *Lactobacillus* strains, adhering to Caco-2 cells, on the growth of bacteria causing infections of the digestive or urogenital system shall be continued by the authors of this study.

Conclusions

Selected biosurfactant synthesising strains: *Lactobacillus fermenti* 126, *Lactobacillus acidophilus* PG 8/4 and *Lactobacillus casei rhamnosus* CCM 1825 show good adhesive properties, which was confirmed by their adhesion to a monolayer of intestinal epithelium cells in vitro, represented by the Caco-2 cell line.

1. Good adhesive properties of *Lactobacillus* strains indicate that due to biosurfactant synthesis, the bacteria can effectively colonise the digestive system, which can reduce the adhesion of pathogenic microflora to the intestinal epithelium cells.

2. The biosurfactants synthesised by *Lactobacillus* can be used for impregnating surfaces, e.g. made of polystyrene, in order to inhibit the adhesion of pathogenic cells, e.g. *Klebsiella pneumoniae* 2. The degree of the adhering cells; population reduction depends on the duration of their contact with the base.

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Reference

BERNET M., BRASSART D., NEESER R., SERVIN A.L. 1994. *Lactobacillus acidophilus LA 1 binds to cultured human intestinal cell lines and inhibits cell attachment and cell invasion by enterovirulent bacteria*. Gut., 35: 483-489.

CAMPO R., BRAVO D., CANTON R., RUIZ-GARBAJOSA P., GARCIA-ALBIACH R., MONTRSI-LIBOIS A., YUSTE F.J., ABRAINA V., BAGUERO F. 2005. *Scarce evidence of yogurt Lactic acid bacteria in human feces after daily yogurt consumption by healthy Volunteers*. Appl. Environ. Microbiol., 71 (1): 547-549.

FORESTIER CH., DE CHAMPS CH.M., VATOUX C., JOLY B. 2001. *Probiotic activities of Lactobacillus casei rhamnosus: in vitro adherence to intestinal cells and antimicrobial properties*. Res. Microbiol., 152: 167-173.

KALLIOMAKI M.J. 2001. *Probiotics in the primary prevention of atopic disease: a randomised, placebo-controled trial*. Lancet, 357: 1076-1079.

LEE Y.K., LIM C.Y., TENG W.L., OUWEHAND A.C., TUOMOLA E.M., SALMINEN S. 2000. *Quantitative approach in the study of adhesion of Lactic Acid Bacteria to intestinal cells and Their Competition with Enterobacteria*. Appl. Environ. Microbiol., 3692-3697.

MATIJASIC B.B., NARAT M., PETERNEL M.Z., ROGELJ J. 2006. *Ability of Lactobacillus gaseri K7 to inhibit Escherichia coli adhesion in vitro on Caco-2 cells and ex vivo on pigs, jejunal tissue*. Int. J. Food Microbiol., 107: 92-96.

REID G. *In vitro* testing of *Lactobacillus acidophilus* NCFM™ *as a possible probiotic for the urogenital tract*. 2000. Int. Dairy J., 10: 415-419.

RODRIGUES L., BANAT I.M., TEIXEIRA J., OLIVEIRA R. 2006. *Biosurfactants: potential applications in medicine*. J. Antimicrob. Chemo., 57: 609-618.

RODRIGUES L., VAN DER MEI H.C., TEIXEIRA J., OLIVEIRA R. 2004. *Influence of biosurfactants from probiotic bacteria on formation of biofilms on voice prostheses*. Appl. Environ. Microbiol., 4408-4410.

SAREM-DARMERDJI L., SAREM F., MARCHAL L., NICOLAS J.P. 1995. *In vitro colonization ability of human colon mucosa by exogenous Lactobacillus strains*. FEMS Microbiol. Lett., 131: 133-137.

SERVIN A.L. 2004. *Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens*. FEMS Microbiol. Lett., 28: 405-440.

SINGH P., CAMEOTRA S.S. 2004. *Potential applications of microbial surfactants in biomedical sciences*. Trends Biotechnol., 22 (3): 142-146.

TUOMOLA E.M., SALMINEM S.J. 1998. *Adhesion of some probiotic and dairy Lactobacillus strains to Caco-2 cell cultures*. Int. J. Food Microbiol., 41: 41-51.

VELRAEDS M.M., BELT G.B., BUSSCHER H.J., REID G., VAN DER MEI H.C. 2000. *Inhibition of uropathogenic biofilm growth on silicone rubber in human urine by Lactobacillus*. World J. Urol., 18: 422-426.

VELRAEDS M.M., VAN DER MEI H.C., REID G. 1997. *Inhibition of initial adhesion of uropathogenic Enterococcus faecalis to solid substrata by an adsorbed biosurfactant layer from Lactobacillus acidophilus*. Urology., 49: 790-794.

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EFFECT OF BASIL, OREGANO AND PAPRIKA ADDITIVES ON THE PROPERTIES AND LIPIDS OXIDATION WHEAT BREAD SUPPLEMENTED WITH FLAX SEEDS

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Key words: bread, flaxseed, oregano, basil, paprika, fat, peroxide value, acid value, anisidine value.

Abstract

The purpose of this study was to evaluate the additives of basil, oregano and paprika on the properties of bread supplemented with flax seeds and on lipid hydrolysis and oxidation. The breads were made from wheat flour type 650, with 5% addition of milled and boiled flax seeds and simultaneous 1% addition of frozen herbs: oregano or basil or 5% addition of dried red paprika fruits. The properties of bread were characterized by: organoleptic analysis and measure of loaf volume, acidity, free lipids content, moisture content and elasticity of bread crumb. Quality of fat extracted from bread crumb was determined by analysis of acid, peroxide and anisidine values.

It was shown that additions of herbs and paprika, introduced for bread supplemented with flax seeds, generally improve quality of bread, but their effect was differentiated. Addition of oregano increase of testiness and volume of bread and elasticity of crumb, however, contents of moisture and free lipids were decreased. This type of bread, in comparison to bread with flax seeds only, contained less of peroxides 24 hour after baking and less of secondary lipid oxidation products 24 and 72 hour after baking. The influence of basil was similar as oregano, however, it was found such differences as: decreasing of bread volume and increasing of peroxide value in bread stored by period 72 hour. The addition of dried paprika fruits although the mostly decreased volume of bread, but substantially increased testiness and the most favorably influenced on fat quality, it in the biggest degree limited contents of peroxides and secondary lipid oxidation products.

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WPŁYW BAZYLII, OREGANO I PAPRYKI NA CECHY ORAZ UTLENIANIE TŁUSZCZU PIECZYWA PSZENNEGO SUPLEMENTOWANEGO NASIONAMI LNU

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Słowa kluczowe: pieczywo, nasiona lnu, oregano, bazylia, papryka, tłuszcz, liczba kwasowa, liczba nadtlenkowa, liczba anizydynowa.

Abstrakt

Celem badań było ustalenie wpływu dodatków mrożonej bazylii i oregano oraz suszu z owoców papryki na cechy pieczywa suplementowanego nasionami lnu oraz stopień utlenienia tłuszczu pieczywa. Badania wykonano na pieczywie sporządzonym z mąki pszennej typu 650, z 5% dodatkiem zmielonych i zaparzonych nasion lnu włóknistego oraz równoczesnymi 1% dodatkami mrożonych ziół: oregano lub bazylii, lub 5% dodatkiem suszu z owoców papryki czerwonej. Cechy pieczywa badano dokonując oceny organoleptycznej, pomiaru objętości bochenków, kwasowości, zawartości tłuszczu wolnego oraz wilgotności i elastyczności miękiszu. Stopień utlenienia tłuszczu miękiszu pieczywa oceniano oznaczając liczby: kwasową, nadtlenkową oraz anizydynową.

Stwierdzono, że dodatki ziół oraz papryki, wprowadzone do pieczywa z lnem, wpłynęły na poprawę jego jakości, aczkolwiek w różnym stopniu. Dodatek oregano zwiększał smakowitość i objętość pieczywa oraz elastyczność miękiszu, natomiast zmniejszał wilgotność, zawartość tłuszczu wolnego, nadtlenków w pieczywie przechowywanym przez 24 h oraz zawartość wtórnych produktów utlenienia, zarówno w pieczywie przechowywanym 24 h, jak i 72 h. Dodatek bazylii oddziaływał dość podobnie do dodatku oregano, jednakże zmniejszał, a nie zwiększał objętość pieczywa oraz zwiększał zawartość nadtlenków w pieczywie przechowywanym 72 h. Dodatek suszu z papryki wprawdzie w największym stopniu wpływał na zmniejszenie objętości pieczywa, ale znacznie podnosił jego smakowitość oraz najkorzystniej wpływał na jakość tłuszczu, ograniczając w największym stopniu zawartość nadtlenków oraz wtórnych produktów utleniania.

Introduction

Supplementation of bread with flax seeds, a rich source of components valuable to health, is the best way to increase their consumption. Compounds that confirm the introduction of these seeds to bread are lipids, phytoestrogens and dietary fiber.

Among lipid compounds, of special importance is α-linolenic acid (18:3 n-3) which constitutes over 50% of the total fatty acids (TAŃSKA, ROTKIEWICZ 2003, ROTKIEWICZ et al. 2003, CUNNANE et al. 1992). In a human body, this fatty acid is desaturated and elongated to more unsaturated acids with a longer carbon chain, i.e. eicosapentaenoic (20:5 n-3), docosapentaenoic (22:5 n-3) and docosahexaenoic (22:6 n-3), from which eicosanoids are formed (SOMMER et al. 2002). Eicosanoids of omega-3 acids protect against diseases of the cardiovascular system owing to, among others, their antisclerotic (DJOUSSE et al. 2003)

and anti-inflammatory activity (TARPILA et al. 2005, after JAMES et al.). The anticarcinogenic action of flax seeds oil has, in turn, been reported in a study by THOMPSON et al. (1996).

Lignans of flax seeds, considered as phytoestrogens, include primarily secoisolariciresinol diglycoside and matairesinol. The content of secoisolariciresinol diglycoside in flax seeds accounts for ca. 5 mg g^{-1} (SPENCE et al. 2003), which is 75-800 times higher than the seeds of other oil plants, cereal grain, legumes as well as vegetables and fruits (THOMPSON et al. 1991). In the colon, lignans are transformed by colonic microflora to enterodiol and enterolactone, which demonstrate biological activity due to structural similarity to estrogens (QU et al. 2005). This activity is manifested by, among others, antioxidative action (DAVIES et al. 1999), which reduces the risk of the development of neoplastic disease. It has been confirmed in clinical studies in cases of breast, prostate and colon carcinomas (BYLUND et al. 2005, QU et al. 2005). SPENCE et al. (2003) suggest that flax seeds may become an alternative to substitutive hormonal therapy in postmenopausal women, because providing the anticarcinogenic and antiatherogenic benefits of estrogen; they do not involve the negative effect, i.e. thrombosis.

Dietary fiber of flax seeds, constituting 35-45% of their mass, is 2/3 composed of insoluble compounds and 1/3 of soluble compounds (CARTER 1993). The soluble compounds of the fiber form viscous solutions in the digestive tract, which regulates defecation, alleviates obstructions and other symptoms of irritable colon, and decreases glucose absorption, which is important for diabetics (STILLER 1994). The insoluble compounds of dietary fiber decrease the risk of diverticular colonopathy (ALDORI et al. 1998) and coronary heart disease reducing cholesterol, triglycerides and arterial blood pressure (PEREIRA et al. 2004). The existence of a negative correlation between the content of dietary fiber in a diet and incidence of colon and rectum carcinomas (BINGHAM et al. 2003) shows that flax seeds can to reduce the risk of the occurrence of these diseases.

The addition of flax seeds up to 13% does not deteriorate the organoleptic properties of bakery products (GAMBUŚ et al. 1999a,b, 2001), but raises doubts linked with an increase of lipid oxidation products content, especially during storage of bread (ROTKIEWICZ et al. 2003). The lipid oxidation can be reduced by, e.g., the application of herbs or vegetables (JULIANI, SIMON 2002). The undertaken study is aimed at determining whether the addition of selected herbs and vegetables, applied at a dose increasing the tastiness of bread with flax seeds, can simultaneously to act as antioxidants. The objective of the first part of the study, presented in this manuscript, was to evaluate the effect of additives of frozen basil and oregano as well as dried paprika fruits on the properties of bread supplemented with flax seeds and on lipid hydrolysis and oxidation in bread crumb.

Materials and Methods

The materials of this study were: wheat flour type 650, flax seeds (fiber-type), frozen herbs: basil and oregano and dried red paprika fruits in cube form about size 0.3 x 0.3 mm.

The wheat flour was characterized by measurements of: moisture content (PN-91/A-74010), acidity (PN-60/A-74007), water absorption (REYES-MORENO et al. 2002), falling number (PN-ISO-3093:1996) and wet gluten content (PN-A-74043-3). Flaxseed seeds were characterized on the basis of organoleptic analysis (PN-R-66149:1997) and determination of moisture content (PN-91/A-74010:1991), mass of 1000 seeds (HABER, HORUBAŁOWA 1992), fat content (PN-EN ISO 734-1:2000), total phenolic compounds content (AOAC 1974) and water absorption (REYES-MORENO et al. 2002). Flaxseed oil, extracted using petroleum ether, characterized by measurements of: acid value (PN-EN 660:2005), peroxide value (PN-EN ISO 3960:2005), anisidine value (PN-EN ISO 6885:2001) and content of conjugated compounds, dienes and trienes, (AOACS 1973). In frozen herbs and dried paprika were analyzed: moisture content (PN-91/R-87019) and total phenolic compounds content (AOAC 1974).

The quality and form of additives were determined on the basis of our early researches (ROTKIEWICZ et al. 2003) and of laboratory baking process results. Based on organoleptic opinion the following four variants of additives were selected: (1) 5% flax seeds, (2) 5% flax seeds + 1% oregano, (3) 5% flax seeds + 1% basil and (4) 5% flax seeds + 5% dried paprika fruits. Flax seeds were added after their milling, boiling and cooling, because bread with seeds prepared in this way not posses foreign flavor. Herbs were added after unfreeze.

Dough was making according to method of Research Institute for Baking Industry, described by JAKUBCZYK and HABER (1981). Water addition was determined on the basis of moisture content in components and water absorption of flour, flax seed seeds and dried paprika fruits.

Bread properties were analyzed after 24 and 72 hours after baking. The organoleptic analysis were done pointwise (PN-A-74108), bread volume was measure in SA-Wy apparatus and were determined: acidity (PN-A-74108:1996), content of free fat by Soxhlet method (PN-73/R-66164), moisture content (PN-91/A-74010:1991) and elasticity of crumb. The crumb elasticity was measured for 25x25x25 mm cubes, sliced from central part of loaf, by using UTM Instron 4301. Parameters of test were following: capacity of head – 1000 N, compressing element – flat pin about diameter 35 mm, speed of compression – 50 mm min^{-1} and range of deformation 25%.

In fat extracted from bread crumb 24 and 72 hours after baking were determined: acid value (PN-ISO-660:1998), peroxide value (PN-EN ISO 3960:2005) and anisidine value (PN-ISO-3960:1996).

Results and Discussion

Characteristics of raw materials

Wheat flour used for bread making was characterized by good technological quality (Table 1), which was indicated by the values of parameters include in range determined by the Polish standard (PN-91/A-74022). It should be point at that the value of the falling number of flour, reaching 453 s, slightly exceeded the maximum value of that factor reported in guidelines for the technological quality of flour for bread making, i.e. 450 s (AMBROZIAK 1998). The high value of the falling number indicates a low activity of α-amylase.

Table 1

Quality of wheat flour

Quality factor	$\bar{x}$	$\hat{s}$
Moisture content (%)	14.4	0.05
Acidity (°)	3.5	0.14
Water absorption (%)	69.3	0.58
Falling number (s)	453	20.5
Wet gluten content (%)	29	1.0

$\bar{x}$ – mean value, $\hat{s}$ – standard deviation

Flax seeds were not impurity, were sound and mature, with a natural color, sheen, natural flavor and moisture content of 10.5% (Table 2), and thus fulfilled the requirements of the qualitative standard of seeds designed for processing (PN-74/R-66200). Flax seeds contained 40% d.m. of fat and were characterized by an acid value of 2.08 mg KOH g^{-1}, a peroxide value of 4.46 mEq O_2 kg^{-1}, an anisidine value of 0.72 and conjugated compounds content of 0% (Table 2). Literature data report that fat content in flax seeds cultivated in Poland may include in range from 36% d.m. (ROTKIEWICZ et al. 2003) to 44% d.m. (GAMBUŚ et al. 2001). The content of total phenolic compounds in flax seeds accounted for 8.6 mg g^{-1} (Table 2) and was higher than that found by VELIOGLU et al. (1998) in seeds of Canadian cultivars and reached 5.09 mg g^{-1}. In turn, in seeds of flax cultivated in Canada, OOMAH and MAZZA (1998) determined the content of total phenolic acids at the level of 13.4 mg g^{-1}. Water absorption capacity of flax seeds reached 278.7% and was over four times higher than that of flour (Tables 1 and 2). It results a high, ca. 10%, content of non-starch polysaccharides, such as: rhamnose, fructose, xylose, galactose and uronic acids in flax seeds, which in the presence of water produces mucilage (CARTER 1993).

Table 2

Quality of additives

Quality factor	$\bar{x}$	$\hat{s}$
Flax seeds		
Organoleptic analysis	natural flavor, color and sheen	
Moisture content (%)	10.5	0.13
Mass of 1000 seed (g)	5.4	0.04
Phenolic compounds content (mg g^{-1} d.b.)	8.6	0.00
Water absorption (%)	279	9.5
Fat content (% d.b.)	40.1	0.11
Acid value of fat (mg KOH g^{-1} fat)	2.08	0.000
Peroxide value of fat (mEq O_2 kg^{-1} fat)	4.46	0.023
Anisidine value of fat (–)	0.72	0.017
Totox value (–)	9.64	0.015
Conjugated compounds in fat (%):		
dienes	0.00	0.000
trienes	0.00	.000
Frozen oregano		
Moisture content (%)	83.0	0.23
Total phenoloic compounds content (mg g^{-1} d.b.)	70.8	0.00
Frozen basil		
Moisture content (%)	86.4	0.32
Total phenolic compounds content (mg g^{-1} d.b.)	50.7	0.00
Dried paprika fruits		
Moisture content (%)	16.9	0.13
Total phenolic compounds content (mg g^{-1} d.b.)	51.7	0.00

$\bar{x}$ – mean value, $\hat{s}$ – standard deviation

The herbs added to bread, i.e. oregano and basil, characterized by moisture content equal 83.0 and 86.4%, respectively, whereas moisture content of paprika was 16.9%. The content of total phenolic compounds in these additives ranged from 50 to 70 mg g^{-1} d.m. (Table 2).

Quality of wheat bread

All types of bread obtained high number of points in the organoleptic analysis (36-39 points), but two types of bread: that with the addition of flax seeds and basil as well as that with the addition of flax seeds and paprika were more accepted by the panelists. The volume of control bread reached 253 cm^3 100 g^{-1}. The addition of flax seeds only affected an increase in bread volume by 5 cm^3 100 g^{-1}, whereas the additions of flax seeds with herbs exerted different effects: oregano was found to increase bread volume and basil decreased it (Table 3). The lowest volume, 214 cm^3 100 g^{-1}, was reported for bread with the

addition of flax seed and paprika. According to Polish Standard (PN-92/A--74105), the volume of 100 g of bread made from 550 type flour should not be lower than 300 cm^3, whereas that of bread made from 720 type flour should not be lower than 230 cm^3. Using the same type of flour, as in presented study, GAMBUŚ et al. (1999) obtained bread with a volume of 486 cm^3 100 g^{-1}. Our previous studies have demonstrated that bread made from type 550 flour was characterized by a volume of 338 cm^3 100 g^{-1} (ROTKIEWICZ et al. 2003). In view of these data, the volume of breads in the reported study should be considered as low. It is probably to be linked with a low amylolytic activity of flour and increased viscosity of starch gel in dough, which seems to be confirmed by the results of crumb elasticity measurement (Table 3).

Table 3

Quality of bread

Specification		Wheat bread with				
		control	5% flax seed	5% flax seed + 1% oregano	5% flax seed + 1% basil	5% flax seed + 5% paprika
Loaf of bread						
Organoleptic note	$\bar{x}$	36^a	37^a	37^a	39^a	39^a
(number of points)	$\hat{s}$	3.0	3.3	3.3	2.6	3.8
Volume of 100 g bread	$\bar{x}$	253^a	258^a	264^a	235^b	214^c
(cm^3)	$\hat{s}$	3.2	7.5	12.8	1.4	3.7
Crumb of bread						
Acidity (°)	$\bar{x}$	1.6^a	1.7^a	1.7^a	1.6^a	1.7^a
	$\hat{s}$	0.06	0.07	0.03	0.07	0.06
Free lipids content	$\bar{x}$	0.54^a	2.71^b	2.53^c	2.12^d	2.08^d
(% d.b.)	$\hat{s}$	0.011	0.021	0.018	0.032	0.021
Moisture content (%)						
24 h after baking	$\bar{x}$	42.6^a	47.3^b	46.4^c	47.5^b	46.0^c
	$\hat{s}$	0.25	0.45	0.38	0.45	0.31
72 h after baking	$\bar{x}$	40.7^a	45.5^b	44.3^c	45.7^b	42.9^d
	$\hat{s}$	0.15	0.41	0.56	0.86	0.14
Maximum compression force (N)						
24 h after baking	$\bar{x}$	5.0^a	4.1^b	3.0^c	3.9^d	4.1^e
	$\hat{s}$	0.01	0.01	0,00	0.01	0.02
72 h after baking	$\bar{x}$	7.4^a	5.6^b	3.7^c	4.7^d	4.6^e
	$\hat{s}$	0.01	0.02	0.01	0.01	0.04

$\bar{x}$ – mean value, $\hat{s}$ – standard deviation. Means with the same letter in the same line are not significantly different ($p = 0.05$)

According to the Polish Standard (PN-92/A-74105), the acidity of bread made from wheat flour type 550, should not be higher than 3°. All types of bread in this study were characterized by lower acidity, ranging from 1.6 to 1.7° (Table 3). The content of free lipids in crumb was differentiated, the lowest

reported in the control (0.54% d.m.), and the highest in the bread with the addition of flax seeds only (2.71% d.m.). The fivefold increase in the content of the free lipids in bread with the addition of flax seeds only is a natural result of introduction fat with the seeds. In contrast, the fact that bread with the addition of flax seeds and simultaneous additions of herbs and paprika contains less free lipids (Table 3) points to the potential effect of these additives on lipids binding in dough. During hydratation of flour and mixing of dough, free lipids, are likely to bind with proteins, e.g. HMW glutenins and LMW protein "S" (ligolin) (ZAWISTOWSKA et al. 1985). This leads to an increase in the number of hydrophobic bonds, substituting a part of hydrogen bonds that stabilize the gluten structure of dough (PASCHKE, JANKIEWICZ 2002). Eventually, fat introduced with flax seeds modifies the physical properties of dough, the structure of bread crumbs and its sensory traits.

An important property of bread is maintaining a state of freshness for a longer period of time. Freshness is determined by, among other things, moisture content which, in turn, depends on drying rate. It has been found that 24 hours after baking, the control breads were characterized by the lowest moisture content of the crumb (42.6%), whereas those with additives were contained more water, ranging from 46.0 to 47.5% (Table 3). Additives of oregano and paprika decreased moisture content of bread crumb, but additive of basil not significantly changed this parameter in comparison to bread supplemented with flax seeds. 72 hours after baking, the moisture content was observed to decrease in all types of bread, however, the bread with additives was still characterized by a higher (by 4-5%) moisture content of crumbs compared to the control bread.

The value of the maximum compressive force, evaluating the elasticity of crumb, in the control bread reached 5.0 N 24 hours after baking and 7.4 N after 72 hours. All additives introduced to bread were found to considerably increase the elasticity of the crumb what result in decreasing of the maximum compressive force: by 18-40% after 24 hours and by 24-50% 72 hours after baking. The most elastic crumb was observed in the case of bread with the addition of flax seeds and oregano. In evaluating the ranges of decreasing bread elasticity between 24 and 72 hours after baking, the greatest decrease in the elasticity was observed in the control bread, whereas the lowest was in the bread supplemented with flax seeds and paprika (Table 3).

The higher elasticity of bread with flax seeds as compared to wheat bread, similar to the moisture content discussed above, results in the high water binding capacity of flax seeds mucilage. FEDENIUK and BILIADERIS (1994) demonstrated that the water binding capacity of flax seeds mucilage ranged from 2500 to 3000 g H_2O 100 g^{-1}. This value is higher than that demonstrated by most plant gums and reaches ≥ 30 g H_2O 100 g^{-1}. Studies by BÁRCENAS and

ROSELL (2005) also found that flax seeds can successively replace hydrocolloids in bakery products and confectionery added to improve their microstructure and rheological properties.

Quality of fat extracted from bread crumb

In the characteristics of the quality of fat extracted from bread crumb, attention is focused on the highest degree of hydrolysis and oxidation of fat from the control bread. Its acid value 24 and 72 hours after baking reached 11.08 and 26.57 mg KOH g^{-1}, respectively (Table 4). Fat of the bread with the addition of flax seeds only was characterized by the acid value a few times lower than that of the control bread, reaching 2.59 mg KOH g^{-1}. An analysis of the effect of the addition of herbs and paprika on the degree of lipid hydrolysis in bread with flax seeds indicated that herbal additives generally enhanced hydrolysis, whereas the paprika additive enhanced the hydrolysis in bread stored for a short period of time (24 hours) and inhibited it in bread stored for a longer period of time (72 hours) – Table 4.

Table 4

Quality of free lipids extracted from bread crumb

Specification		Wheat bread with				
		control	5% flax seed	5% flax seed + 1% oregano	5% flax seed + 1% basil	5% flax seed + 5% paprika
Acid value (mg KOH g^{-1} fat)						
24 h after baking	$\bar{x}$	11.08^a	2.59^b	2.49^b	4.56^c	3.61^d
	$\hat{s}$	0.534	0.211	0.341	0.119	0.210
72 h after baking	$\bar{x}$	26.57^a	9.06^b	10.15^c	11.63^d	6.33^e
	$\hat{s}$	0.887	0.567	0.774	0.727	0.512
Peroxide value (mEq O_2 kg^{-1} fat)						
24 h after baking	$\bar{x}$	88.55^a	64.81^b	42.86^c	49.17^d	22.13^e
	$\hat{s}$	3.987	2.876	2.765	1.349	4.649
72 h after baking	$\bar{x}$	218.75^a	116.58^b	121.72^b	133.05^c	37.59^d
	$\hat{s}$	3.978	5.878	7.646	9.345	6.248
Peroxide value (mEq O_2 100 g^{-1} bread crumb)						
24 h after baking		0.027^a	0.093^b	0.058^c	0.055^d	0.025^e
72 h after baking		0.070^a	0.172^b	0.171^b	0.153^c	0.045^d
Anisidine value (–)						
24 h after baking	$\bar{x}$	97.32^a	48.71^b	31.45^c	36.86^c	24.95^d
	$\hat{s}$	7.348	3.211	5.479	2.411	3.028
72 h after baking	$\bar{x}$	118.11^a	69.64^b	45.23^c	58.72^d	40.16^c
	$\hat{s}$	6.413	5.211	5.349	4.286	4.694

$\bar{x}$ – mean value, $\hat{s}$ – standard deviation. Means with the same letter in the same line are not significantly different ($p = 0.05$).

The lipid peroxide values appeared to depend on the type of additive and time of bread storage. The highest peroxide values, accounting for 88.55 and 218.75 mEq O_2 kg^{-1}, respectively 24 and 72 hours after baking, were observed in the control bread (Table 4). Fat of breads with additives was characterized by lower (by 35-83%) values of this parameter. The evaluation of the effect of herbal and paprika supplements on the lipid oxidation in breads with flax seeds indicate that the antioxidative effect of herb additives occurred only in the breads analyzed 24 hours after baking. In turn, in bread 72 hours after baking, the herbs stimulated the lipid oxidation, which was indicated by values of the peroxide number higher by 4-14% (Table 4). The addition of paprika was found to significantly (by nearly 70%) inhibit lipid oxidation both 24 and 72 hours after baking. Distinct inhibition of the lipid oxidation in bread crumb by paprika and the slight short-term inhibition by herbs are most likely due to the unequal quantity of phenolic compounds with an antioxidative potential introduced by them to bakery products (JULIANI, SIMON 2002). The 1% supplement of herbs delivered 7.10-12.04 mg of phenolic compounds, whereas the 5% supplement provided 214.55 mg of these compounds to 100 g of bread.

The content of secondary products of the lipid oxidation in the control bread crumb, irrespective of its storage time, was the highest, which was indicated by the anisidine values reaching 97.32 and 118.11 respectively 24 and 72 hours after baking (Table 4). Fat of bread supplemented only with flax seeds was characterized by twice as low values of the anisidine value. The herbal and paprika additives substantially inhibited the formation of secondary lipid oxidation products both 24 and 72 hours after baking: oregano by 35%, basil by ca. 20%, and paprika by over 40% (Table 4). An analysis of peroxides in 100 g of bread crumb indicates that both 24 and 72 hours after baking, the highest content of peroxides was found in bread with the addition of flax seeds only, whereas the lowest was in bread supplemented with dried paprika fruits (Table 4). Herbal supplements appeared to substantially decrease the content of peroxides in bread with flax seeds 24 hours after baking, whereas after 72 hours only the basil additive was efficient. The addition of paprika was identically efficient 24 and 72 hours after baking and reduced the content of peroxides in bread by 75% (Table 4).

Apart from phenolic compounds, the most inhibition of lipid oxidation in bread due to the addition of dried paprika fruits is certainly evoked upon the activity of carotenoids. In red paprika, their content may range from 0.35 to 0.99%, with capsanthin and capsorubin (70-80%) and lycopene predominating (KOSTRZEWA 1997). These carotenoids are acknowledged as the most active in quenching singlet oxygen (BEUTNER et al. 2001). In addition, lycopene is capable of scavenging free radicals (CICHOCKA 1998), thus demonstrating stronger antioxidant properties than other carotenoids (BOBROWSKA, OLĘDZKA 2002).

Conclusions

The results of these study show that content of lipid oxidation products in bread supplemented with flax seeds increase, and point to both the need for reducing lipid oxidation as well as the possibility of applying seasonings to this end. Seasonings, e.g. frozen basil and dried red paprika fruits, applied at doses which increase the tastiness of bread, generally not worsen properties of bread with flax seeds, and decrease the content of lipid oxidation products. A 5% addition of dried paprika fruits appeared to be especially efficient and was found to inhibit lipid oxidation in bread, independently to time of their storage. In bread with addition of paprika content of primary (expressed as peroxide value) and secondary (expressed as anisidine value) lipid oxidation products was above 30% and 50% lower, respectivly. The addition of herbs inhibited lipid oxidation in the slightest degree.

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References

ALDORI W.H., GIOVANNUCCI E.L., ROCKETT H.R., SAMPSON L., RIMM E.B., WILLETT W.C. 1998. *A prospective study of dietary fiber types and symptomatic diverticular disease in men*. J. Nutr., 128: 714-719.

AMBROZIAK Z. 1998. Produkcja piekarsko-ciastkarska. WSiP, Warszawa.

AOAC 9.110, *Association of the Official Analytical Chemists*. 1974. Washington, DC.

AOACS. 1973. *Official Methods Cd 7-58. Ultraviolet spectrophotometric method. Poly-unsaturated acids*.

BÁRCENAS M.E., ROSELL C.,M. 2005. *Effect of HPMC addition on the microstructure, quality and aging of wheat bread*. Food Hydrocolloids, 19: 1037-1043.

BEUTNER S., BLOEDORN B., FRIXEL S., HERNÁNDEZ BLANCO I., HOFFMANN T., MARTIN H.-D., MAYER B., NOACK P., RUCK CH., SCHMIDT M., SCHÜLKE I, SELL S., ERNST H., HAREMZA S., SEYBOLD G., SIES H., STAHL W., WALSH R. 2001. *Quantitative assessment of antioxidant properties of natural colorants and phytochemicals: carotenoids, flavonoids, phenols and indigoids. The role of β-carotene in antioxidant functions*. J. Sci. Food Agric., 81: 559-568.

BINGHAM S.H., DAY N.E., LUBEN R., FERRARI P., SLIMANI N., NORAT T., CLAVEL-CHAPELON F., KESSE E., NIETERS A., BOEING H., TJOENNELAND A., OVERVAD K., MARTINEZ C., DORRONSORO M., GONZALEZ C.A., KEY T.J., TRICHOPOULOU A., NASKA A., VINEIS P., TUMINO R., KROGH V., BUENO-DE-MESQUITA H.B., PEETERS P.H.M., BERGLUND G., HALLMANS G., LUND E., SKEIE J., KAAKS R., RIBOLI E. 2003. *Dietary fibre in food and protection against colorectal cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC): an observational study*. Lancet, 361: 1496-1501.

BOBROWSKA B., OLĘDZKA R. 2002. *Współczesne poglądy na rolę żywieniową luteiny i likopenu*. Bromat. Chem. Toksykol., 35 (3): 289-296.

BYLUND A., SAARINEN N., ZHANG J.-X., BERGH A., WIDMARK A., JOHANSSON A., LUNDIN E., ADLERCREUTZ H., HALLMANS G., STATTIN P., MAKELA S. 2005. *Anticancer effects of plant lignan 7-hydroxymatairesinol on a prostate cancer model in vivo*. Exp. Biol. Med., 230: 217-223.

CARTER J.F. 1993. *Potential of flaxseed and flaxseed oil in baken goods and other products in human nutrition*. Cereal Foods World, 38 (10): 753-759.

CICHOCKA A. 1998. *Nie tylko na długowieczność*. Kuchnia, 11: 64-66.

CUNNANE S.C., GANGULI S., MEMARD CH. LIEDE A.C., HAMADEH M.J., CHEN Z.-Y., WOLEVER T.M.S.,

JENKINS D.J.A. 1993. *High α-linolenic acid flaxeed* (*Linum usitatissimum*): *some nutritional properties in humans*. Br. J. Nutr., 69: 443-453.

DAVIES M.J., BOWEY E.A., ADLERCREUTZ H., ROWLAND I.R., RUMSBY P.C. 1999. *Effect of soy or rye supplementation of high-fat diets on colon tumour development in azoxymethane-treated rats*. Carcinogenesis 20: 927-931.

DJOUSSE L., FOLSOM A.R., PROVINCE M.A., HUNT S.C., ELLISON R.C. 2003. *Dietary linolenic acid and carotid atherosclerosis: the National Heart, Lung, and Blood Institute Family Heart Study*. Am. J. Clin. Nutrit., 77: 819-825.

FEDENIUK R.W., BILIADERIS C.G. 1994. *Composition and physicochemical properties of linseed (Linum usitatissimum L.) mucilage*. J. Agric. Food Chem., 42: 240-247.

GAMBUŚ H., BOROWIEC F., GAMBUŚ F., ZAJĄC T. 1999a. *Zdrowotne aspekty chleba z dodatkiem nasion lnu oleistego*. Żywność, 4(21): 185-193.

GAMBUŚ H., GAMBUŚ F., BOROWIEC F., ZAJĄC T. 1999b. *Możliwość zastosowania nasion lnu oleistego w piekarstwie.* Zesz. Nauk. AR w Krakowie, 360: 83-94.

GAMBUŚ H., MIKULEC A., PISULEWSKI P., BOROWIEC F., ZAJĄC T., KOPEĆ A. 2001. *Hipocholesterolemiczne właściwości chleba z nasionami lnu oleistego*. Żywność, 3 (28) Supl.: 54-64.

HABER T., HABEROWI H., LEWCZUK J., SIWOSZEK K. 1993. *Zioła jako dodatki do chleba o obniżonej zawartości soli*. Przem. Spoż., 1/93: 17-20.

JAKUBCZYK T., HABER T. 1993. *Analiza zbóż i przetworów zbożowych*. Wyd. SGGW-AR, Warszawa, ss. 279-285.

JULIANI H.R., SIMON J.E. 2002. *Antioxidant activity of basil*. In: Eds. *Trends in new crops and new uses*. J. JANICK and A. WHIPKEY. ASHS Press, Alexandria, VA, Proc. of the 5th Nat. Symp., 575-579 pp.

KOSTRZEWA E. 1997. *Aromatyzujące, konserwujące i barwiące właściwości przypraw ziołowych*. Wiad. Zielar., 39 (3): 17-19.

OOMAH B.D., MAZZA G. 1998.*Compositional changes during commercial processing of flaxseed*. Ind. Crops Prod., 9: 29-37.

PASCHE H., JANKIEWICZ M. 2002. *Wpływ dodatku tłuszczu na właściwości lepkosprężyste ciasta pszennego*. Żywność, Supl., 3 (32): 127-133.

PEREIRA M.A., O;REILLY E., AUGUSTSSON K., FRASER G.E., GOLDBOURT U., HEITMANN B.L., HALLMANS G., KNKT P., PITINEN P., SPIEGELMAN D., STEVENS J., VIRTAMO J., WILLETT W.C., ASCHERIO A. 2004. *Dietary fiber and risk of coronary heart disease: a pooled analysis of cohort studies*. Archives of Int. Med., 164: 370-376.

PRZYBYLSKI R., ESKIN N.A.M. 1995. *Methods to measure volatile compounds and the flavor significance of volatile compounds*. Chapter 6. In: *Methods to assess quality and stability of oils and fat-containing foods*. Eds. by WARNER K., ESKIN N.A.M. AOCS Press, Champaign, IL, 107-133 pp.

QU H., MADL R.L., TAKEMOTO D.J., BAYBUTT R.C., WANG W. 2005. *Lignans are involved in the antitumor activity of wheat bran in colon cancer SW480 cells*. J. Nutr., 135: 598-602.

REYES-MORENO C., MILAN-CARRILLO J., ROUAUD-SANDEZ O., GARZON-TIZNADO J., MORA-ESCOBEDO R. 2002. *Dehulling/softening/extrusion (DSE): technological alternative to improve nutritional quality of chickpea*. Agrociencia, 36: 181-189.

ROTKIEWICZ D., KONOPKA I., TAŃSKA M. 2003. *Comparison of the influence of two forms (oil – and fibre – type) of flaxseeds on selected wheat dough and bread properties*. Pol. J. Natur. Sc., 14 (2): 545-555.

SOMMER E., SOMMER S., SKOPIŃSKA-RÓŻEWSKA E. 2002. *Nienasycone kwasy tłuszczowe a nowotworzenie*. Współczesna Onkologia, 6 (2): 60-63.

SPENCE J.D., THORNTON T., MUIR A.D., WESTCOTT N.D. 2003. *The effect of flax seed cultivars with differing content of* α-linolenic acid and lignans on responses to mental stress. J. Amer. Coll. Nutrit., 22(6): 494-501.

SPILLER R.C. 1994. *Pharmacology of dietary fiber*. Pharmacol. Ther., 62: 407-427.

TAŃSKA M., ROTKIEWICZ D. 2003. *Stopień przemian lipidów wybranych olejów roślinnych i konsumpcyjnych nasion oleistych*. Tłuszcze Jadalne, 38 (3-4): 147-161.

TARPILA A., WENNBERG T., TARPILA S. 2005. *Flaxseed as a functional food*. Curr. Topics Nutraceutic. Res., 3 (3): 167-188.

THOMPSON L.U., RICHARD S.E., ORCHESON L.J., SEIDL M.M. 1996. *Flaxseed and its lignan and oil components reduce mammary tumor growth at a late stage of carcinogenesis*. Carcinogenesis, 17: 1373-1376.

THOMPSON L.U., ROBB P., SERRAINO M., CHENG F. 1991. *Mammalian lignan production from various foods*. Nutr. Cancer, 16: 43-52.

VELIOGLU Y.S., MAZZA G., GAO L., OOMAH B.D. 1998. *Antioxidant activity and total phenolics in selected fruits vegetables, and grain products*. J. Agric. Food Chem., 46: 4113-4117.

ZAWISTOWSKA U., BEKES F., BUSHUK W. 1985. *Gluten proteins with high affinity to flour lipids*. Cereal Chem., 62 (4): 284-289.