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WEED INFESTATION OF SPRING BARLEY IN CROP ROTATIONS WITH ITS DIFFERENT SHARE

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K e y w o r d s: spring barley, crop rotation, previous crops, weed infestation, biological indicators.

A b s t r a c t

The paper presents the results of a 3-year-long (2008–2010) study on the dynamics of weed infestation of spring barley sown after different previous crops (potato, spring wheat and spring barley, in 1- and 2-year sequences of the same crop) in four-field crop rotation systems with 25, 50 and 75% shares of spring barley. Weed infestation was evaluated at the barley tillering stage and before harvesting, with a focus on the number and species composition of weeds, as well as weed dry matter during harvest. The results were used for the calculation of the Shannon-Wiener diversity index and evenness index, Simpson's dominance index and Sørensen similarity index. Previous crops and share of spring barley in crop rotation significantly differentiated the infestation of spring barley with weeds at the tillering stage. The lowest weed infestation was found on the site of spring barley grown after potato in crop rotations with a 25 and 50% share of spring barley. Growing spring barley after spring wheat and in monoculture, and a 75% share of spring barley in crop rotation promoted the emergence of weeds. At the end of vegetation the number of annual and biennial weeds decreased by 45.3–79%, and the number of perennial weeds increased almost 3-fold with respect to their numbers in spring. The highest weed infestation was found in a four-field crop rotation with a 25% share of barley and on fields where spring barley was sown without an intercrop, and after spring wheat in crop rotations with a 75% share of spring barley. The highest biomass of weeds was produced in four-field with a 50% share of barley, where barley was grown after itself. Crop rotation had no effect on the species richness of weed communities. Lower diversity and evenness, and higher dominance of weed populations were found in communities on fields where barley followed potato in crop rotation with a 25% share of barley, and where barley was grown for two seasons without an intercrop in a crop rotation with a 75% share of barley.

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ZACHWASZCZENIE JĘCZMIENIA JAREGO W PŁODOZMIANACH Z RÓŻNYM JEGO UDZIAŁEM

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S ł o w a k l u c z o w e: jęczmień jary, płodozmian, przedplony, zachwaszczenie, wskaźniki biologiczne.

A b s t r a k t

W pracy przedstawiono 3-letnie (2008–2010) wyniki badań nad dynamiką zachwaszczenia jęczmienia jarego wysiewanego po różnych przedplonach (ziemniaku, pszenicy jarej oraz jęczmieniu jarym, w 1 i 2-krotnym następstwie po sobie) w czteropolowych płodozmianach z 25, 50 i 75% jego udziałem. Ocenę zachwaszczenia przeprowadzono w fazie krzewienia jęczmienia i przed jego zbiorem. Uwzględniała ona liczebność i skład gatunkowy chwastów, a podczas zbioru również ich suchą masę. Wyniki te posłużyły do obliczeń wskaźników różnorodności i równomierności gatunkowej Shannona-Wienera, dominacji Simpsona i współczynnika podobieństwa Sørensena. Przedplony i udział jęczmienia jarego w płodozmianie istotnie różnicowały jego zachwaszczenie w fazie krzewienia. Najmniejsze zachwaszczenie stwierdzono w stanowisku jęczmienia jarego po ziemniaku w płodozmianach z 25 i 50% jego udziałem. Uprawa po pszenicy jarej i po sobie oraz 75% udział jęczmienia w płodozmianie sprzyjały wschodom chwastów. Pod koniec wegetacji, w stosunku do stanu wiosennego, liczebność chwastów krótkotrwałych zmniejszyła się o 45,3–79%, a wieloletnich prawie 3- krotnie wzrosła. Najwięcej chwastów stwierdzono w czteropolówce z 25% udziałem jęczmienia oraz na polach po jęczmieniu jarym i pszenicy jarej w płodozmianach z 75% jego udziałem. Najobfitszą biomasę chwasty wykształciły w czteropolówce z 50% udziałem jęczmienia, w stanowisku jęczmienia po sobie. Nie stwierdzono wpływu płodozmianów na bogactwo gatunkowe zbiorowisk chwastów. Mniejszą różnorodność i wyrównanie, a zarazem większą dominację populacji odnotowano w zbiorowiskach pola po ziemniaku w płodozmianie z 25% udziałem jęczmienia oraz po jęczmieniu uprawianym dwa razy po sobie w płodozmianie z 75% jego udziałem.

Introduction

Specialised plant production increasingly frequently leads to simplification in crop rotation systems. This is reflected, for example, in a reduced number of grown plants and shorter intervals between the return of the same crop on the field. In most farms production methods are driven by economic factors. Farmers deliberately limit or even eliminate labour-intensive crops, and replace them with cereals, which are popular mainly because of economic reasons and usability. By exceeding a 70% share of cereals in the crop structure farmers switch from natural, correct crop rotation to its simplified forms, or even monoculture. This approach leads to increased abundance and biomass of weeds, with the simultaneous compensation of selected species (CAVERS and BENOIT 1989, STEVENSON et al. 1997, THOMPSON 1992). This results in lower

yields of individual crops and whole cropping systems (MARSHAL et al. 2003, OERKE et al. 1994).

Many studies have demonstrated a significant role of the proper choice and sequence of plants in crop rotation in limiting weed infestation in cereals (SEIBUTIS and FEIZA 2008). The scale of weed pressure depends on the species and cultivar of cereals, but also on their natural competitiveness with segetal flora. Spring barley has a low tolerance to sowing without an intercrop (JOHNSTON 1997, ROUS 1992, STRNAD 1993), and its incorrect position in crop rotation creates favourable conditions for increased weed infestation of its canopy (JASTRZĘBSKA et al. 2012). ZAWIŚLAK and SADOWSKI (1992) concluded that only natural, correct crop rotation with a suitable interval between growing spring barley after itself helps to maintain weed density at the level not decreasing barley yield, while KOSTRZEWSKA and WANIC (2005) indicated that it also promotes the preservation of biodiversity in agrophytocenoses.

Considering the above, a research hypothesis was proposed that weed infestation of spring barley depends on its position in crop rotation. To verify the hypothesis a field experiment was established in order to evaluate the effect of position in crop rotations with 25, 50 and 75% shares of spring barley on the number and biomass of weeds and diversity of their communities.

Materials and Methods

The analysed data were obtained from 3-year-long (2008–2010) studies carried out in a strict static field experiment established in 2005 at the Production and Experimental Station in Bałcyny, near Ostróda (53°36' N, 19°51' E), an experimental centre of the University of Warmia and Mazury in Olsztyn. The experiment was carried out using the random block method in 4 replicates, on typical lessive (Systematyka gleb Polski 2011), Haplic Luvisol (Loamic) (IUSS 2015) soil formed from sandy clay loam. The topsoil (0–20 cm) contained 8.9 to 10.4 $g \cdot kg^{-1}$ C_{org}, was acidic (pH_{KCl} 5.5–5.7), and contained from high to very high levels of phosphorus and potassium (80 to 99 $mg \cdot 100\ g^{-1}$P of soil and 182 to 233 $mg \cdot 100g^{-1}$ K) and low levels of magnesium (36 to 47 $mg \cdot 100\ g^{-1}$). The studied crop was spring barley, Rastik cultivar, grown after different previous crops in the following crop rotations:

A (25% of barley – control site): potato – spring barley$^{(2)}$ – peas – spring wheat

B (50% of barley): potato – spring barley$^{(2)}$ – spring wheat – spring barley$^{(4)}$

C (50% of barley): potato – spring wheat – spring barley$^{(3)}$ – spring barley$^{(4)}$

D (75% of barley): potato – spring barley$^{(2)}$ – spring barley$^{(3)}$ – spring barley$^{(4)}$.

Soil for the experiment was prepared using a traditional tillage. In spring the soil was cultivated, treated with mineral fertilizers and harrowed. The barley seeding rate was 500 germinating kernels per m^2. The field was harrowed after sowing. Mineral fertilization did not differ depending on previous crops, and was adjusted to the content of nutrients in the soil. The dose of pure NPK component was 161 $kg \cdot ha^{-1}$ (N – 60; P- 35 and K – 66). Manure at a dose of 30 t ha^{-1} was applied once in autumn before planting potatoes in a four-year rotation. Barley was protected against mono- and dicotyledonous weeds from the tillering stage (BBCH 23–29) to the shooting stage (BBCH 30–32). Monocotyledonous weeds were controlled with a herbicide containing fenoxaprop-P-ethyl (Puma Universal 069 EW at a dose of 1.0 $l \cdot ha^{-1}$), and dicotyledonous with Mustang 306 SE (florasulam + 2,4D EHE) at a dose of 0.5 $l \cdot ha^{-1}$. Current weed infestation was assessed annually before the application of herbicides at the initial stage of barley tillering (BBCH 21–22) and before the barley harvest (BBCH 89–92). The assessment was focused on the number and species composition of weeds per m^2, as well as weed dry matter during the barley harvest. Measurements were taken using the frame technique in two replicates on each plot. The results were used for the calculation of Simpson's dominance index (1949) and the Shannon-Wiener diversity index and evenness index (1948). Weed communities were compared using the Srrensen similarity index (1948). Data on the number and biomass of weeds were processed statistically by using the analysis of variance and Duncan's test at a significance level of $p = 0.05$. Nomenclature of weed species was adopted after MIREK et al. (1995).

Results

Previous crops and the share of spring barley in crop rotation significantly differentiated barley weed infestation at the tillering stage (Table 1). Significantly lower weed density was found on the field after potato in four-field rotation A (control site) and the same sequence in crop rotation B with a 50% share of barley. Growing spring barley in crop rotations with a 50% of its share on the fields after spring wheat (B and C) and without an intercrop (C), as well as in rotation with a 75% share of the spring barley after potato and without an intercrop (D) significantly increased weed infestation. The number of taxa in the analysed plots in relation to the control crop rotation (A) was on average 27.2% higher, with the greatest difference (36.5%) in crop rotation B, with a 50% share of barley on the site after spring wheat. Weed communities were formed by 15–20 species. The highest weed richness was found in four-field crop rotation B, where barley was grown without an intercrop, and the lowest

Table 1

Weed infestation of spring barley at the tillering stage, plants · m^{-2}

Weed species	Crop rotation/field								Mean
	A/2	B/2	B/4	C/3	C/4	D/2	D/3	D/4	
Annuals and biennials									
Thlaspi arvense L.	29.7	10.0	54.7	28.0	41.0	36.9	26.2	24.2	31.34
Chenopodium album L.	43.9	23.0	33.4	36.3	32.2	25.8	31.3	17.3	30.40
Fallopia convolvulus (L.) A. Love	17.7	22.8	19.7	19.3	16.0	26.7	24.9	34.8	22.74
Veronica arvensis L.	4.4	18.3	11.4	21.6	22.0	22.5	25.0	15.3	17.56
Stellaria media (L.) Vill.	7.9	5.7	8.7	7.4	6.7	13.1	4.7	5.2	7.43
Galium aparine L.	0.9	1.3	1.7	7.3	4.0	7.3	5.4	5.8	4.21
Viola arvensis Murray	4.4	6.3	5.0	6.8	–	4.8	3.3	3.0	4.20
Spergula arvensis L.	–	6.1	3.7	6.0	8.7	–	3.8	3.7	4.00
Polygonum aviculare L.	3.2	4.7	5.5	3.4	7.0	1.4	–	2.7	3.49
Capsella bursa-pastoris (L.) Medicus	2.5	5.3	5.5	1.4	6.0	3.3	–	2.8	3.35
Polygonum lapathifolium L.	1.3	0.7	4.0	3.8	–	2.1	4.0	10.8	3.34
Galinsoga parviflora Cav.	3.3	–	2.2	0.7	–	4.7	7.7	3.0	2.70
Fumaria officinalis L.	2.0	0.4	4.7	–	–	5.8	3.3	1.8	2.25
Raphanus raphanistrum L.	2.0	2.7	–	4.3	1.1	–	3.3	1.8	1.90
Mentha arvensis L.	–	–	–	2.7	5.3	–	3.3	3.3	1.83
Vicia hirsuta (L.) S.F. Gray	–	5.0	0.7	2.0	1.8	–	–	–	1.19
Lamium amplexicaule L.	–	2.5	–	–	1.7	2.7	0.7	–	0.95
Lycopsis arvensis L.	–	2.9	2.2	–	–	–	1.2	1.2	0.94
Echinochloa crus-galli (L.) P.B.	1.8	–	–	–	3.7	1.3	–	–	0.85
Myosotis arvensis (L.) Hill	–	–	3.8	–	–	–	–	–	0.48
Veronica persica Poir.	–	–	–	–	–	3.2	–	–	0.40
Matricaria maritima (L.)	–	–	–	2.2	–	–	–	–	0.28
Erodium cicutarium (L.) L'Hérit.	–	–	–	–	–	–	–	1.6	0.20
Galeopsis tetrahit L.	–	–	1.0	–	–	–	–	–	0.13
Total annuals and biennials	125.0	117.7	167.9	153.2	157.2	161.6	148.1	138.3	146.1
Perennials									
Equisetum arvense L.	1.0	1.8	0.9	5.3	–	1.0	2.0	2.8	1.85
Sonchus arvensis L.	–	1.4	2.2	–	–	–	–	3.8	0.93
Cirsium arvense (L.) Scop	0.7	–	2.0	–	–	2.9	–	–	0.70
Agropyron repens (L.) Beauv.	–	–	–	–	1.4	–	–	–	0.18
Total perennials	1.7	3.2	5.1	5.3	1.4	3.9	2.0	6.6	3.65
Total per m^2	126.7^{c*}	120.9^{c}	173.0^{a}	158.5^{ab}	158.6^{ab}	165.5^{ab}	150.1^{b}	144.9^{bc}	149.78
Number of species	16	18	20	17	15	17	16	19	17

*a, b, c – values marked with the same letter do not differ significantly at $p = 0.05$

on field C, with the same cropping sequence. The analysed phytocoenoses were formed mainly by annual and biennial weeds, typical spring and wintering species (more than 90% of all weeds). Of these, 4 species were dominant: *Thlaspi arvense, Chenopodium album, Fallopia convolvulus* and *Veronica arvensis*. Their share in weed communities ranged from 63.2% (crop rotation D on a field where barley was grown without an intercrop) to 75.5% (control

four-field crop rotation). The higher weed infestation of barley grown after spring wheat in crop rotation B was mainly attributed to the greater abundance of *Thlaspi arvense* (density greater by 84.2% than in the control four-field crop rotation), the presence of *Myosotis arvensis* (not recorded on other sites) and the presence of *Sonchus arvensis* and *Agropyron repens*, while on site C – to *Veronica arvensis, Galium aparine, Viola arvensis* and *Matricaria maritima* (the latter taxon was not found on other fields). The higher weed infestation of barley grown after itself in crop rotation C was caused by the more abundant presence of *Thlaspi arvense, Spergula arvensis, Polygonum aviculare, Capsella bursa-pastoris* and *Mentha arvensis*, and on field D after potato because of *Thlaspi arvense, Fallopia convolvulus, Veronica arvensis, Stellaria media, Galinsoga parviflora* and *Fumaria officinalis.*

The number of weeds at the end of vegetation was 45.3–79% lower than in spring (Table 2). However, the decrease concerned annuals and biennials, while an almost 3-fold increase was found for perennial weeds. The highest weed density was found on the field where barley was grown after potato in the control crop rotation, and after spring wheat and without an intercrop in the four-field crop rotations B and C. On these fields the number of weeds was significantly greater (almost 2-fold) than on the other low-diversified sites. The analysed communities were formed by annual and biennial taxa, i.e. typical spring weeds and wintering weeds (69.3 to 86.8% of the total number of weeds) and perennials. *Galissoga parviflora, Chenopodium album, Fallopia convolvulus, Equisetum arvense, Veronica arvensis*, *Agropyron repens* and *Thlaspi arvense* were the dominant species. Perennial weeds not encountered in spring were also found, i.e. *Taraxacum officinale, Plantago lanceolata* and *Plantago major*. The highest species richness was found in four-field crop rotation B, on the site where barley was grown after wheat, and the lowest in the control crop rotation. Higher weed infestation resulted from the more abundant presence of *Galinsoga parviflora, Chenopodium album, Thlaspi arvense* and *Cirsium arvense* in the control crop rotation*, Galinsoga parviflora, Fallopia convolvulus, Polygonum aviculare* and *Equisetum arvense* in barley grown after wheat on site B, and *Galinsoga parviflora, Chenopodium album*, *Polygonum aviculare* and *Agropyron repens* on site C, where barley was grown without an intercrop.

The largest biomass of weeds was produced in four-field crop rotation C, on the site where barley was grown after itself (Table 3), and it was significantly greater than on other fields of the analysed cropping systems (almost 3-fold greater than in the control site). Weed biomass was also significantly greater in the field in crop rotation B, where barley was grown after wheat, than on the sites with potato as an intercrop in crop rotation C and both fields of crop rotation D. Differences between weed infestation on other fields were not

Table 2

Weed infestation of spring barley before harvest, plants · m^{-2}

Weed species	Crop rotation/field								Mean
	A/2	B/2	B/4	C/3	C/4	D/2	D/3	D/4	
Annuals and biennials									
Galinsoga parviflora Cav.	18.7	8.2	15.8	8.7	16.7	5.4	21.1	12.5	13.39
Chenopodium album L.	22.0	1.8	5.0	0.9	8.3	3.3	1.8	2.4	5.69
Fallopia convolvulus (L.) A.Love	1.0	5.8	10.3	6.1	6.0	4.6	3.3	7.2	5.54
Veronica arvensis L.	4.7	4.3	5.7	4.9	3.3	3.4	2.0	2.0	3.79
Thlaspi arvense L.	10.6	–	2.9	–	11.7	–	–	–	3.15
Echinochloa crus–galli (L.) P.B.	3.1	5.3	2.5	0.4	4.0	3.3	3.3	–	2.74
Polygonum aviculare L.	–	1.3	6.0	4.8	5.8	1.2	–	1.5	2.58
Veronica persica Poir.	–	2.7	3.3	1.7	2.3	1.4	4.7	2.0	2.26
Polygonum lapathifolium L.	–	0.7	0.8	2.4	–	–	0.7	0.8	0.68
Spergula arvensis L.	–	–	–	1.8	1.0	0.3	–	–	0.39
Mentha arvensis L.	–	–	1.0	1.2	–	–	–	–	0.28
Galium aparine L.	–	–	–	–	–	0.4	0.8	0.5	0.21
Capsella bursa-pastoris (L.) Medicus	–	–	–	–	–	0.8	–	0.9	0.21
Lamium amplexicaule L.	–	–	0.7	0.8	–	–	–	–	0.19
Galeopsis tetrahit L.	–	–	1.3	–	–	–	–	–	0.16
Viola arvensis Murray	–	0.7	–	–	–	–	–	0.5	0.15
Stellaria media (L.) Vill	–	–	1.4	–	0.8	–	–	–	0.28
Total annuals and biennials	60.1	30.8	56.7	33.7	59.9	24.1	37.7	30.3	41.69
Perennials									
Equisetum arvense L.	3.3	2.8	8.4	4.3	3.0	8.0	3.2	3.8	4.60
Agropyron repens (L.) Beauv.	1.8	1.3	5.0	2.5	11.2	2.7	0.7	1.5	3.34
Cirsium arvense (L.) Scop.	2.6	1.3	1.2	0.5	1.5	–	2.0	–	1.14
Sonchus arvensis L.	0.6	2.5	1.8	1.9	–	–	0.4	1.8	1.13
Taraxacum officinale F.H. Wigg.	–	1.0	–	–	–	–	1.0	–	0.25
Plantago major L.	–	–	–	–	0.7	–	–	–	0.10
Plantago lanceolata L.	0.8	–	–	–	–	–	–	–	0.09
Total perennials	9.1	8.9	16.4	9.2	16.4	10.7	7.3	7.1	10.65
Total per m^2	69.2^{a*}	39.7^{b}	73.1^{a}	42.9^{b}	76.3^{a}	34.8^{b}	45.0^{b}	37.4^{b}	52.34
Number of species	11	14	17	15	14	12	13	13	14

*a, b – values marked with the same letter do not differ significantly at $p = 0.05$

significant. The dominant species in dry weed matter were *Chenopodium album, Thlaspi arvense, Galinsoga parviflora, Equisetum arvense* and *Agropyron repens*. The greater mass of dry weeds from the field of barley grown after itself in four-field rotation C was associated with the large biomass of *Chenopodium album*, *Thlaspi arvense* and *Equisetum arvense*. These weeds together formed 70% of the total biomass of the weed community. Growing barley after wheat in four-field crop rotation B promoted the development of *Agropyron repens;* the mass of this weed was almost 15-fold greater than in the control crop rotation, where barley was grown after potato. *Agropyron repens* was also abundant on the field where barley was grown after itself (site C).

Table 3

Weed infestation of spring barley before harvest, $g \cdot m^{-2}$

Weed species	Crop rotation/field								Mean
	A/2	B/2	B/4	C/3	C/4	D/2	D/3	D/4	
Annuals and biennials									
Chenopodium album L.	8.0	3.6	3.7	1.4	20.5	3.1	1.2	4.4	5.74
Thlaspi arvense L.	4.3	-	3.1	-	21.5	-	-	-	3.61
Galinsoga parviflora Cav.	3.0	2.7	2.7	1.3	6.0	3.7	5.9	1.9	3.40
Fallopia convolvulus (L.) A.Love	1.5	1.1	3.4	1.9	2.1	2.1	1.0	1.6	1.84
Veronica arvensis L.	0.2	1.2	2.8	2.6	0.8	1.7	0.4	0.9	1.33
Echinochloa crus-galli (L.) P.B.	2.8	2.5	0.6	0.5	0.8	1.0	1.5	-	1.18
Veronica persica Poir.	–	1.0	1.6	0.5	1.6	0.2	2.2	0.7	0.98
Polygonum aviculare L.	–	0.2	2.3	1.5	2.5	1.0	–	0.1	0.95
Polygonum lapathifolium L.	–	0.2	0.2	0.9	–	–	0.1	0.3	0.21
Galium aparine L.	–	–	–	–	–	0.2	0.6	0.2	0.12
Spergula arvensis	–	–	–	0.4	0.2	0.1	–	–	0.09
Mentha arvensis L.	–	–	0.1	0.4	–	0.1	–	–	0.06
Viola arvensis Murray	–	–	–	–	–	–	–	0.2	0.03
Galeopsis tetrahit L.	–	–	0.4	–	–	–	–	–	0.05
Avena fatua L.	–	–	0.3	–	–	–	–	–	0.04
Stellaria media (L.) Vill	–	–	0.1	–	0.1	–	–	–	0.03
Lamium amplexicaule L.	–	–	–	0.4	–	–	–	–	0.05
Capsella bursa-pastoris (L.) Medicus	–	–	–	–	0.01	–	–	0.3	0.05
Viola arvensis L.	–	0.1	–	–	–	–	–	–	–
Total annuals and biennials	19.8	12.6	21.4	11.8	56.1	13.0	12.9	10.6	19.76
Perennials									
Equisetum arvense L.	3.8	2.9	6.4	4.2	12.0	10.7	4.2	4.8	6.13
Agropyron repens (L.) Beauv.	0.7	5.0	10.7	0.5	6.7	1.5	0.2	0.6	3.24
Cirsium arvense (L.) Scop.	2.8	2.6	0.7	0.3	2.3	–	5.9	–	1.83
Taraxacum officinale F.H. Wigg.	–	0.9	–	–	–	–	0.2	–	0.14
Sonchus arvensis L.	0.1	4.0	–	0.1	–	–	0.1	1.8	0.90
Plantago major L.	–	–	0.7	–	–	–	–	–	0.09
Plantago lanceolata L.	0.4	–	–	–	–	–	–	–	0.05
Total perennials	7.8	15.4	18.5	5.1	21.0	12.2	10.6	7.2	12.38
Total per m^2	27.6^{bc*}	27.9^{bc}	39.9^{b}	17.5^{c}	77.1^{a}	25.2^{bc}	23.5^{c}	17.8^{c}	32.06

*a, b, c – values marked with the same letter do not differ significantly at $p = 0.05$

Biological indicators reflecting the diversity of weed communities calculated based on their abundance demonstrated that both in spring and at the end of vegetation the barley canopy in the control crop rotation (grown after potato) was characterised by a greater dominance of weeds and a lower diversity and evenness of their individual populations (Table 4). Before harvest, populations less diversified in terms of their size were also recorded on the field cultivated in crop rotation D, where barley was grown after itself. There were no significant differences between other sites in terms of diversity. However, the values of indicators calculated based on weed biomass were different. The highest species dominance and the lowest diversity and even-

Table 4

Biological indicators for weed communities

Index	Crop rotation/field							
	A/2	B/2	B/4	C/3	C/4	D/2	D/3	D/4
Barley tillering – based on the number of weeds								
Dominance (λ)	0.21	0.12	0.16	0.13	0.15	0.13	0.14	0.12
Diversity (H')	1.99	2.43	2.27	2.35	2.20	2.31	2.25	2.43
Evenness (J')	0.72	0.84	0.76	0.83	0.81	0.81	0.81	0.83
Before barley harvest – based on the number of weeds								
Dominance (λ)	0.21	0.11	0.11	0.11	0.12	0.13	0.26	0.18
Diversity (H')	1.83	2.37	2.34	2.43	2.29	2.19	1.89	2.10
Evenness (J')	0.76	0.90	0.81	0.88	0.87	0.88	0.74	0.82
Before barley harvest – based on the air-dry matter of weeds								
Dominance (λ)	0.17	0.11	0.14	0.12	0.19	0.24	0.18	0.17
Diversity (H')	1.98	2.32	2.30	2.40	1.93	1.80	1.96	2.38
Evenness (J')	0.83	0.88	0.80	0.87	0.73	0.73	0.77	0.80

ness of weed populations were found for the field in crop rotation D, where barley was grown after potato. A lower evenness in comparison to other sites was also found for barley grown after itself in four-field crop rotation C.

The assessment of similarity for the analysed phytocenoses demonstrated significant differences between weed communities in barley grown after various previous crops (Table 5). At the tillering stage the greatest similarity was found for both fields of crop rotation: C – barley after wheat and after itself, C – barley after wheat and D – barley after barley, D – barley after potato and barley and D – after barley grown without an intercrop for one or two seasons. At the end of vegetation the greatest similarity in the population size and biomass was found for communities on the field in crop rotation B after potato and D – barley grown without an intercrop for two seasons, and C – after spring wheat and D – after barley. High levels of similarity in terms of population size were also found for the following pairs: B – after potato and D – after barley grown twice after itself, B – after wheat and C – after barley grown twice after itself and B – after wheat and C – after wheat, and in terms of biomass for D – after potato and D – after barley grown twice after itself.

Discussion

The effects of previous crops and share of spring barley in crop rotation on weed infestation of barley has been investigated by many authors (GAWĘDA at al. 2014, LÈGÈRE et al. 2005, LIEBMAN and STAVER 2001, O'DONOVAN et al. 2007). In our study the position and share of barley in crop rotation significant-

Table 5

Similarity index for weed communities, %

Compared weed communities in barley after different previous crops	Similarity based on		
	number	number	biomass
	at the barley tillering stage	before barley harvest	
A2/B2	61.3	43.4	59.1
A2/B4	74.3	54.3	50.7
A2/C3	76.2	39.5	42.7
A2/C4	69.1	67.8	47.2
A2/D2	71.4	39.4	50.5
A2/D3	70.9	56.4	55.5
A2/D4	64.0	44.1	55.7
B2/B4	65.5	60.9	58.5
B2/C3	73.0	70.6	46.6
B2/C4	68.0	55.3	38.6
B2/D2	66.5	67.8	51.8
B2/D3	69.9	66.7	53.8
B2/D4	68.9	73.2	61.1
B4/C3	73.0	66.4	54.4
B4/C4	75.1	70.6	52.2
B4/D2	74.3	60.2	59.0
B4/D3	70.4	59.1	40.2
B4/D4	65.6	64.2	52.8
C3/C4	78.8	55.1	27.7
C3/D2	74.6	63.0	59.7
C3/D3	83.1	51.4	47.1
C3/D4	72.4	70.4	64.9
C4/D2	73.5	51.3	46.4
C4/D3	73.3	57.1	34.9
C4/D4	63.1	54.3	31.9
D2/D3	79.0	54.1	49.4
D2/D4	70.7	65.2	61.5
D3/D4	78.5	65.9	47.1

ly determined its infestation with weeds. At the tillering stage (before treatments with herbicides) barley grown on the field after potato in four-field crop rotations A and B was infested by a significantly lower number of weeds than in other positions. However, in crop rotations with a 50 and 75% share of barley, growing barley without an intercrop (crop rotations C and D), after spring wheat (four-field crop rotation B), and after potato (system D) resulted

in increased weed infestation. The lower weed infestation of barley grown after potato was associated with the use of well-decomposed manure in autumn and intensive soil management on that site (harrowing, cultivating, earthing), which destroyed the emerging weeds, thus reducing the size of the soil seed bank. The positive role of potato in reducing weed infestation of following crops was also emphasized by JASTRZĘBSKA et al. (2012) and of other root crops by MAJCHRZAK and PIECHOTA (2013).

Growing spring barley after itself and after spring wheat significantly increased weed infestation. Such a crop sequence promotes the emergence of weeds that have a developmental cycle similar to spring cereals (ZAWIŚLAK and SADOWSKI 1992). Moreover, it causes negative changes in the soil system that weaken the growth of the crop. This is reflected in a thinner canopy, and shorter plants with weaker foliage, which creates favourable conditions for the development of weeds producing greater numbers of seeds enlarging their soil bank (CAVERAS and BENOIT 1989, RIEMENS at al. 2007, ROBERTS 1981). In crop rotation D, higher weed infestation of barley grown after potato indicates that despite the positive effect of potato in other cropping systems reducing the infestation of barley with weeds, the one-year break after three seasons of growing barley on the same field was insufficient. Different findings were made by KOSTRZEWSKA and WANIC (2005), who reported no effect of growing barley without an intercrop in crop rotations systems with a 75% share of cereals on the number of weeds in the barley canopy during spring.

The use of herbicides, combined with the competitive effect of barley clearly reduced the number of weeds at the end of the growing season, eliminating the differences resulting from the crop position in the rotation system in most sites. DERKSEN et al. (1995) concluded that the use of herbicides has a stronger effect than the share of species in crop rotation. WOŹNIAK (2004) also found that weeding methods can significantly affect the density and biomass of weeds. In the analysed experiment higher weed infestation was only found for barley grown after spring wheat (four-field crop rotation B) and after barley following wheat (C). Greater weed biomass was also noted on site C. Importantly, growing barley after itself and a 75% share of barley in crop rotation was not associated with a significant increase in weed biomass, which was comparable to that in the control crop rotation, where barley followed potato. JASTRZĘBSKA et al. (2012) reported that the weed biomass was lower in barley grown after potato, and was higher in crop rotation with a 75% share of barley grown continuously on the same site for 3 seasons. Similar findings on the role of potato as a previous crops in limiting weed biomass were reported by KOSTRZEWSKA et al. (2011) and ORZECH and WANIC (2014).

In our experiment the position of barley in crop rotation did not significantly differentiate the species richness of weed communities. A slightly greater

number of taxa was recorded only in crop rotation B, where barley was grown after spring wheat. MAJCHRZAK and PIECHOTA (2013) also concluded that the position in crop rotation has no significant effect on the number of weed species. The minor effect of crop rotation on the number of weed species in the barley canopy was also reported by LÈGÈRE et al. (2005). According to ZAWIŚLAK and SADOWSKI (1992), the sequence of cereals (including spring barley) has a stronger differentiating effect on the dominance of weeds than their species richness.

In the analysed communities the dominant species were typical spring annual and biennial weeds characteristic for spring cereals: *Thlaspi arvense*, *Chenopodium album*, *Fallopia convolvulus* and *Veronica arvensis,* and also *Galinsoga parviflora* at the end of vegetation. A greater share of perennial weeds (particularly *Equisetum arvense* and *Agropyron repens*) was also noted at the end of vegetation. Generally, the presented results are consistent with those reported by other authors. JASTRZĘBSKA et al. (2012) and KOSTRZEWSKA et al. (2011) indicated *Chenopodium album* as the dominant weed in the barley canopy. Similar conclusions were reached by ORZECH and WANIC (2014).

In our study the diversity index (H') was similar in spring and at the end of vegetation, regardless of the herbicide treatments. However, LÈGÈRE et al. (2005) demonstrated reduced values of this index when intensive agronomic methods were used. Contrasting findings were made by WILSON et al. (2003), who stated that data on the significant effects of herbicides on the diversity of weed communities are either limited or lacking, as demonstrated in our study.

In the presented study the values of the evenness index were moderate (0.72–0.90), suggesting a slight dominance of species in weed communities (LÈGERÈ et al. 2005).

Potato as a previous crops in crop rotation with a 25% share of barley caused a slight increase in the dominance and a decrease in the diversity and evenness of species in weed populations (calculated based on the population size). Before harvest the highest diversity in the population size was found in the crop rotation with a 75% share of barley grown without an intercrop. KOSTRZEWSKA and WANIC (2005) also found greater dominance and lower evenness of distribution for individual weed species in a crop rotation with a 50% share of barley on the site after potato. Moreover, KOSTRZEWSKA et al. (2011) documented minor differences in the diversity of weed species in spring barley as the effect of previous crops, while MAJCHRZAK and PIECHOTA (2013) demonstrated that a previous crops has no significant effect on the values of diversity and dominance indices. LÈGERÈ et al. (2005) found no significant effects of crop rotation on species diversity, but reported a clear trend towards reduced diversity in a monoculture and increased diversity in a crop rotation system, which was also proven by STEVENSON et al. (1997).

Conclusions

1. At the tillering stage (before herbicide treatment) the lowest weed infestation was found on fields where spring barley was grown after potato in crop rotations with 25 and 50% shares of barley. Growing barley after spring wheat and without an intercrop, and a 75% share of barley in crop rotation promoted the emergence of weeds.

2. At the end of vegetation the number of weeds was 45–79% lower than in spring. The highest weed infestation was found in a four-field crop rotation with a 25% share of barley, and on fields after spring barley and spring wheat in crop rotations with a 75% share of barley.

3. Crop rotation had no effect on the species richness of weed communities. Lower species diversity and evenness and higher species dominance were found in weed communities on fields where barley was grown after potato in crop rotation with a 25% share of barley and after barley grown twice after itself in a crop rotation with a 75% share of barley.

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INFLUENCE OF APPLICATION MINERAL SORBENTS IN SOIL CONTAMINATED WITH NICKEL ON THE CONTENT OF SOME ELEMENTS IN INDIAN MUSTARD

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K e y w o r d s: halloysite, Indian mustard, macroelements, Ni-contamination, zeolite.

A b s t r a c t

The effects of increasing nickel contamination of soil on selected macroelement uptake by Indian mustard (*Brassica juncea* L. Czern) and application of natural zeolite, raw and modified halloysite were investigated in this experiments. In a vegetative-pot experiment, four different level of nickel contamination, i.e., 0 (control), 80, 160, 240, 320 $mg \cdot kg^{-1}$ were applied in an analytical grade $NiSO_4 \cdot 7H_2O$ solution mixed thoroughly with the soil. The content of nitrogen, phosphorus, sodium, calcium, potassium and magnesium in Indian mustard depended on the dose of nickel and type of neutralizing substance. The average accumulation of tested elements in Indian mustard grown in nickel contaminated soil were found to follow the decreasing order Na>P>Ca>Mg>K>N. The application of MH turned out to be most advantageous, resulting in a small increase in the average content of Na and Mg. Addition of RH and NZ led to the highest increase in the average content of the P.

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WPŁYW DODATKU SORBENTÓW MINERALNYCH DO GLEBY ZANIECZYSZCZONEJ NIKLEM NA ZAWARTOŚĆ WYBRANYCH MAKROELEMENTÓW W GORCZYCY SAREBSKA

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S ł o w a k l u c z o w e: haloizyt, gorczyca sarepska, zeolit, zanieczyszczenie niklem, makroelementy.

A b s t r a k t

Przedmiotem badań było określenie wpływu wzrastającego zanieczyszczenia gleby niklem oraz dodatku naturalnego zeolitu, modyfikowanego i surowego haloizytu na zawartość wybranych makroelementów w gorczycy sarebska (*Brassica juncea* L. Czern.). W doświadczeniu wazonowym zastosowano cztery wzrastające dawki niklu 0 (kontrola), 80, 160, 240, 320 $mg \cdot kg^{-1}$ wprowadzonych w formie związku $NiSO_4 \cdot 7H_2O$ cz.d.a, który wymieszano z glebą. Zawartość azotu, fosforu, sodu, wapnia, potasu i magnezu w gorczycy sarepskiej zależała od wielkości dawki niklu oraz typu substancji neutralizujących. średnia zawartość badanych makroelementów w gorczycy sarepskiej rosnącej na glebie zanieczyszczonej niklem przyjmowała następującą kolejności Na>P>Ca>Mg>K>N. Aplikacja MH okazała się najbardziej korzystna, powodując wzrost średniej zawartości Na i Mg. Dodanie RH i NZ wywołały największy wzrost średniej zawartości w przypadku P.

Introduction

Nickel (Ni) has two main oxidation states (+2 and +3) and five natural isotopes. Especially dangerous for living organisms as well as plants is the cation Ni^{2+}, occurring in many salts of mineral and organic acids. Nickel has been listed as a priority control pollutant by the United States Environmental Protection Agency (US EPA), and is present in plant tissues in the range of 0.05–10 $\mu g \cdot g^{-1}$ of dry matter (JAIME et al. 2012, Singh and PRASAD 2015). This element is sorbed by hydrated Al and Fe oxides, organic substance and clay minerals, which results in its accumulation in soil. What is more, nickel from a natural source, in contaminated soils, is less soluble and moves less readily than the remaining elements. Mineral fertilizers, especially phosphorus ones, organic fertilizers, compost, organic waste, mineral waste used to lime soils can be a source of nickel in the natural environment; moreover, volcanic eruptions and wind-blown dust also play a role (BUEKERS et al. 2015, ESTRADE et al. 2015). Additionally, nickel can also be derived from anthropogenic activities, e.g. metal mining, vehicle emissions, industrial wastes, paint manufacturing,

coal combustion (ELOUEAR et al. 2009, VAVERKOVÁ and ADAMCOVÁ 2014, SCHORNÍK et al. 2015).

Another effect of the harmful influence of nickel are disturbances in the proportions of the chemical composition of plants, which can lead to the excessive, toxic accumulation of this element and reduction in the general sum of cations in plant tissues and an increase in the amount of calcium and phosphorus (RADZIEMSKA et al. 2013).

The effectiveness of phytoremediation methods largely depends on the choice of an appropriate plant species, characterized by a high tolerance and high accumulation capability of heavy metals in its mass (LIU et al. 2010). *B. juncea* (L.) is a high biomass crop species – at least 10 times higher than other hyper accumulators, able to tolerate and accumulate high concentrations of potentially toxic trace elements (NOVO and GONZÁLEZ 2013, NOVO et al. 2013a, 2013b, KARAK et al. 2013). MINGLIN et al. (2005) reported that it is the most promising and interesting plant model for phytoremediation, as it accumulates more than 400 $\mu g \cdot g^{-1}$dry weight (DW) of cadmium in its shoot. *B. juncea* (L). might also be a plant suitable for phytoremediation of Pb-contaminated soils and waters in spite of high mercury phototoxicity (SHIYAB et al. 2009).

The present research objective was to investigate the effects of mineral sorbents, i.e. raw halloysite (RH), modified halloysite (MH), and natural zeolite (NZ) on the content of selected elements in above-ground parts of Indian mustard *B. juncea* (L.) grown in Ni-contaminated soil.

Materials and Methods

Experimental system description

The experiment was assessed under the conditions of a pot experiment in an acclimatized greenhouse, with two factors and fourfold replication. The first factor was the addition of increased doses of Ni to soil (0, 80, 160, 240, and 320 $mg \cdot kg^{-1}$), introduced in the form of chemically pure aqueous solutions of nickel sulphate heptahydrate ($NiSO_4 \cdot 7H_2O$) (Sigma-Aldrich). The second factor consisted of the addition of three mineral adsorbents, i.e., raw halloysite, modified halloysite, and natural zeolite (3.0% w/w). Soils without nickel and amendments (0.0%) were designated as the control. Non-polluted soil for the pot experiment were collected at a depth of 0–20 cm from farmland in the vicinity of Olsztyn, Poland, (53°35'45''N, 19°51'06'E). The chemical properties of the soil are shown in Table 1. The soil was air-dried, passed through a 1-cm sieve and packed into 20-cm-diameter and 26-cm-height experimental pots

Table 1

Physical and chemical parameters of the experimental soil

Soil chemical parameters	
pH	4.80
Hydrolytic acidity (mmol · kg^{-1})	33.75
Sum of exchangeable bases Ca^{++}, Mg^{++}, K^{+}, Na^{+} (mmol · kg^{-1})	62.20
Cation exchange capacity (mmol · kg^{-1})	95.95
Base saturation (%)	64.80
Organic matter	
Organic carbon (g · kg^{-1})	7.13
Total N (g · kg^{-1})	1.04
Carbon:Nitrogen	6.85
N-NH_4^+ (mg · kg^{-1})	21.18
N-NO_3^- (mg · kg^{-1})	9.88
Soil texture (%)	
Fractions 2.0–0.05 mm	86.6
Fractions 0.05–0.002 mm	11.2
Fractions 0.002 mm	2.2
Trace metal (mg kg^{-1})	
Nickel	4.05
Copper	8.49
Chromium	10.95
Zinc	24.21
Lead	16.33
Manganese	210.9
Available forms (mg kg^{-1})	
Phosphorous	46.6
Potassium	8.20
Magnesium	33.9

(10 kg soil per pot); it was then used for physical and chemical analysis, as well as N, P, K, Mg, Ca, Na concentration analysis.

The polyethylene pots were maintained under natural day/night conditions; during the day (14h), the air temperature was 26±3°C and approximately ten degrees lower (16±2°C) at night (10h), with a relative humidity of 75±5%. The plants were watered every other day with distilled water to 60% of the maximum water holding capacity of the soil. The plants were harvested after 100 days, and soil and sorbents were collected.

The seeds of *B. juncea* (L.) cv. Małopolska, were obtained from an authorized Seed Production Centre in Olsztyn, Poland (OLZNAS-CN Sp. z o.o.), and were planted at the quantity of $n=5$ per pot. Soil was fertilized with a macro- and micronutrient fertilizer mixture (g kg^{-1}) containing N-26%, K_2O-26%, B-0.013%, Cu-0.025%, Fe-0.05%, and Mn-0.025%. The above-part of Indian mustard was harvested in the flowering phase and plant material samples were collected for laboratory tests.

Sample preparation and element content analysis

In the laboratory, plant samples were thoroughly rinsed, first with tap water and then with deionized water to remove dust and soil particles. After oven drying (60°C, 48h) the plants were weighed (DW) and powdered using an analytical mill (A11 IKA, Germany) preceding the chemical analyses. The samples were kept at an ambient temperature until analysis. All reagents were of analytical reagent grade unless otherwise stated. Ultra-pure (UP) water (Millipore System, USA) of 0.055 $\mu S \cdot cm^{-1}$ resistivity was used for preparing the solutions and dilutions.

Total nitrogen content was tested for by means of Kjedahl's method after mineralization in concentrated sulfuric (VI) acid using hydrogen peroxide as a catalyst (BREMNER 1965). Phosphorus (P) was assessed by colorimetric analysis, using the vanadium-molybdenum method (Cavell 1955); sodium (Na), calcium (Ca), potassium (K) – atomic emission spectrometry, AES method (SZYSZKO 1982), magnesium (Mg) – atomic absorption spectrometry, AAS method (SZYSZKO 1982).

Statistical analysis

Statistical analysis was performed using the software Statistica (StatSoft, 2010). Differences of means between treatments were tested by ANOVA and comparisons of means using LSD test, at $p=0.05$. The means and standard deviations (±SD) of five replications are reported.

Results and Discussion

In the presented research, nitrogen (N) content in the above-ground plant mass of *B. juncea* (L.) was determined by the dose of nickel as well as the addition of natural zeolite (NZ) as well as raw (RH) and modified (MH) halloysite (Table 2). In the control series (without additives), the differences in nitrogen content were additionally correlated with the increasing doses of this element. Soil contamination amounting to 320 mg $Ni \cdot kg^{-1}$ soil caused the highest increase in nitrogen in the analyzed plant. Studies by Karimi et al. (2003) also indicated that the nitrogen concentration of *Vicia faba* L. and *Brassica arvensis* L. was not reduced by nickel at these levels, in contrast to previous studies conducted by ARDUINI et al. (2006). In the presented studies, all of the immobilizing additives applied in the experiment (NZ, RH and MH) induced an increase in the nitrogen content of above-ground parts of Indian

mustard plants. However, this effect was most significant in the case of the raw halloysite (RH) additive. Modified halloysite (MH) in soils with a dose of 320 mg Ni · kg^{-1} soil resulted in an over threefold increase in the nitrogen content of *B. juncea* (L.) as compared to the group to which neutralizing substances had not been applied. In an experiment conducted by WYSZKOWSKI and RADZIEMSKA (2010), the applied contamination alleviating substances (especially natural zeolite) stimulated an increase in the total nitrogen content, more evidently in *Zea mays* L. than in *Lupinus luteus* L. In another study carried out by WYSZKOWSKI and RADZIEMSKA (2013), zeolite and CaO generally caused concentrations of nitrogen compounds to increase in oat grain and straw, in contrast to the roots, where they were typically lower. The average share of nitrogen in the analyzed macronutrients increased from 30.62% (control) to 48.23% (320 mg Ni · kg^{-1} soil) (Fig. 1). Differences between the mineral sorbents and the control were not noted at the same dose of Ni ($P<0.05$).

Table 2
Effect of nickel and various mineral sorbents on N, P, K, Ca, Na and Mg concentration in Indian mustard, (g · kg^{-1} dry mass)

Treatment		Nickel concentration (mg · kg^{-1})					Mean
		0	80	160	240	320	
N	Control	11.44 ± 0.81^{a}	13.48 ± 1.15^{a}	19.40 ± 2.32^{a}	25.31 ± 3.91^{a}	29.1 ± 5.42^{a}	19.75
	NZ	10.54 ± 0.72^{a}	12.73 ± 1.82^{a}	17.92 ± 1.99^{a}	25.74 ± 4.08^{a}	30.1 ± 6.10^{a}	19.41
	RH	11.05 ± 1.04^{a}	13.24 ± 1.52^{a}	18.51 ± 2.81^{a}	27.32 ± 4.44^{a}	32.1 ± 6.04^{a}	20.44
	MH	11.32 ± 0.78^{a}	13.44 ± 1.15^{a}	18.46 ± 2.45^{a}	26.23 ± 4.08^{a}	31.0 ± 5.79^{a}	20.09
P	Control	3.66 ± 0.21^{a}	3.79 ± 0.32^{a}	4.12 ± 0.41^{a}	4.48 ± 0.47^{a}	3.66 ± 0.54^{a}	3.94
	NZ	3.82 ± 0.19^{a}	3.81 ± 0.38^{a}	4.05 ± 0.43^{a}	4.66 ± 0.46^{a}	4.51 ± 0.61^{a}	4.17
	RH	3.18 ± 0.15^{b}	3.98 ± 0.42^{a}	4.01 ± 0.45^{a}	4.73 ± 0.44^{a}	4.02 ± 0.77^{a}	3.98
	MH	3.28 ± 0.22^{b}	3.32 ± 0.29^{b}	3.89 ± 0.33^{a}	4.44 ± 0.39^{a}	4.29 ± 0.68^{a}	3.84
K	Control	12.52 ± 0.65^{a}	14.11 ± 0.73^{a}	17.56 ± 1.46^{a}	20.04 ± 1.52^{a}	21.05 ± 2.06^{a}	17.06
	NZ	13.11 ± 0.96^{a}	15.08 ± 1.01^{a}	18.11 ± 1.14^{a}	20.52 ± 1.43^{a}	16.61 ± 1.75^{b}	16.69
	RH	13.62 ± 1.11^{a}	15.31 ± 1.32^{a}	19.62 ± 1.05^{b}	21.14 ± 1.52^{a}	19.62 ± 2.00^{a}	17.86
	MH	12.94 ± 0.99^{a}	14.86 ± 1.22^{a}	17.98 ± 0.99^{a}	18.49 ± 1.48^{a}	18.90 ± 1.93^{a}	16.63
Ca	Control	3.92 ± 0.24^{a}	4.03 ± 0.32^{a}	3.86 ± 0.40^{a}	4.08 ± 0.32^{a}	3.71 ± 0.33^{a}	3.92
	NZ	3.97 ± 0.33^{a}	3.98 ± 0.41^{a}	4.00 ± 0.44^{a}	4.14 ± 0.42^{a}	4.24 ± 0.49^{b}	4.07
	RH	3.59 ± 0.25^{a}	3.38 ± 0.32^{b}	3.78 ± 0.45^{a}	4.12 ± 0.54^{a}	4.28 ± 0.55^{b}	3.83
	MH	3.73 ± 0.21^{a}	3.39 ± 0.29^{b}	3.89 ± 0.33^{a}	4.14 ± 0.49^{a}	4.24 ± 0.41^{b}	3.88
Na	Control	1.32 ± 0.11^{a}	1.46 ± 0.16^{a}	1.60 ± 0.20^{a}	1.51 ± 0.22^{a}	1.73 ± 0.21^{a}	1.52
	NZ	1.20 ± 0.17^{b}	1.44 ± 0.27^{a}	1.59 ± 0.27^{a}	$1.70 \pm 0.36^{a,b}$	1.73 ± 0.31^{a}	1.53
	RH	1.32 ± 0.12^{a}	1.48 ± 0.25^{a}	1.51 ± 0.32^{a}	1.50 ± 0.27^{a}	1.64 ± 0.40^{a}	1.49
	MH	1.29 ± 0.19^{a}	1.40 ± 0.31^{a}	1.43 ± 0.29^{a}	1.82 ± 0.24^{b}	1.80 ± 0.31^{a}	1.55
Mg	Control	3.32 ± 0.11^{a}	3.03 ± 0.32^{a}	3.01 ± 0.41^{a}	3.15 ± 0.57^{a}	3.06 ± 0.47^{a}	3.11
	NZ	3.45 ± 0.19^{a}	3.51 ± 0.43^{a}	3.61 ± 0.53^{b}	3.60 ± 0.46^{a}	3.91 ± 0.61^{b}	3.62
	RH	3.55 ± 0.35^{a}	3.72 ± 0.48^{a}	3.72 ± 0.55^{b}	3.57 ± 0.68^{a}	4.02 ± 0.83^{b}	3.72
	MH	3.66 ± 0.36^{a}	3.62 ± 0.29^{a}	3.86 ± 0.46^{b}	3.60 ± 0.59^{a}	4.05 ± 0.61^{b}	3.76

Mean values for five samples (±standard deviation) are shown. Data within columns that do not have common indices are significantly different at $p<0.05$

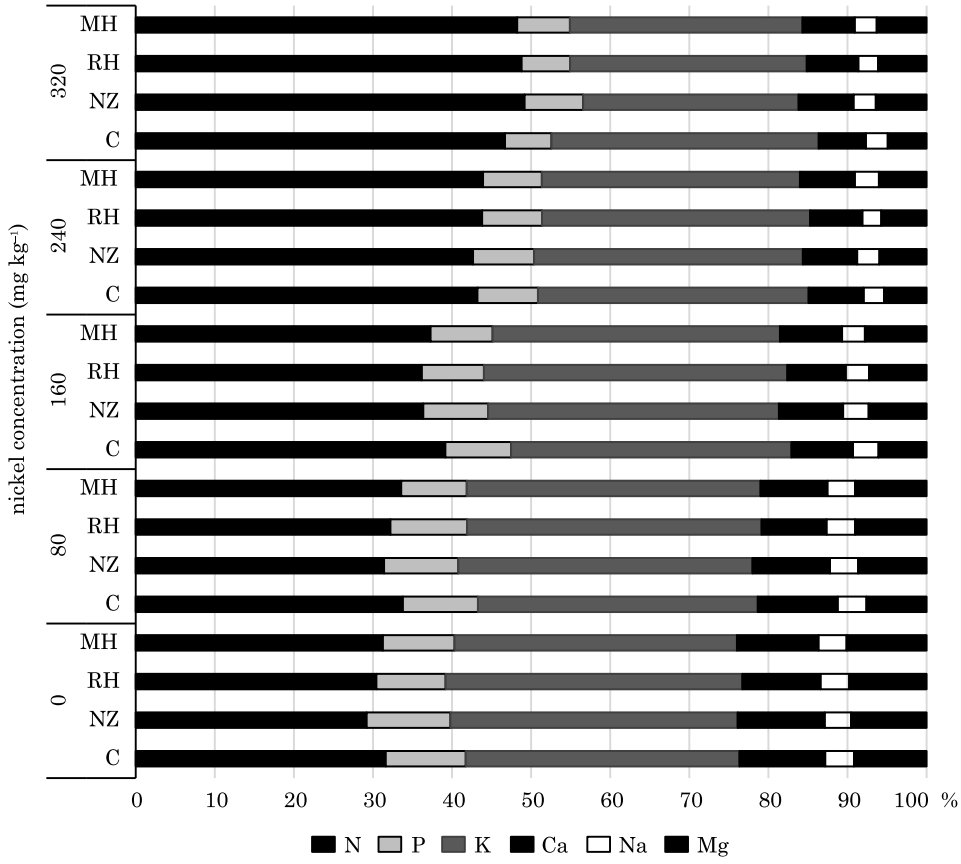

Fig. 1. The average percentage share of N, P, K, Ca, Na and Mg in above-ground parts of Indian mustard cultivated in Ni-polluted soil

Phosphorus content (P) in the above-ground parts of Indian mustard was significantly influenced by: the dose of soil contamination with nickel as well as the mineral substances in the form of natural zeolite, and raw and modified halloysite (Table 2). In the series without neutralizing additives, increasing doses of Ni only insignificantly influenced phosphorus content in the analyzed plant. Contamination of soil at 240 mg Ni · kg^{-1} soil caused the highest increase in phosphorus content (+22%) in *B. juncea* (L.). The addition of raw halloysite (+13%) and natural zeolite (9%) led to the highest increase in the average content of the analyzed element in relation to pots without neutralizing additives. In another study of RADZIEMSKA et al. (2013), modified halloysite in crops subjected to doses of 160 and 240 mg Ni · kg^{-1} soil led to a nearly twofold increase in the phosphorus content of *Zea mays* L. in relation to plants without the addition of neutralizing substances. The average percentage share of P in

the total of the analyzed macronutrients decreased along with the nickel content of soil, ranging from 9.63-6.52% (Fig. 1). The increase in the concentration of phosphorus in plants contaminated with nickel was confirmed by studies of MATRASZEK et al. (2002) and KARIMI et al. (2013).

The application of nickel to soil on the whole led to increased potassium contents in plants as compared to the control series – without alleviating substances (Table 2). An almost twofold increase in K concentration was confirmed in *B. juncea* (L.) grown in pots with the highest dose of Ni (320 mg · kg^{-1}). Research by PALOCIS et al. (1998) confirm increased contents of potassium in tomatoes under the influence of nickel contamination. In the presented studies, among the substances added to the soil to alleviate nickel contamination, raw halloysite (RH) turned out to be the best, leading to a 31% increase in the average content of the described element in the test plant as compared to the control series. An analogical situation was observed in the case of natural zeolite (NZ) and modified halloysite (MH) additives, although their influence was smaller. In soils contaminated at the level of 240 and 320 mg Ni · kg^{-1} with the addition of MH, the content of the analyzed element in Indian mustard plants was approximately 10% lower than in plants grown in soil free of contamination and additives. The percentage share of potassium in the sum of N, P, K, Ca, Mg and Na was similar and ranged from 36.94–36.72% with the exception of the group with the highest dose of Ni in soil, where its share was lower, i.e. 30.08% (Fig. 1). The variable influence of the different doses of Ni in soil on K content in plants was reported by MATRASZEK et al. (2002) and KARIMI et al. (2013).

The Ni dose as well as the addition of NZ, RH and MH shaped Ca content in Indian mustard, (Table 2). In the series lacking additives (control) and increasing nickel contamination, calcium content in the tested plant was not characterized by a clear direction of values. In this series, plants grown in soil contaminated by nickel at a level of 240 mg Ni · kg^{-1} was characterized by the highest calcium content. Studies of CROOKE (1955) and PALOCIS et al. (1998) also confirm the stated dependency, observing an increase in Ca content in the biomass of oats under the influence of increasing doses of nickel. The carried out studies indicate that using substances (NZ, RH and MH) that alleviate nickel contamination had an influence on the average calcium content in the above-ground mass of Indian mustard. The listed additives had the highest influence in the case of study groups containing the highest doses of nickel. In relation to the control group, the application of RH and MH turned out to be most beneficial, leading to an 8% increase in average calcium content in the test plant. The average percentage share of calcium decreased along with the increase in Ni concentration in soil and ranged from (0 mg Ni · kg^{-1}) to 6.51% (320 mg Ni · kg^{-1} soil) (Fig. 1). A decrease in the percentage share of Ca in the

leaves of plants in the *Brassicaceae* family under the influence of nickel was confirmed by PUTNIK-DELIĆ et al. in their studies (2014).

Based on the carried out studies, it turns out that using alleviating substances (natural zeolite, raw and modified halloysite), the contamination of soil with nickel had a significant influence on Na content in the above-ground mass of Indian mustard, (Table 2). In the control series (without additives), a positive correlation between the sodium content of the tested plant and increasing soil contamination with Ni occurred. The alleviating substances used in the experiment had a beneficial effect on the average sodium content in Indian mustard plants. Among the substances alleviating nickel contamination added to soil, the application of modified halloysite (MH) turned out to be most advantageous, resulting in a small increase in the average content of Na. The average percentage share of this element ranged from 2.71% in the group with a dose of 240 mg $Ni \cdot kg^{-1}$ soil, to 3.54% in group 0 (Fig. 1). Insignificant percentage changes in Na content in the sum of macroelements (N, P, K, Ca, Mg and Na) in the leaves of wild mustard (*Brassica arvensis* L.) growing on soil contaminated with various doses of nickel were obtained by KARIMI et al. (2013).

Natural and modified halloysite, and zeolite, as well as increasing doses of nickel, significantly affected the magnesium content of Indian mustard, (Table 2). Nickel can displace Mg from chrolorphyll and enzymes (FURINI 2012). The content of Mg in research by PUTNIK-DELIĆ et al.(2014) increased in several brassica species tested under conditions of excessive Ni contamination. The content of Mg in the described plant in the control series – without alleviating additives, was negatively correlated with increasing doses of nickel. The opposite dependency was noted by MATRASZEK et al. (2002), in whose studies magnesium content in the leaves of spinach increased along with the influence of increasing doses of nickel. On the other hand, studies by PALOCIS et al. (1998) prove that Mg content in oats under the influence of increasing doses of nickel also exhibited a decreasing tendency, as in the present research. Applying alleviating substances had a positive effect on average magnesium content in above-ground parts of *B. juncea* (L.). The addition of modified halloysite (MH) was found to have the most beneficial effect, leading to the highest increase in the average magnesium content of the test plants as compared to the control series. Row halloysite (RH) and natural zeolite (NZ) also had a positive effect, although to a lesser degree. The lowest average percentage share of magnesium (5.77%) in the sum of analyzed macronutrients in the above-ground parts of mustard was noted in the group treated with a 240 mg $Ni \cdot kg^{-1}$ soil dose of nickel, whereas the highest (9.66%) occurred in the control group (Fig. 1). A changeable percentage share of Mg, depending on the part of the plant, the plant species and Ni concentration of soils, was noted by KARIMI et al. (2013) and PUTNIK DELIĆ et al. (2014) in their research.

Conclusions

The content of macroelements (N, P, Na, Ca, K and Mg) in Indian mustard depended on the dose of the nickel contaminant and the application of alleviating substances incorporated into the soil. In the control series (without the addition of natural zeolite, row halloysite, modified halloysite), the differences in nitrogen, phosphorous, potassium, and sodium content were positively correlated with the increasing doses of Ni-contamination. Soil with 320 mg of Ni per 1 kg of soil led to the highest increase in nitrogen, phosphorous, potassium and mgnesium content in the above-ground parts of Indian mustard. The application of modified halloysite (MH) turned out to be most advantageous, resulting in a small increase in the average content of sodium and magnesium. Moreover, the addition of raw halloysite (RH) and natural zeolite (NZ) led to the highest increase in the average content of the phosphorus, in relation to pots without neutralizing additives.

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THE YIELD AND GRAIN QUALITY OF WINTER RYE (*SECALE CEREALE L.*) UNDER THE CONDITIONS OF FOLIAR FERTILIZATION WITH MICRONUTRIENTS (Cu, Zn and Mn)

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K e y w o r d s: foliar fertilization, nutrients, protein, starch, yield components.

A b s t r a c t

The grain of winter rye (*Secale cereale L.*) originated from a field experiment conducted in the years 2011–2013 at the Didactic-Experimental Centre of the University of Warmia and Mazury in Tomaszkowo (53°72 N; 20°42 E) in Poland. The aim of the study was to determine the yield of winter rye and its components and the content of proteins, starch and selected mineral components (P, K, Ca, Mg, Cu, Fe, Zn, Mn) in grain under the conditions of NPK fertilization and foliar feeding with micronutrients applied separately or in combination. Fertilization with mineral fertilizers (NPK) and with mineral fertilizers with micronutrients applied separately or in combination resulted in a significant increase in grain yield, as compared to the plot with no fertilization. Fertilization with mineral fertilizers and foliar feeding with micronutrients (except for Cu) increased protein content in rye grain. Foliar application of nitrogen of basic fertilization with copper increased the concentration of starch in the grain. The mineral fertilization (NPK) with micronutrients applied in combination (NPK+Cu, Zn, Mn) increased the content of phosphorus (as compared to the plot with no fertilization and fertilized with mineral fertilizers NPK) and potassium (as compared to the NPK plot). Supplementation of basic fertilization with zinc or manganese or a combination of micronutrients resulted in an increase in Mn content in the grain as compared to the plot with no fertilization and as compared to the plot fertilized with mineral fertilizers.

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PLONOWANIE I JAKOŚĆ ZIARNA ŻYTA OZIMEGO (*SECALE CEREALE L.*) W WARUNKACH DOLISTNEGO NAWOŻENIA MIKROELEMENTAMI (Cu, Zn i Mn)

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Słowa kluczowe: nawożenie dolistne, składniki pokarmowe, białko, skrobia, składowe plonu.

Abstrakt

Ziarno żyta ozimego (*Secale cereale L.*) pochodziło z doświadczenia polowego przeprowadzonego w latach 2011–2013 w Zakładzie Dydaktyczno-Doświadczalnym UWM w Tomaszkowie (53°72 N; 20°42 E) Polska. Celem pracy było określenie plonu żyta ozimego i jego komponentów oraz zawartości białka, skrobi i wybranych składników mineralnych (P, K, Ca, Mg, Cu, Fe, Zn, Mn) w ziarnie pod wpływem dolistnego dokarmiania mikroelementami stosowanymi pojedynczo lub łącznie. Pod wpływem nawożenia nawozami mineralnymi (NPK) oraz mineralnie łącznie z mikroelementami stosowanymi pojedynczo lub łącznie stwierdzono istotny wzrost plonu ziarna względem obiektu bez nawożenia. Nawożenie nawozami mineralnymi oraz dolistne dokarmianie mikroelementami (za wyjątkiem Cu) zwiększyło zawartość białka w ziarnie żyta. Dolistne dokarmianie azotem i miedzią zwiększyło koncentrację skrobi w ziarnie. Wspomaganie nawożenia NPK mikroelementami stosowanymi łącznie (NPK+Cu, Zn, Mn) zwiększyło zawartość fosforu (w porównaniu z obiektem bez nawożenia i nawożonym nawozami mineralnymi NPK) oraz potasu (względem obiektu NPK). Uzupełnienie podstawowego nawożenia cynkiem lub manganem lub łącznie mikroelementami wpłynęło na wzrost zawartości Mn w ziarnie względem obiektu bez nawożenia i w porównaniu z obiektem nawożonym nawozami mineralnymi.

Introduction

Winter rye (*Secale cereale L.*) is a cereal whose grain is used mainly for human consumption (BUSHUK 2001). Therefore, the content of micronutrients and macronutrients in the grain is as important as the yield (BEDNAREK et al. 2006, KOWIESKA et al. 2011). In the agronomical practises of cereals the main factors which allow for the achievement of high yield of plants having favourable quality properties include the level of agronomical practises, habitat conditions and genetic determinants of the varieties (JANKOWSKI et al. 2003, HANSEN et al. 2004, NEDZINSKIENE 2006, JASKULSKI and JASKULSKA 2009, CIOROMELE and CONTOMAN 2015). It is suggested that the yield and the nutritional and technological value of the grain is determined by satisfying nutritional needs by fertilization (STĘPIEŃ and WOJTKOWIAK 2015). The dose and form of fertilizer components and the method of fertilization are very

important. The treatment of intervention foliar feeding of plants with micronutrients at the time of critical demand for nutrients is applied more and more frequently (FEGARIA et al. 2009). Foliar application of fertilizers is recommended in the phase of shooting when the plant is in a period of intensive cell divisions. In practice, three most important elements considered during feeding of cereals are: Mn, Cu and Zn. These micronutrients are involved in many physiological processes, including their presence in the composition of various enzymes and their role as enzyme activators (HANSCH and MENDEL 2009). There elements also affect the effectiveness of fertilization with macronutrients and influence the yield and chemical composition of the grain. The economic and environmental reason for the foliar application of certain nutrients is also their high productive efficiency (JASKULSKI and JASKULSKA 2009).

The effect of fertilization with nitrogen fertilizers on the quality characteristics of the plants was a subject of many studies; however the effect of nitrogen together with foliar application of micronutrients is less investigated.

The aim of the study was to determine the yield and its components, the content of proteins, starch and selected mineral components (P, K, Ca, Mg, Cu, Fe, Zn, Mn) in grain of winter rye under the conditions NPK fertilization and of foliar feeding with micronutrients applied separately or in combination.

Materials and Methods

The field experiment was conducted at the Didactic-Experimental Centre in Tomaszkowo (53°72 N; 20°42 E) in Poland. Winter rye (*Secale cereal L.*) of the Dańkowskie Diament variety was cultivated in 2011/2012 and 2012/2013 growing seasons on a lessive soil with a granulometric composition of a medium silty loam of complex 4, class IIIb. The soil characteristics presented in the Table 1. The experiment was established using a method of randomised blocks, in triplicate. The plot size was 6.25 m^2, the harvested plot area was 4.0 m^2. Winter rye was cultivated after winter triticale, sowing 160 kg ha^{-1} in 2011 and 172 kg ha^{-1} in 2012 (5.00 million grains ha^{-1}), spacing between plant rows of 120 mm.

In the experiment, the following variants of fertilization were taken into account:

1. „Without fertilization" (control I).
2. „NPK" (control II) – At all sites, nitrogen fertilization was applied at an amount of 90.0 kg · ha^{-1} with the doses divided as follows: in-soil application of 54.0 kg · ha^{-1} (urea 46%) at the tillering stage (at Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie scale: BBCH 22–23), and foliar

Table 1
Physical and chemical soil properties before the experiment started (average 2011–2012)

Measured parameters	Corresponding values
Soil type (WRB 2014)	Haplic Cambisol
Soil texture	loam
pH in KCl	6.89
Total organic C, g kg^{-1} DM	7.93
Total N, g kg^{-1} DM	0.95
P, mg kg^{-1}	76.4
K, mg kg^{-1}	195.1
Mg, mg kg^{-1}	74.0
Mn, mg kg^{-1}	214
Cu, mg kg^{-1}	2.8
Zn, mg kg^{-1}	9.0
Fe, mg kg^{-1}	1700

application of 36.0 kg N ha^{-1} (10% urea solution) at the stem elongation stage (BBCH 30–31). Triple superphosphate (46%) at a dose equivalent to 30.2 kg $P \cdot ha^{-1}$, and potassium salt (56%) at a dose equivalent to 83.1 kg K ha^{-1}, as a pre-sowing application.

3. „NPK+Cu" – Mineral fertilization as in the „NPK" variant + foliar fertilization with 0.2 kg Cu ha^{-1} (1% solution of $CuSO_4$ – with 10% urea solution).

4. „NPK+Zn" – Mineral fertilization as in the „NPK" variant + foliar fertilization with 0.2 kg Zn ha^{-1} (1% solution of $ZnSO_4$ – with 10% urea solution).

5. „NPK+Mn" – Mineral fertilization as in the „NPK" variant + foliar fertilization with 0.2 kg Mn ha^{-1} (0.5% solution of $MnSO_4$ – with 10% urea solution).

6. „NPK+Cu,Zn,Mn" – Mineral fertilization as in the „NPK" variant + foliar fertilization with: 0.2 kg Cu ha^{-1}; 0.2 kg Zn ha^{-1}; 0.2 kg Mn ha^{-1} – with 10% urea solution.

Cu, Zn and Mn (individually or in combination) were applied to leaves as aqueous solutions at the stem elongation stage (BBCH 30–31).

Soil tillage treatments included first ploughing performed after harvesting the forecrop. In order to cover crop residues before sowing of winter rye, a pre-sowing plough and harrowing were performed. Directly before sowing, a combined cultivator and seed drill was applied to all plots in order to mix mineral fertilizers and prepare the soil for sowing. Weeds were destroyed using herbicides in 2012 – Mustang Forte 195 SE (a.i. florasulam 5 g, aminopyralid

10 g, 2.4 D 180 g) 1.0 dm^{-3} ha^{-1} and Puma Universal 069 WG (a.i. fenoksaprop-P-etyl 69.0 g) 1.2 dm^{-3} ha^{-1}; in 2013 – Atlantis 12 OD (a.i. jodosulfuron methyl sodium 2 g; mesosulfuron methyl 10 g) 0.45 dm^{-3} ha^{-1} + Sekator 125 OD (a.i.: jodosulfuron methyl sodium 25 g, amidosulfuron 100 g) 0.15 dm^{-3} ha^{-1}, in spring after resumption of rye vegetation (BBCH 21–29). Protection against pests and diseases was not performed.

Every year, in the course of the experiment the grain was collected, dried and purified. The amount of yield was determined and the samples were collected and subjected to chemical analysis for the content of macro- and micronutrients according to the methods used in agricultural chemistry. The samples of grain were mineralised under wet conditions in a mixture of HNO_3 and $HClO_4$ acids at a ratio of 4:1. The content of mineral components (Ca, Mg, K, Cu, Fe, Mn, Zn) was determined by flame atomic absorption spectrometry method (flame: acetylene-air). The analyses were performed using an iCE 3000 SERIES atomic absorption spectrometer provided by THERMO, equipped with a GLITE data station, working in emission system (WHITESIDE and MINER 1984). Phosphorus was determined using a vanadium-molybdenum method, in the material previously mineralised with H_2SO_4 with the addition of H_2O_2 as an oxidant.

For the measurements of protein and starch content in the grain an Infratec™ 1241 grain analyser, which employs the near-infrared analysis within the wavelength range 570–1100 nm was applied.

During the entire field experiment a monitoring of temperature and precipitation amount was conducted (Table 2). The mean monthly air temperatures during the growing season of winter rye were similar and did not differ from multi-year average values. The amount of precipitation was higher by 21.8 mm and 10.6 mm in September 2011 as compared to the multi-year average. October and November 2011 were characterised by an amount of precipitation lower by 39.0 and 31.1 mm as compared to 1981–2010, and by 13.1 and 30.7 mm as compared to the multi-year period. In April 2012 a twofold excess of the multi-year precipitation amount was recorded. In the years of the study, at the beginning of heading ray phase (May) the amounts of precipitation were similar. In June 2012 the amount of precipitation significantly exceeded the amount of precipitation in the following year of the study (2013). In July 2012 and 2013 the amounts of precipitation were similar, but they exceeded by 39% on average the multi-year amount of precipitation.

The results were statistically processed with STATISTICA 10.0 software (StatSoft, Tulsa, Oklahoma, USA). The statistical calculations were performed using a one-way ANOVA. Apart from the basic parameters, standard deviation and statistically homogenous groups were determined with Duncan's test at $\alpha = 0.05$. Coefficients of linear correlation (Pearson's r) were calculated.

Table 2

Weather conditions in 2011–2013 and the multi-year average of 1981–2010

Year	Month												
	IX	X	XI	XII	I	II	III	IV	V	VI	VII	VIII	IX–VIII
Temperature (°C)													average
2011/2012	14.1	8.3	3.1	2.3	-1.7	-7.5	3.0	7.8	13.4	15.0	19.0	17.7	7.9
2012/2013	13.5	7.4	4.9	-3.5	-4.6	-1.1	-3.5	5.9	14.8	17.5	18.0	17.4	7.2
1981/2010	12.8	8.0	2.9	-0.9	-2.4	-1.7	1.8	7.7	13.5	16.1	18.7	17.9	7.9
Precipitation (mm)													sum
2011/2012	67.5	29.5	14.1	25.8	61.8	27.7	24.1	73.1	51.7	103.2	121.0	45.1	644.6
2012/2013	45.7	68.5	45.2	11.8	44.1	22.6	18.1	28.5	54.5	61.2	121.9	37.6	559.7
1981/2010	56.9	42.6	44.8	38.2	36.4	24.2	32.9	33.3	58.5	80.4	74.2	59.4	581.8

Results and Discussion

Winter rye (*Secale cereale L.*) of the Dańkowskie Diament variety belongs to the group of the highest-yield rye variety in the area of Poland. According to COBORU (2014), the yield of grain for this variety at an average level of fertilization and protection equals 6.74 t ha^{-1} on average, while intensive fertilization and protection increased the yield to 8.39 t ha^{-1}. In this study the average grain yield of the study variety of winter rye was 7.98 t ha^{-1} (Table 3). In comparison to the study of NOGALSKA et al. (2012), the grain yield of winter rye for the Dańkowskie Diament variety was higher and varied in the study years. The statistical analysis confirmed the effect of years of the study on grain yield and its components. In the first year the grain yield was higher by 14.2%, the length of the ear by 28.0%, the number of grains per ear by 44.3% and the grain weight per ear by 33.0%. The 1000 grain weight was higher in the second year of the study by 8.9%. The obtained results of the impact of weather conditions in growing seasons 2011/2012 and 2012/2013 on the described parameters confirm results previously obtained by CHMIELEWSKI and KOHN (2000). The study uses data of a long-term field experiment at Berlin-Dahlem for the period between 1962 and 1996. According to them higher temperatures in the winter and an earlier start of the growing season favour the grain yield. Moderate temperatures before starting the shooting phase prolong the period of spikelet formation, which leads to an increase in the number of spikelets. High temperatures and the drought in the ripening phase have a negative impact on the 1000 grain weight. According to JASKULSKI and JASKULSKA (2009), the beneficial effect of foliar fertilization (multifertilizers SONATA ZBOŻE) on grain yield of winter wheat is the higher, the higher is the amount of precipitation in April and June.

Table 3

Grain yield and yield components of winter rye (average 2012–2013)

Fertilisation treatments		Grain yield [t ha^{-1}]	Ear length [mm]	Number of grains per ear	Weight of grains per ear [g]	Weihgt of 1000 grains [g]
Without fertilization	average	7.00^{b}	80.9^{a}	43.2^{a}	1.34^{a}	32.8^{a}
	SD	1.08	10.9	11.0	0.22	1.61
NPK	average	8.48^{a}	82.4^{a}	47.7^{a}	1.44^{a}	32.5^{a}
	SD	1.35	16.09	10.8	0.30	1.12
NPK+Cu	average	8.08^{a}	83.4^{a}	44.2^{a}	1.32^{a}	33.1^{a}
	SD	0.49	10.0	8.3	0.22	1.79
NPK+Zn	average	8.23^{a}	81.2^{a}	35.7^{a}	1.21^{a}	33.4^{a}
	SD	1.20	11.1	18.3	0.26	3.33
NPK+Mn	average	7.98^{a}	79.1^{a}	36.3^{a}	1.40^{a}	32.7^{a}
	SD	1.00	13.3	16.1	0.31	1.40
NPK+Cu,Zn,Mn	average	8.11^{a}	78.5^{a}	42.7^{a}	1.30^{a}	32.4^{a}
	SD	0.41	13.1	12.2	0.34	1.70
Average for years						
2012	average	8.51^{a}	90.9^{a}	49.2^{a}	1.53^{a}	31.4^{b}
	SD	1.11	7.1	12.5	0.22	0.87
2013	average	7.45^{b}	71.0^{b}	34.1^{b}	1.15^{b}	34.2^{a}
	SD	0.59	5.4	8.5	0.15	1.45

Note. NPK – mineral fertilizers, Cu, Zn, Mn – micronutrients
Averages in columns (separately for fertilization treatments and years) followed by the same letter are insignificant according to Duncan's test ($\alpha < 0.05$), SD – standard deviation

According to JANKOWSKI et al. (2003), NEDZINSKIENE (2006), CIOROMELE and CONTOMAN (2015) and WOJTKOWIAK et al. (2015), grain yield is determined by the nitrogen dose. LEWANDOWSKI and KAUTER (2003) suggest that a significant increase in the yield of all cereal species can be obtained after the application of 70 kg N ha^{-1} (in conditions of south western Germany).

In this study, in case of mineral fertilization (NPK) and mineral fertilization combined with micronutrients applied separately (NPK+Cu, NPK+Zn, NPK+Mn) or in combination (NPK+Cu,Zn,Mn), a significant increase in grain yield was observed, as compared to the plot with no fertilization. BLECHARCZYK et al. (2004) found that the NPK fertilization (N-90 kg ha^{-1}, P-26 kg ha^{-1}, K-100 kg ha^{-1}) in crop rotation and monoculture of rye resulted in an increase in the grain yield by 102.9%, in the number of ears m-2 by 38.1%, in the number of grains per ear by 41.9%, in the grain weight per ear by 46.9%, and in the 1000 grain weight by 13.9%, as compared to the control plot (with no fertilization). In this study, mineral fertilizers did not significant affect the yield components. MALAKOUTI (2008), KUMAR et al. (2009), NADIM et al. (2012) suggest that micronutrients and mutual relationships between elements posi-

tively influence physiological processes of plants, which is reflected in improved yield. In this study, foliar feeding in order to supplement the basic fertilization with mineral fertilizers (NPK) did not affect grain yield and its components. According to NOGALSKA et al. (2012) fertilization with compound fertilizers, especially with AMOFOSMAG 3, in conditions of north-eastern region of Poland, has the effect on the increase in grain yield, as compared with one-component fertilizers. JASKULSKI and JASKULSKA (2009) in four out of 9 years of their research found a significant increase in the grain yield of winter wheat by 5.0–6.7% as a result of the application of SONATA ZBOŻE compound fertilizer, as compared to the yield of the wheat which was not subjected to foliar fertilization.

The content of protein and starch belongs to important criteria for the quality of cereals (RAGAEE et al. 2006, WOJTKOWIAK et al. 2015). NOWOTNA et al. (2006) showed that the average content of protein (for five test varieties of winter rye) is 9.64%. SKUODIENE and NEKROŠEINE (2009) indicate a broader range of protein content from 9 to 19% and of starch content from 49 to 66% in rye grain obtained in west Lithuania region. In this study the grain of winter rye contained from 10.1 to 10.6% of protein (Table 4). The content of protein in the grain was differentiated by weather conditions in given years and variants of fertilization. The content of protein in the second year of the study was higher by 2.9% as compared with the first year. Higher protein content corresponded to lower grain yields of rye. The year 2013 with a lower amount of precipitation and a higher mean air temperature in the spring growing season (May-June) favoured a greater concentration of protein. Similar dependencies of protein accumulation on weather conditions were confirmed by the study of LOPES-BELLIDO et al. (2000) and GARRIDO-LESTACHE et al. (2004). As a result of mineral fertilization (NPK) and supplementation with foliar feeding with manganese (NPK+Mn), zinc (NPK+Zn) and micronutrients applied in combination (NPK+Cu,Zn,Mn), protein content in grain increased by 5.0%, 4.0%, 3.0% and 2.0%, respectively, as compared to the plot with no fertilization. Supplementation of basic fertilization with copper (NPK+Cu) or micronutrients in combination (NPK+Cu,Zn,Mn) resulted in a decrease in protein content in grain by 3.8% and 2.8%, as compared to the mineral fertilization (NPK).

In this study the starch content in the grain of winter rye of the Dańkowskie Diament variety ranged from 64.4 to 64.9%. According to ZIELIŃSKI et al. (2007) starch content in three winter rye varieties (Amilo, Warka and Dańkowskie Złote – obtained from a local plant breeding station in Poland) ranged from 53.3% to 55.7%. According to NOWOTNA et al. (2007) among 6 analyzed rye varieties, only the Walet and Dańkowskie Złote varieties contained more starch than indicated in the present study.

Table 4

The content of proteins and starch in grain of winter rye (average 2012–2013)

Fertilisation treatments		Protein [%]	Starch [%]
Without fertilization	average	10.1^{d}	64.7^{ab}
	SD	0.74	0.61
NPK	average	10.6^{a}	64.4^{b}
	SD	0.10	0.15
NPK+Cu	average	10.2^{cd}	64.9^{a}
	SD	0.23	0.30
NPK+Zn	average	10.4^{ab}	64.7^{ab}
	SD	0.23	0.30
NPK+Mn	average	10.5^{ab}	64.5^{ab}
	SD	0.19	0.21
NPK+Cu,Zn,Mn	average	10.3^{c}	64.8^{ab}
	SD	0.08	0.38
Average for years			
2012	average	10.2^{b}	64.7^{a}
	SD	0.41	0.40
2013	average	10.5^{a}	64.6^{a}
	SD	0.22	0.35

Note. NPK – mineral fertilizers, Cu, Zn, Mn – micronutrients
Averages in columns (separately for fertilization treatments and years) followed by the same letter are insignificant according to Duncan's test ($\alpha < 0.05$), SD – standard deviation

Table 5

Content of macronutrients in the grain of winter rye (average 2012–2013)

Fertilisation treatments		Macronutrients [g kg^{-1} DM]			
		P	K	Ca	Mg
Without fertilization	average	3.61^{b}	4.45^{a}	0.59^{a}	0.92^{a}
	SD	0.08	0.07	0.03	0.06
NPK	average	3.66^{b}	4.29^{b}	0.56^{a}	0.91^{a}
	SD	0.08	0.04	0.06	0.04
NPK+Cu	average	3.51^{b}	4.42^{ab}	0.57^{a}	0.96^{a}
	SD	0.06	0.06	0.02	0.01
NPK+Zn	average	3.52^{b}	4.39^{ab}	0.55^{a}	0.94^{a}
	SD	0.07	0.09	0.06	0.03
NPK+Mn	average	3.21^{c}	4.37^{ab}	0.52^{a}	0.89^{a}
	SD	0.05	0.06	0.05	0.02
NPK + Cu,Zn,Mn	average	3.92^{a}	4.47^{a}	0.52^{a}	0.94^{a}
	SD	0.08	0.10	0.05	0.01
Average for years					
2012	average	3.28^{b}	3.85^{b}	0.65^{a}	0.98^{a}
	SD	0.10	0.08	0.07	0.02
2013	average	3.86^{a}	4.95^{a}	0.47^{b}	0.88^{b}
	SD	0.13	0.11	0.03	0.01

Note. NPK – mineral fertilizers, Cu, Zn, Mn – micronutrients
Averages in columns (separatly for fertilization treatments and years) followed by the same letter are insignificant according to Duncan's test ($\alpha < 0.05$), SD – standard deviation

Years of the study did not influence the concentration of starch. Among the fertilized plots, only supplementation of mineral fertilization with copper (NPK+Cu) caused an increase in starch content by 0.78%, as compared to the NPK plot. Higher starch content corresponded to a lower protein content, and the correlation coefficient equalled r = -0,639 (data not shown in Table 7).

The present study demonstrated that the years of the study have an impact on the content of P, K, Ca and Mg in the grain of winter rye (Table 5). In the first year of the study more Mg (by 11.4%) and Ca (by 38.3%), and in the second year more P (by 17.7%) and K (by 28.6%) was found. The study of MALAKOUTI (2008) shows that the use of combined mineral fertilization with microelements not only increases grain yield, but also improves nutritional value of cereal grain.

In this study winter rye grain contained 3.57 g P kg^{-1}, 4.40 g K kg^{-1}, 0.56 g Ca kg^{-1}, 0.93g Mg kg^{-1}on average. In comparison to studies of NOGALSKA et al. (2012), conducted at the same localization and conditions, a higher content of Mg was found in the grain of winter rye of the Dańkowskie Diament variety. The amounts of other mineral components (K, P, Ca) were similar. In the present study the combination of mineral fertilization with foliar feeding with micronutrients (NPK+Cu,Zn,Mn) contributed to a significant increase in phosphorus content in grain (from 6.6% to 18% in comparison with individual fertilizer variants).

The highest amount of potassium was found after the application of mineral fertilizers in combination with three micronutrients (NPK+Cu,Zn,Mn) and in the plot with no fertilization. In these variants the increase in K content (by 4.0% on average) was achieved only for the plot fertilized exclusively with mineral fertilizers (NPK).

In the experiment no increase in Ca and Mg content as a result of fertilization with macro- and micronutrients was achieved. Further, in a study of LEWANDOWSKI and KAUTER (2003) increasing doses of nitrogen (0, 70 and 140 kg ha^{-1}) did not result in clear changes in calcium content in the grain of winter rye.

According to RAGAEE et al. (2006) rye grain (obtained from Experimental Farm in UAE University) is rich in iron (43.0 mg kg) and manganese (24.4 mg kg). In turn Kan (2015) indicates that rye (cultivated on sand-loam) grain has the highest content of Zn among major cereal species. In the present study the content of micronutrients in the grain of winter rye was characterised by variability in individual years of the study (Table 6). In the first year of the study more Fe (by 1.5% respectively), and in the second year more Zn and Mn (by 8.5% and 7.0%, respectively) was found. The combination of fertilization with mineral fertilizers with manganese (NPK+Mn) favoured the increase in Cu in grain by 14.2%, as compared to the plot with no fertilization. The

supplementation of mineral fertilization (NPK) with copper (NPK+Cu) or a combination of micronutrients (NPK+Cu,Zn,Mn) resulted in an increase in iron content in grain by 9.1%; 9.0% and 5.3%, respectively, as compared to the control plot (no fertilization). Foliar spraying with zinc (NPK+Zn), manganese (NPK+Mn) and a combination of micronutrients (NPK+Cu,Zn,Mn) resulted in a reduction in Fe content, as compared to the control plot fertilized with mineral fertilizers (NPK). Foliar feeding with zinc (NPK+Zn) and a combination of micronutrients (NPK+Cu,Zn,Mn) decreased the content of Zn in the grain of winter rye, as compared with fertilization with mineral fertilizers without micronutrients (NPK). Supplementation of basic fertilization with zinc (NPK+Zn), manganese (NPK+Mn) and a combination of micronutrients (NPK+Cu,Zn,Mn) resulted in an increase in Mn content in the grain (by 33.9, 30.2 and 23.1%, respectively), as compared to the plot with no fertilization and (by 25.7%, 22.2% and 15.5%, respectively), as compared with the plot fertilized with mineral fertilizers (NPK).

Table 6

Content of micronutrients in the grain of winter rye (average 2012–2013)

Fertilisation treatments		Macronutrients [mg kg^{-1} DM]			
		Cu	Fe	Zn	Mn
Without fertilization	average	2.88^{bc}	24.13^{c}	29.77^{ab}	10.70^{c}
	SD	0.07	0.19	0.41	0.41
NPK	average	3.05^{abc}	26.33^{a}	32.57^{a}	11.40^{c}
	SD	0.01	0.19	1.04	0.41
NPK+Cu	average	3.15^{ab}	26.30^{a}	32.83^{a}	11.20^{c}
	SD	0.06	0.27	3.59	0.47
NPK+Zn	average	2.80^{c}	23.83^{c}	27.63^{b}	14.33^{a}
	SD	0.03	0.14	0.40	0.68
NPK+Mn	average	3.29^{a}	24.17^{c}	32.10^{a}	13.93^{ab}
	SD	0.34	0.19	0.18	0.27
NPK + Cu,Zn,Mn	average	3.12^{abc}	25.40^{b}	27.47^{b}	13.17^{b}
	SD	0.12	0.45	0.27	0.60
Average for years					
2012	average	2.99^{a}	25.21^{a}	28.68^{b}	12.04^{b}
	SD	0.08	0.22	2.01	0.39
2013	average	3.11^{a}	24.83^{b}	31.12^{a}	12.88^{a}
	SD	0.22	0.55	3.00	0.44

Note. NPK – mineral fertilizers, Cu, Zn, Mn – micronutrients
Averages in columns (separatly for fertilization treatments and years) followed by the same letter are insignificant according to Duncan's test ($\alpha < 0.05$), SD – standard deviation

Correlation analysis showed a positive correlation between Fe content and the content of Zn (r=0.457) and a negative one with Mn content (r=-0491) in winter rye grain (Table 7).

Table 7
Correlations between content of micronutrients, macronutrients, proteins and starch in grain of winter rye (average 2012–2013)

Specification	Fe	Zn	Mn	P	K	Proteins
Cu	n.s.	0.405	n.s.	n.s.	n.s.	n.s.
Fe	–	0.457	-0.491	0.390	n.s.	n.s.
Zn	0.457	–	-0.423	-0.427	n.s.	n.s.
Mn	-0.491	-0.423	–	n.s.	n.s.	n.s.
Ca	n.s.	n.s.	-0.357	n.s.	n.s.	n.s.
Mg	n.s.	n.s.	n.s.	0.419	n.s.	n.s.
Starch	n.s.	n.s.	n.s.	n.s.	0.358	-0.639

Correlations are significant at $p<0.05$, N=36

The increase in Zn content in the grain was accompanied not only by an increase in Fe content, but also by an increase in Cu content (r=0.405) and a decrease in Mn content (r=-0.423). A negative correlation between Mn content and Fe, Zn and also Ca content (r=-0,357) was observed. With the increase of P content in rye grain, the content of Fe (0.390) and Mg (r=0.419) increased, while the concentration of Zn decreased (r=-0.427). Among mineral components only potassium (K) contributed to the increase in starch content in winter rye grain (r=0.358). The conducted correlation analysis revealed a reduction in starch content with increasing protein content (r=-0.639).

Conclusion

Fertilization with mineral fertilizers (NPK) and with mineral fertilizers with micronutrients applied separately or in combination resulted in a significant increase in grain yield (by 0.98 t ha^{-1} to 1.48 t ha^{-1}), as compared to the plot with no fertilization. Fertilization with mineral fertilizers and foliar feeding with micronutrients (except for Cu) increased protein content in rye grain (NPK 5.0%, NPK+Mn 4.0%, NPK+Zn 3.0% and NPK+Cu,Zn,Mn 2.0%). Foliar application of nitrogen of basic fertilization with copper increased the concentration of starch in the grain by 0,78% as compared to the NPK plot. The mineral fertilization (NPK) with micronutrients applied in combination (NPK+Cu,Zn,Mn) increased the content of phosphorus (by 8.6% as compared to the plot with no fertilization and by 7.1% fertilized with mineral fertilizers NPK) and potassium (by 4.2% as compared to the NPK plot). Supplementation of basic fertilization with zinc or manganese or a combination of micronutrients resulted in an increase in Mn content in the grain (by 33.9, 30.2 and 23.1%, respectively) as compared to the plot with no fertilization and (by

25.7%, 22.2% and 15.5%, respectively) as compared to the plot fertilized with mineral fertilizers.

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THE EFFECTIVENESS OF THE PREPARATION MEDIUM-CHAIN FATTY ACIDS (MCFA) AND A HERBAL PRODUCT ON THE GROWTH PERFORMANCE OF TURKEYS

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Key words: medium-chain fatty acids, herbal additives, growth performance, turkeys.

A b s t r a c t

The goal of the experiment was to specify the impact of glycerides of medium-chain fatty acid (AveMix MCT) additives and a herbal additive (AdiSan) on production results in turkeys. The research material covered 160 BIG-6 turkeys (hens) divided into 2 groups, 4 series in each group. The experiment lasted 15 weeks. The control group received compound feed, without additives, of organic acids and herbs. The compound feed for turkeys in the second group was enriched with a preparation of glycerides of medium-chain fatty acids (C_6+C_8) *(AveMix MCT sil- AVEVE Biochem)* in the amount of 1.4 (starter 1), 1.2 (starter 2), 1.0 (grower 1 and 2), 0.8 (finisher) kg/t and the additional herbal preparation AdiSan (AdiFeed), containing natural essential oils (thymol, cinnamon oil and eucalyptus oil) in the amount of 0.1 kg/t of the compound feed.

Supplementing the feed ration for turkeys with the AveMix MCT and AdiSan additive highly significantly improved the final body mass compared to animals in the control group (9592.1 vs 9109.7 g, $P \leq 0.01$). The Feed Conversion Ratio (FCR) was lower for birds in the control group compared to experimental turkeys (2.35 vs 2.27 kg/kg), although these differences were not statistically significant. The European Production Index was lower by 45 points ($P \leq 0.01$) in turkeys from the group receiving in their ration the additives AveMix MCT and AdiSan.

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WPŁYW DODATKU ŚREDNIOŁAŃCUCHOWYCH KWASÓW TŁUSZCZOWYCH (MCFA) I PREPARATU ZIOŁOWEGO NA WYNIKI PRODUKCYJNE INDYKÓW

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S ł o w a k l u c z o w e: średniołańcuchowe kwasy tłuszczowe, dodatki ziołowe, wyniki produkcyjne, indyki.

A b s t r a k t

Celem badań było określenie wpływu dodatku glicerydów średniołańcuchowych kwasów tłuszczowych (AveMix MCT) oraz preparatu ziołowego (AdiSan) na wyniki produkcyjne indyków. Materiał badawczy obejmował 160 indyczek BIG-6. Ptaki podzielono losowo na 2 grupy, po 4 powtórzenia w każdej. Odchów ptaków prowadzono do wieku 15 tygodni. Ptaki z grupy kontrolnej (I) żywiono mieszankami paszowymi bez dodatku kwasów organicznych i ziół. Mieszanki dla ptaków z grupy II wzbogacono dodatkiem glicerydów średniołańcuchowych kwasów tłuszczowych (C_6+C_8; *AveMix MCT sil- AVEVE Biochem)* w ilościach (kg/t mieszanki):1,4 (starter 1), 1,2 (starter 2), 1,0 (grower 1 i 2), 0,8 (finiszer) oraz 0,1 preparatu ziołowego AdiSan (AdiFeed), zawierającego naturalne olejki eteryczne (tymol, olejek cynamonowy i olejek eukaliptusowy).

Końcowa masa ciała ptaków żywionych z zastosowaniem dodatku preparatów AveMix MCT i AdiSan, była istotnie większa w porównaniu z masą ciała ptaków z grupy kontrolnej (wiek 15. tyg.; 9592,1g vs 9109,7g; $P \leq 0{,}01$), podobnie Europejski Wskaźnik Wydajności (387,2 vs 342,6; $P \leq 0{,}01$). Współczynnik wykorzystania paszy (FCR), u ptaków z grupy kontrolnej był gorszy w porównaniu ze wskaźnikiem (FCR) w grupie doświadczalnej (2,27 kg/kg i 2,35 kg/kg), przy czym różnica nie była istotna statystycznie.

Introduction

Withdrawing antibiotic growth promoters, intensification of production, and genetic progress in poultry still require improving zoohygienic conditions, increasing purity of feed, and changes in meal programmes. Using feed additives, e.g. pro- and prebiotics and organic acids, aims at improving production results, strengthening natural immunity, and the stabilisation of adequate microflora of the digestive tract, ensuring animal health (ALLOUI et al. 2013, HUYGHEBAERT et al. 2011).

The pH of the intestinal contents plays a crucial role in maintaining the intestinal microbiological balance. Clostridia and other pathogenic bacteria responsible for enteric diseases do not grow at low pH levels. This indicates that reducing the pH of the intestinal contents contributes to the proper function of the digestive tract (MILCZAREK et al. 2012, DHAMA et al. 2011). Organic acids are known for their strong bacteriostatic properties. Commercial

preparations (acidifiers) appear to enhance digestibility and diet palatability, thus improving feed conversion and the growth of animals, including pigs and poultry. The use of short chain fatty acids (SCFA), medium-chain fatty acids (MCFA), and other organic acids was largely based on their antimicrobial activity in the intestinal tract (DHAMA et al. 2014, GANGULY 2013, HUTH et al. 2010).

Medium-chain triglycerides have been shown to be good alternatives to nutritional antibiotics in poultry, due to the high antibacterial activity of the medium-chain fatty acids (MCFA). Free MCFA (C6:0 to C12:0) have been shown to be more bactericidal to numerous gram-negative and gram-positive bacteria than SCFA. The strength of the antimicrobial activity towards specific groups of bacteria varies according to the chain length of the MCFA (SHOKROLLAHI et al. 2014, HERMANS et al. 2012, ROSSI et al. 2010).

Moreover, fitobiotics constitute an alternative to antibiotic growth promoters in poultry feeding. Herbs and essential oils improve digestion and the conversion of feed ingredients, consequently improving livestock rearing results. At the same time they have antibacterial and antioxidative properties, and they contribute to improving the dietetic and palatability value of compound feed (ALP et al. 2012, LIPINSKI et al. 2011). One herb, *Origanum hyrtium,* contains phenolic compounds (carvacrol and thymol) showing very strong antibacterial properties against such strains as *Escherichia coli, Staphylococcus aureus, Salmonella typhimurium* and *Listeria monocytogenes*. Garlic has similar properties, inhibiting the growth of *E. coli* and other *Enterobacteria, S. typhimurium* (BENTO et al. 2013, KIRKPINAR et al. 2011). Moreover, herbs and spices such as ginger, pepper, rosemary, thyme, clove, coriander, mustard and cinnamon participate in the elimination of other pathogenic bacteria (HIPPENSTIEL et al. 2011, BRENES and ROURA 2010).

Feed additives often allow growers to achieve production results comparable to those obtained with the use of antibiotic growth promoters, especially when accompanied by changes in environmental and husbandry conditions. The best results have been reported in the case of mixtures of different additives, e.g. oligosaccharides or plant extracts with organic acids. Combinations of feed additives that would be equally effective as antibiotics are at present the object of interest for many researchers (KRISHAN and NARANG 2014, BOZKURT at al. 2012).

The aim of this study was to determine the effect of the application of glycerides of medium-chain fatty acids (AveMix MCT) and a herbal preparation (AdiSan) on the growth performance of turkeys.

Materials and Methods

The experiment involved 160 BIG 6 turkeys (females), divided into two groups, with four replicates per group. The birds from each subgroup (20) were placed in separate pens. The turkeys were bred on litter in typical conditions. The experiment lasted for 15 weeks.

The complete diets for turkeys used in the trial were produced in the mash form. These were standard diets whose nutritional value and composition were adapted to the requirements of the intensively-growing birds (Table 1). The grain component in the diets (wheat) was supplemented with high-protein feeds (soybean meal, fish meal-starter diets), with soybean oil as an additional source of energy. In order for the diets to contain the required amount of exogenous amino acids, synthetic methionine, lysine and threonine were added. The diets contained mineral-vitamin premixes with coccidiostatic (Clinacox- starter 1, 2 and grower 1 diets). The diets also contained an enzyme preparation (xylanase, phytase).

These diets were applied in the feeding of turkeys from group I (control). Similar diets were used in the experimental group II. The diets from group II additionally contained glycerides of medium-chain fatty acids (C_6+C_8) *(AveMix MCT sil – AVEVE Biochem)*. The tested dosages were: 1.4 (starter 1 diet), 1.2 (starter 2 diet), 1.0 (grower 1 and 2 diets) and 0.8 (finisher diet) kg of AveMix MCT sil per tonne of complete feed. The diets from group II additionally contained a herbal preparation, AdiSan (AdiFeed). It contains natural essential oils: thymol, cinnamon oil and eucalyptus oil. The tested dosage was: 0.1 kg of AdiSan per tonne of complete feed. The Weende method was used to determine ash, crude protein, ether extract and crude fibre in the diets (AOAC, 2005) – Table 2.

The body weights of turkeys were measured weekly. Feed intake and mortality rates were also analysed. The data were used to calculate the feed conversion ratio (FCR), measured as kg feed intake per kg body weight gain The effectiveness of production was based on the European Efficiency Index (EEI), calculated from the average body weight, liveability, number of production days and feed utilisation (FCR).

All data were analysed using a one-way analysis of variance and Duncan's test. The results obtained were characterized with an arithmetic mean (x), a standard error of the mean (*SEM*) and *P* value. All calculations were made with STATISTICA 10 software.

Table 1

Composition and nutritional value of control diets for turkeys

Specification	Starter 1 0–3 weeks	Starter 2 4–6 weeks	Grower 1 7–9 weeks	Grower 2 10–12 weeks	Finisher 13–15 weeks
Ingredients [g/kg, as-fed basis]					
Wheat	513.7	544.9	561.7	611.6	691.3
Soybean meal	394.8	356.9	356.8	301.8	225.8
Fish meal	30.0	30.0	–	–	–
Soybean oil	10.0	18.4	37.0	41.8	42.9
L-lysine HCl	4.1	3.7	2.4	2.4	3.5
DL-methionine	2.7	2.5	2.5	2.5	2.4
L-threonine	1.0	0.4	1.0	0.7	1.0
Limestone	15.6	15.1	11.8	12.5	9.6
Monocalcium phosphate	20.5	20.6	18.1	17.9	14.6
Sodium bicarbonate	1.0	1.0	1.0	1.0	1.0
Salt	1.4	1.3	2.5	2.6	2.7
Feed enzymes	0.2	0.2	0.2	0.2	0.2
Premix*	5.0	5.0	5.0	5.0	5.0
Nutritional value					
ME, [kcal/kg]	2770	2850	2970	3050	3150
CP, g	27.50	25.50	24.00	22.00	19.00
Lys, [%]	1.78	1.65	1.40	1.26	1.15
Met+Cys, [%]	1.10	1.05	0.98	0.93	0.85
Ca, [g]	1.35	1.30	1.15	1.14	0.95
Available P, [g]	0.70	0.69	0.58	0.57	0.50
Na, [g]	0.14	0.14	0.15	0.15	0.15

* Premix provided per kilogram of diets: Starter – 12 500 IU vitamin A, 4 500 IU vitamin D_3, 87.5 mg vitamin E, 3.75 mg vitamin K_3, 3.5 mg vitamin B_1, 10 mg vitamin B_2, 75 mg niacin, 22.5 mg pantothenic acid, 6.0 mg vitamin B_6, 30 μg vitamin B_{12}, 2.5 mg folic acid, 400 μg biotin, 800 mg choline chloride, 92.5 mg Fe, 130 mg Mn, 20 mg Cu, 105 mg Zn, 2.5 mg J, 0.3 mg Co; Grower – 11 500 IU vitamin A, 4 140 IU vitamin D_3, 80.5 mg vitamin E, 3.45 mg vitamin K_3, 3.22 mg vitamin B_1, 9.2 mg vitamin B_2, 69 mg niacin, 20.7 mg pantothenic acid, 5.52 mg vitamin B_6, 37.6 μg vitamin B_{12}, 2.3 mg folic acid, 368 μg biotin, 600 mg choline chloride, 85.1 mg Fe, 120 mg Mn, 18.4 mg Cu, 96.6 mg Zn, 2.3 mg J, 0.28 mg Co; Finisher – 10 500 IU vitamin A, 3 780 IU vitamin D_3, 66.5 mg vitamin E, 2.85 mg vitamin K_3, 2.66 mg vitamin B_1, 7.6 mg vitamin B_2, 57 mg niacin, 17.1 mg pantothenic acid, 4.6 mg vitamin B_6, 22.8 μg vitamin B_{12}, 1.9 mg folic acid, 304 μg biotin, 400 mg choline chloride, 70.3 mg Fe, 98.8 mg Mn, 15.2 mg Cu, 79.8 mg Zn, 1.9 mg J, 0.23 mg Co.

Table 2

Chemical composition of diets

Item	Diets				
	Starter	Grower I	Grower I	Grower II	Finisher
Dry matter, %	88.71	88.57	88.94	89.15	88.96
Crude ash, %	6.70	6.22	5.66	5.08	4.57
Crude protein, %	27.20	25.97	24.05	22.22	18.93
Ether extract,%	2.35	3.23	4.63	5.22	5.46
Crude fibre,%	2.01	2.40	2.49	2.90	2.73
N-free extractives, %	50.45	50.75	52.11	53.73	57.27

Results and Disccusion

The turkey hen body weights are shown in table 3. After the first week of life the control group birds weighed 155.5 g, on average. The birds in the group fed on diets with AveMix MCT and AdiSan weighed a similar amount (154.1 g). In the subsequent 8 weeks of life the body weights of the birds from the experimental groups (II) were slightly lower than in the control birds. However, these differences were found to be statistically non-significant.

Table 3

Average body weight (BW) of turkey females, g

Period, week	Groups		*SEM*	*P*
	I – K	II (AveMix MCT +AdiSan)		
1	155.5	154.1	2.227	0.778
2	326.3	320.4	4.267	0.529
3	609.1	582.8	10.514	0.236
4	1034.9	983.8	17.763	0.163
5	1469.2	1417.5	20.778	0.240
6	2139.3	2060.0	31.292	0.230
7	2866.0	2758.8	33.517	0.112
8	3763.2	3666.3	39.835	0.251
9	4333.0	4311.3	48.410	0.841
10	5114.5	5285.5	80.333	0.323
11	5854.0	6052.0	85.598	0.279
12	6733.8	6896.7	61.419	0.206
13	7714.9 B	8026.3 A	69.935	0.009
14	8406.4 b	8776.3 a	92.672	0.031
15	9109.7 B	9592.1 A	107.063	0.007

a, b – $P \leq 0.05$, A, B – $P \leq 0.01$

In the 10th week of life the body weight of the turkey hens in group II (MCFA product and herbal additive) was 5285.5 g, and that of the birds in the control group was 5114.5 g. In subsequent weeks of life the body weights of the birds in the experimental group II were also greater than in the control birds. The turkeys from group II (9592.1 g) were characterized by a significantly higher body weight as compared to the birds from the control group (9109.7 g, $P \leq 0.01$). The birds fed the diets containing AveMix MCT and AdiSan were therefore heavier by about 5.3%. The differences noted in reference to the control group were highly statistically significant. Others received different results: HEJDYSZ et al., (2012) did not observe a statistically significant impact of short- and medium-chain fatty acids on the improvement of poultry body mass gain, similarly to MILBRADT et al. (2014), after enriching the ration for turkeys with organic acids (a mixture of short- and medium-chain fatty acids).

Moreover, KIRKPINAR et al. (2011) did not notice statistically important differences in the body mass of chickens for fattening after administering a mixture of essential oils from oregano and garlic. The mixture of organic acids and plant extract feed (carvacrol and thymol) in the compound feed for chickens for fattening caused a significant reduction in body weight compared to the control group at the age of 21 days (641.49 vs 766.92 g) (AKYUREK and YEL 2011). MIKULSKI et al. (2008) observed that supplementing compound feed for turkeys with a mixture of organic acids and essential oils (citric acid, fumaric acid, orthophosphoric acid, malic acid with a mixture of hydrogenated essential oils from citrus fruits, cinnamon, oregano and thyme) did not have a significant impact on the final body weight of animals, but in the period from the 56th to the 84th day of the experiment a significant increase in this indicator took place compared to birds from the control group (9.58 vs 9.07 kg).

The total feed intake by the growing turkeys was a little diverse, and accounted for 21.32 kg in the control group, and for 21.77 kg in group II (Table 4). The differences observed between the groups were not statistically significant. The differences observed between the groups were statistically significant only in the 4th and 7th week. Analyses of the results from the entire fattening period indicate that the application of the MCFA preparation AveMix MCT and herbal preparation AdiSan in diets for turkeys had no pronounced effect on their feed intake. HEJDYSZ et al. (2012) in their research found that the addition of capric acid and a mixture of three organic acids caused a significant drop in feed consumption in chickens (3335; 3345 vs 3456 g). Moreover, enriching the compound feed for turkeys with organic acids caused a significant drop in feed intake compared to groups which were supplemented with lincomycin 44% as the antibiotic growth promoter or probiotic (5383 vs 6135; 6520 g) (MILBRADT et al., 2014). Similar results ($P \leq 0.05$) were acquired in chicks in 35 up to 42 days of experiments after enriching the ration with a mixture of essential oils made of garlic and oregano (1225 vs 1446 g) (KIRKPINAR et al., 2011) or an additive of a mixture of organic acids and plant extract feed (967.51 vs 1070.43 g) (AKYUREK and YEL 2011).

Analyses of feed intake per kg of body weight gain during the first 8 weeks demonstrated that the control turkeys were characterized by similar FCR in comparison with birds from group II (MCFA product and herbal additive) – Table 5.

Table 4

Feed intake (cumulative), kg

Age, week	Groups		*SEM*	*P*
	I – K	II (AveMix MCT +AdiSan)		
1	0.22	0.21	0.004	0.416
2	0.57	0.56	0.005	0.414
3	1.03	0.99	0.010	0.501
4	1.67 a	1.61 b	0.014	0.031
5	2.41	2.33	0.026	0.107
6	3.51	3.37	0.038	0.065
7	4.79	4.61	0.046	0.043
8	6.55	6.35	0.064	0.120
9	8.21	8.01	0.108	0.398
10	9.96	10.14	0.119	0.492
11	11.70	12.00	0.117	0.588
12	13.90	14.09	0.177	0.623
13	16.74	16.84	0.152	0.774
14	19.21	19.42	0.203	0.634
15	21.32	21.77	0.256	0.410

a, b – $P \leq 0.05$

Table 5

Feed conversion ratio (FCR) – cumulative, kg/kg

Age, week	Groups		*SEM*	*P*
	I – K	II (AveMix MCT +AdiSan)		
1	1.42	1.41	0.028	0.921
2	1.76	1.76	0.022	0.964
3	1.69	1.70	0.032	0.886
4	1.61	1.64	0.023	0.634
5	1.64	1.64	0.021	0.977
6	1.64	1.64	0.019	0.937
7	1.67	1.67	0.017	0.991
8	1.74	1.73	0.013	0.754
9	1.90	1.86	0.018	0.347
10	1.95	1.92	0.021	0.557
11	2.00	1.98	0.024	0.745
12	2.06	2.04	0.025	0.703
13	2.17	2.10	0.022	0.094
14	2.28	2.21	0.023	0.116
15	2.34	2.27	0.022	0.102

In the following weeks the MCFA product and herbal additive applied in the diets of turkeys improved the feed conversion ratio (FCR) only to a negligible extent. The differences observed were not statistically significant. In the last two weeks of the trial the best feed utilization was reported for the birds from group II (AveMix MCT + AdiSan), and the differences

observed between them and the control birds were as follows: 14 week – 2.21 vs. 2.28 and 15 week – 2.27 vs 2.23. The differences observed were, however, not statistically significant. Irrespective of a lack of statistically-confirmed differences, it should be emphasized that the best feed utilization was observed for the turkeys from group II (MCFA and herbal product). In the experiment by HEJDYSZ et al. (2012), a significant drop in the coefficient of feed intake was observed in chickens for fattening with compound feed enriched with a mixture of MCFA single acids – caprylic and capric compared to the control group (1.50; 1.51; 1.51 vs 1.55 g/kg). Similar results were achieved for turkeys in the 28th to the 70th day of the experiment after enriching the ration with a mixture of organic acids compared to animals from the control group receiving an addition of lincomycin or probiotic (1.71 vs 1.88; 1.87; 1.89 g/kg, $P \leq 0.05$) (MILBRADT et al., 2014). Other results were achieved with the use of the addition of a mixture of oregano and garlic essential oils, or an additive of organic acids and plant extract, and they did not influence feed conversion in chickens for fattening (KIRKPINA et. al. 2011, AKYUREK and YEL 2011, MIKULSKI et al. 2008).

The turkeys' survivability during the experiment was quite high (Table 6). Neither the deaths of animals nor the effects of the feeding applied on their general health condition were observed in the study. Throughout the fattening period the highest mortality was recorded in the control group – 7.50%. In the group where AveMix MCT and AdiSan were applied in feeding the mortality was lower – 3.75%. However, these differences were found to be statistically non-significant. MIKULSKI et al. (2008) showed other results, from the 1st up to the 56th day of the experiment the mortality of turkeys dropped from 3% in the control group to 1% in animals receiving a ration enriched with organic acids and essential oils.

The effectiveness of the turkeys' production is shown in Table 6. The average duration of the fattening period in the experimental groups was 105 days. The feed conversion ratio (FCR), measured as feed consumption per kilogram of body weight, was the worst in the control group (2.35). In group II it was 2.27 kg/kg. The differences observed were, however, not statistically significant.

The European Effectiveness index (EEI) was higher by 44.6 points in the group of birds fed on the diets with AveMix MCT and AdiSan. The differences observed between this group and the control birds were highly statistically significant.

Table 6

Production results of the turkeys over the trial period

Age, week	Groups		*SEM*	*P*
	I – K	II (AveMix MCT +AdiSan)		
Duration of trial, d	105	105		
Final body weight, g	9109.7 B	9592.1 A	107.063	0.007
FCR, kg/kg	2.34	2.27	0.022	0.102
Mortality rate, %	7.50	3.75	1.133	0.097
EEI*	342.6 B	387.2 A	10.032	0.009

* European Efficiency Index

Conclusions

Supplementing the feed ration for turkeys with the preparation of glycerides of medium-chain fatty acids (AveMix MCT) and the additional herbal preparation (AdiSan), containing natural essential oils additive highly significantly improved the final body weight (BW) compared to animals in the control group (9592.1 vs 9109.7 g, $P \leq 0.01$). The Feed Conversion Ratio (FCR) was lower for birds in the control group compared to experimental turkeys (2.35 vs 2.27 kg/kg), although these differences were not statistically significant. The European Production Index was higher by 45 points ($P \leq 0.01$) in turkeys from the group receiving in their ration the additives AveMix MCT and AdiSan.

Translated by Peter Foulds

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HERBICIDE RESISTANCE OF MICROORGANISMS

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Key words: herbicide, microorganisms, sensitivity, resistance, PEC.

Abstract

The aim of study was to evaluate the sensitivity of selected microbial groups cultured on solid media and soil-dwelling microorganisms to metazachlor (Fuego 500 SC), a mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium (Alister Grande 190 OD), and a mixture of terbuthylazine + mesotrione + s-metolachlor (Lumax 537.5 SE). The tested microorganisms were: *Azotobacter* spp., *Arthrobacter* spp., *Bradyrhizobium* spp. (lupini), *Rhizobium leguminosarum* bv. *viciae*, *Streptomyces intermedius*, *Streptomyces viridis*, *Streptomyces longisporoflavus*, *Streptomyces odorifer*, *Fusarium* spp., *Aspergillus* spp., *Penicillum* spp., *Rhizopus* spp. The results indicate that fungi were more sensitive to herbicides than bacteria and actinomycetes. The tested microbes were most resistant to increased doses of the mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium. Predicted environmental concentrations (PEC) calculated on day 160 indicate that increased doses of metazachlor posed the greatest threat for soil-dwelling microorganisms. The applied doses of metazachlor resulted in the highest PEC values, which points to a high risk of soil contamination with this weed control agent.

OPORNOŚĆ DROBNOUSTROJÓW NA HERBICYDY

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Słowa kluczowe: herbicydy, drobnoustroje, wrażliwość, oporność, PEC.

Abstrakt

Celem badania była ocena wrażliwości wybranych grup mikroorganizmów hodowanych na podłożach stałych i w środowisku glebowym na metazachlor (Fuego 500 SC), mieszaninę diflufenikanu + mezosulfuroun metylowego + jodosulfuronu metylo-sodowego (Alister Grande 190 OD)

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i mieszaninę terbutylazyny + mezotrionu + s-metolachloru (Lumax 537.5 SE). Testowanymi drobnoustrojami były: *Azotobacter* spp., *Arthrobacter* spp., *Bradyrhizobium* spp. (lupini), *Rhizobium leguminosarum* bv. *viciae*, *Streptomyces intermedius*, *Streptomyces viridis*, *Streptomyces longisporoflavus*, *Streptomyces odorifer*, *Fusarium* spp., *Aspergillus* spp., *Penicillum* spp., *Rhizopus* spp. Badania te wykazały, że grzyby charakteryzowały się większą wrażliwością na herbicydy niż bakterie i promieniowce. Badane drobnoustroje najbardziej oporne były na zwiększone dawki mieszaniny diflufenikanu + mezosulfuronu metylowego + jodosulfuron metylo-sodowego. Obliczone przewidywane stężenie preparatów w glebie (PEC) w 160 dniu potwierdza, że metazachlor zastosowany w dawkach zanieczyszczających stanowi największe zagrożenie dla bytujących w niej drobnoustrojów. Wartość PEC dla zastosowanych dawek była najwyższa, co dowodzi o możliwości wystąpienia wysokiego ryzyka zanieczyszczenia gleby tym preparatem.

Introduction

The natural environment is increasingly often subjected to anthropogenic contamination, including with herbicides (BAĆMAGA et al. 2014a, KUCHARSKI and WYSZKOWSKA 2008, KUCHARSKI et al. 2009). Due to their widespread use, herbicides are present in various elements of the natural environment, mainly soil and water. Herbicides disrupt the biochemical and physiological responses of weeds, but they can also exert harmful effects on non-target organisms (BAĆMAGA et al. 2012, BAĆMAGA et al. 2014b, WYSZKOWSKA and KUCHARSKI 2004), including microorganisms which quickly respond to environmental changes. Microbes have varied sensitivity to herbicides, and species or strains sensitive to weed control agents are likely to be eliminated from the environment. Resistant organisms are generally characterized by high levels of activity and rapid growth. The responses of microorganisms to herbicides can be indicative of changes taking place in different ecosystems. Variations in microbial activity can be estimated with the use of various tests. Microorganisms play an important role in herbicide degradation, and even the most persistent compounds can be decomposed to forms that are less toxic than the initial substance (DAS and DEY 2013). Microbes can rely on herbicides as sources of nutrients and energy. Microbial consortia decompose herbicides into harmless products more readily than individual species (CASTILLO et al. 2006). Microbes are among the few organisms that can absorb nutrients from various organic and inorganic compounds, which enabled them to colonize all ecosystems and adapt to local conditions. Microorganisms should be used in the process of neutralizing herbicides and other xenobiotics that pose a threat to the environment. Herbicides are generally evaluated for their toxic effects on humans and animals, whereas their impact on microorganisms is rarely investigated. In this study, two laboratory experiments were carried out to evaluate the sensitivity of selected microbial groups cultured on solid media and soil-dwelling microorganisms to metazachlor, a mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium, and a mixture of

terbuthylazine + mesotrione + s-metolachlor. The tested substances can exert different effects on microbes cultured under controlled laboratory conditions and soil-dwelling microorganisms. Soil microbes can grow on soil colloids, which can minimize the negative impact of chemical compounds on microbial development. Based on the results of this study, the tested microorganisms could be used in the process of neutralizing pesticides in soil.

Materials and Methods

The responses of microorganisms cultured on solid media to herbicides

A laboratory experiment was carried out to analyze the effect of metazachlor (active ingredient in the Fuego 500 SC herbicide), a mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium (active ingredients in the Alister Grande 190 OD herbicide) and a mixture of terbuthylazine + mesotrione + s-metolachlor (active ingredients in the Lumax 537.5 SE herbicide) on the growth of the following microorganisms: *Azotobacter* spp., *Arthrobacter* spp., *Bradyrhizobium* spp. (lupini), *Rhizobium leguminosarum* bv. *vicie*, *Streptomyces intermedius, Streptomyces longisporoflavus, Streptomyces odorifer, Streptomyces viridis*, *Rhizopus* spp., *Aspergillus* spp., *Penicillum* spp. and *Fusarium* spp. The tested herbicides are characterized in Table 1. The herbicides were applied in four different doses (Table 2).

Pure microbial cultures were cultivated on agar slants in a thermostat at 28°C (fungi and *Azotobacter* spp. – for 48 h, the remaining bacteria – for 72 h, actinomycetes – for 168 h). The resulting cultures were transferred to agar slants with different media and incubated under identical conditions. The cultures were rinsed off agar slants with 5 cm^3 of aqueous solution of 0.85% NaCl, and they were placed in flasks containing different media in the amount of 1 cm^3 of microorganisms per 100 cm^3 of the medium. Culture media with the microorganisms were poured onto Petri plates in the amount of 15 cm^3. After media solidification, three filter paper discs saturated with different herbicide doses were placed on the plates. Each disc, 6 mm in diameter, was saturated with 5 cm^3 of aqueous herbicide solution. Petri plates were incubated at 28°C (fungi – for 24 h, bacteria – for 48 h, actinomycetes – for 72 h). After incubation, the zones of inhibition created by the tested herbicides for each microbial group were measured in mm. The experiment was performed *in vitro* by the disc diffusion method described by BOROS et al. (2007), in three replications, on six strains of each tested microorganism from the collection of the Department of Microbiology. This qualitative method relies on herbicide

Table 1

General characteristics of the tested herbicides

Name of herbicide	Active ingredient	Chemical group	Action	Dose recommended by manufacturer [dm^3 ha^{-1}]	Manufacturer
Fuego 500 SC	metazachlor	chloroacetanilides	Inhibits mitosis and cell division	2.00	Feinchemia Schwebda GmbH
Alister Grande 190 OD	diflufenican	phenoxy nicotinic acid-amides	Inhibits carotenoid biosynthesis	0.90	BayerCrop Science
	mesosulfuron--methyl	sulfonylureas	Inhibits acetolactate synthase		
	iodosulfuron--methyl-sodium	sulfonylureas	Inhibits acetolactate synthase		
Lumax 537.5 SE	terbuthylazine	triazines	Inhibits photosynthesis in photosystem II A	3.75	Syngenta
	mesotrione	triketones	Inhibits carotenoid and chlorophyll biosynthesis		
	s-metolachlor	chloroacetamides	Inhibits chlorophyll, protein and lipid synthesis		

Table 2

Active ingredient doses applied to filter paper discs on solid media, mg $disc^{-1}$

Name of herbicide	Active ingredient	Active ingredient dose			
		1	2	3	4
Fuego 500 SC	metazachlor	2.500	1.2500	0.6250	0.4160
Alister Grande 190 OD	diflufenican	0.9000	0.4500	0.3000	0.2250
	mesosulfuron-methyl	0.0300	0.0150	0.0100	0.0075
	iodosulfuron-methyl-sodium	0.0225	0.0112	0.0075	0.0056
Lumax 537.5 SE	terbuthylazine	0.9375	0.4687	0.3125	0.2344
	mesotrione	0.1875	0.0937	0.0625	0.0469
	s-metolachlor	1.5625	0.7812	0.5208	0.3906

diffusion from a saturated filter paper disc to a solid culture medium. Herbicides are diffused in a radial pattern and create zones with a concentration gradient. The larger the zone of inhibition, the more sensitive the analyzed microorganism.

Microorganisms were grown and proliferated on the following solid artificial media: *Azotobacter* spp. – on Fenglerowa's medium (1965), *Arthrobacter* spp. – on the medium developed by MULDER and ANTHEUMISSE (1963), *Bradyrhizobium* spp. (lupini) and *Rhizobium leguminosarum* bv. *viciae* – on the YEMB – Vincent medium (1970), *Streptomyces intermedius*, *Streptomyces viridis*, *Streptomyces longisporoflavus* and *Streptomyces odorifer* - on the medium developed by Küster and Williams (PARKINSON et al. 1971), and *Fusarium* spp., *Aspergillus* spp., *Penicillum* spp. and *Rhizopus* spp. on Martin's medium (1950).

Herbicide resistance of soil-dwelling microorganisms

The experiment was performed on sandy loam (Table 3) classified as Eutric Cambisol by the World Reference Base of Soil Resources (2014). Soil samples were collected from the humus horizon at a depth of 0-20 cm, in Tomaszkowo near Olsztyn in north-eastern Poland. Air-dried soil samples of 100 g were passed through a sieve with 2 mm mesh size, placed in 150 cm^3 beakers and combined with different doses of the tested herbicides (Table 1). The applied doses are described in Table 4, and the predicted environmental concentrations (PEC) of active ingredients on day 160 are given in Table 5. Soil was combined with herbicides and brought to 50% capillary capacity with the use of distilled water. Beakers were covered with perforated film and incubated at 25°C for 160 days. After incubation, the counts of organotrophic bacteria were determined on the Bunt and Rovira medium with the addition of soil extract (ALEXANDER 1973), the counts of actinomycetes were determined on the Küster and Williams medium with the addition of antibiotics nystatin and actidione (PARKINSON et al. 1971), and fungal counts were determined on Martin's glucose-peptone agar (1950) with the addition of rose bengal and aureomycin. Petri plates were incubated at 28°C for 7 days (organotrophic bacteria and actinomycetes) and 5 days (fungi). After incubation, the number of colony forming units (CFU) was determined in nine replications. The results were used to calculate the index of microbial resistance (RS) to soil contamination with herbicides according to the formula developed by ORWIN and WARDLE (2004):

$$RS = 1 - \frac{2|D_0|}{C_0 + |D_0|}$$

where:
C_0 – is the soil resistance under natural conditions over time t_0;
P_0 – is the resistance of soil subjected to pressure over time t_0;
$D_0 = C_0 - P_0$.

Table 3

General characteristics of experimental soil

Parameter	Value
sand [2000–50 μm] %	72.00
silt [50–2 μm] %	21.00
clay [<2 μm] %	7.00
pH_{KCl}	7.00
HAC [mmol(+) kg^{-1}]	8.00
TEB [mmol(+) kg^{-1}]	111.00
C_{org} kg^{-1}]	7.05
N_{total} kg^{-1}]	0.86

Explanation: HAC – hydrolytic acidity, TEB – total exchangeable bases, C_{org} – organic carbon content, N_{total} – total nitrogen content

Table 4

Active ingredient doses applied to soil, mg kg^{-1}

Name of herbicide	Active ingredient	Active ingredient dose				
		1	20x	40x	80x	160x
Fuego 500 SC	metazachlor	0.4150	8.3000	16.600	33.200	66.400
Alister Grande 190 OD	diflufenican	0.0540	1.0800	2.1600	4.3200	8.6400
	mesosulfuron-methyl	0.0018	0.0360	0.0720	0.1440	0.2880
	iodosulfuron-methyl-sodium	0.0013	0.0260	0.0520	0.1040	0.2080
Lumax 537.5 SE	terbuthylazine	0.2344	4.6887	9.3750	18.750	37.500
	mesotrione	0.0471	0.9425	1.8850	3.7700	7.5400
	s-metolachlor	0.3906	7.8125	15.625	31.250	62.500

Explanation: 1 – dose recommended by the manufacturer, doses 20-, 40-, 80- and 160-higher than recommended by the manufacturer

Table 5

Predicted environmental concentrations (PEC) in soil on day 160, mg kg^{-1}

Name of herbicide	Active ingredient	Active ingredient dose				
		1	20x	40x	80x	160x
Fuego 500 SC	metazachlor	0.0258	0.5154	1.0309	2.0618	4.1335
Alister Grande 190 OD	diflufenican	0.0144	0.2877	0.5755	1.1509	2.3019
	mesosulfuron-methyl	0.0002	0.0035	0.0070	0.0140	0.0280
	iodosulfuron-methyl--sodium	6.6068E-07	1.3213-05	2.6427E-05	5.2854E-05	0.0001
Lumax 537.5 SE	terbuthylazine	0.0114	0.2283	0.4568	0.9135	1.8270
	mesotrione	0.0013	0.0263	0.0525	0.1051	0.2102
	s-metolachlor	0.0090	0.1822	0.3643	0.7287	1.4573

Explanation: 1 – dose recommended by the manufacturer, doses 20-, 40-, 80- and 160-higher than recommended by the manufacturer

Statistical analyses

The results were processed in the Statistica 10.0 application (StatSoft, Inc. 2011). Homogeneous groups were identified by Tukey's test at a significance level of $p = 0.01$. Microbial responses to the tested herbicides were described by hierarchical cluster analysis (CA) with the use of Ward's method and Euclidean distance. Microbial resistance to herbicides was compared by principal component analysis (PCA). CA and PCA were conducted by exploring multidimensional data sets. Pearson's coefficients of correlation between herbicide dose and microbial resistance were calculated.

Results and Discussion

The responses of microorganisms cultured on solid media to herbicides

Widespread pesticide use in agriculture poses a serious environmental problem. New research is needed to determine the impact of those chemical substances on various organisms, including soil-dwelling microbes, which are reliable indicators of changes in soil environments exposed to stressors (WYSZKOWSKA 2002, ZHANG et al. 2012). Pesticides are degraded in the soil environment, mainly by soil-dwelling microorganisms. Selected bacterial strains, such as *Azotobacter*, *Arthrobacter*, *Pseudomonas* and *Rhodococcus*, are capable of decomposing pesticides, and they are frequently used in bioremediation of pesticide-contaminated soil (PAL et al. 2006). In this study, microbial responses to higher herbicide concentrations were determined by the dose and type of the applied product (Figure 1). In the group of the analyzed bacteria, *Azotobacter* was most resistant to herbicides, and its growth was inhibited only by a mixture of terbuthylazine + mesotrione + s-metolachlor. CHENNAPPA et al. (2014) demonstrated that selected *Azotobacter* species can survive and proliferate in the presence of pesticides. The cited authors tested five *Azotobacter* species (*Azotobacter vinelandii*, *Azotobacter salinestris*, *Azotobacter* sp., *Azotobacter nigricans* subsp. *nigricans*, *Azotobacter tropicalis*) to determine their ability to proliferate on culture media containing pendimethalin, glyphosate, chlorpyrifos and phorate. Thirteen of the 14 evaluated strains proliferated on pesticide-containing media. In our study, bacteria of the genus *Arthrobacter* were most sensitive to the tested herbicides. When the highest herbicide doses were applied, the zone of inhibition for *Arthrobacter* spp. was determined at 22.056 mm (metazachlor), 24.500 mm (diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium) and 24.389 mm (terbuthylazine + mesotrione +

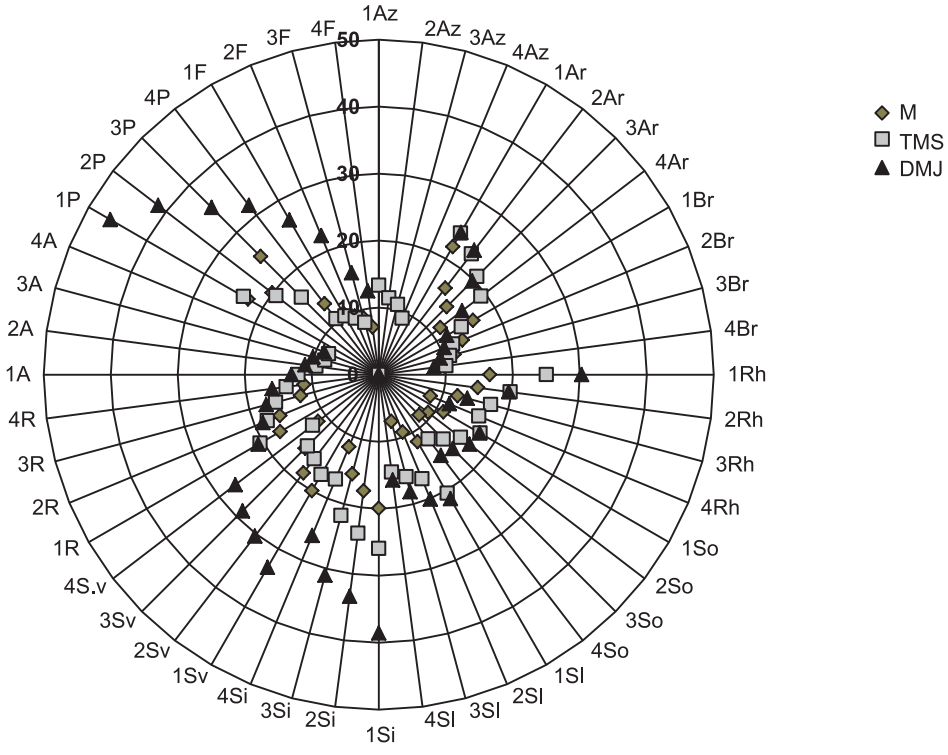

Fig. 1. The effect of herbicides on the growth of microorganisms cultured on solid media
Explanation: 1–4 – herbicide dose, M – metazachlor, DMJ – diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium, TMS – terbuthylazine + mesotrione + s-metolachlor, 0–50 – zone of growth inhibition [mm], *Az – Azotobacter* spp., *Ar – Arthrobacter* spp., *Br – Bradyrhizobium* spp. *(lupine), Rh – Rhizobium leguminosarum* bv. *vicie, Sl – Streptomyces longisporoflavus, Si – Streptomyces intermedius, Sv – Streptomyces viridis, So – Streptomyces odorifer, R – Rhizopus* spp., *A – Aspergillus* spp., *P – Penicillium* spp., *F – Fusarium* spp.

s-metolachlor). *Rhizobium leguminosarum* bv. v*icie* and *Bradyrhizobium* spp. (*lupini*) responded similarly to all evaluated herbicides. The most notable changes in *Rhizobium leguminosarum* bv. v*icie* were observed after the addition of the diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium mixture to the culture medium, and in *Bradyrhizobium* spp. (*lupini*) – after the addition of metazachlor. The varied influence of the analyzed herbicides can be attributed to differences in dose as well as microbial species or strain. SHARMA and KHANNA (2011) compared the impact of two herbicides, fluchloralin and pendimethalin, on the growth of bacteria of the genus *Rhizobium*. They concluded that fluchloralin ($20.25 \cdot 10^4$ mg kg^{-1}) and the lowest dose of pendimethalin ($9.00 \cdot 10^4$ mg kg^{-1}) did not exert a negative influence on the analyzed bacteria, but higher doses of pendimethalin ($15.9 \cdot 10^4$ mg kg^{-1}) inhibited the

growth of *Rhizobium* bacteria. An in vitro experiment conducted by ALLIEVI and GIGLIOTTI (2001) demonstrated that cinosulfuron applied in the amount of 100 mg dm^{-3} had an adverse effect on the growth of *Rhizobium leguminosarum* and *Bradyrhizobium japonicum*.

Actinomycetes were also sensitive to herbicides that diffused from filter paper discs into culture media, in particular at the highest doses. *Streptomyces odorifer* and *Streptomyces longisporoflavus* were most resistant, whereas *Streptomyces intermedius* and *Streptomyces viridis* were most sensitive to the tested substances. The mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium had the most inhibitory effect on the growth of actinomycetes, and it produced inhibition zones with an average diameter of: 16.32 mm for *Streptomyces odorifer*, 18.88 mm for *Streptomyces longisporoflavus*, 29.85 mm for *Streptomyces viridis,* and 32.24 mm for *Streptomyces intermedius*. Metazachlor had the least inhibitory effect on the tested species of actinomycetes. According to SETTE et al. (2004), actinomycetes are characterized by considerable physiological and metabolic diversity, which is why they play an important role in the degradation of chemical compounds that are released into the environment. The cited authors demonstrated that *Streptomyces* spp. strains are resistant to increased doses of alachlor. An alachlor dose of 144 mg dm^{-3} was degraded in 60–75% in 14 days.

In the present study, the tested herbicides also induced changes in the growth pattern of molds. Metazachlor did not exert a negative effect on *Aspergillus* spp., and it had only a minor influence on *Fusarium* spp. (the average zone of inhibition in response to the highest metazachlor dose was 10.56 mm). The mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium had the least toxic effect on *Aspergillus* spp., whereas the mixture of terbuthylazine + mesotrione + s-metolachlor was least toxic for *Fusarium* spp. *Penicillium* spp. was most sensitive to all of the analyzed herbicides, and the diameter of its inhibition zone was determined at 22.556 mm for metazachlor, 46.278 mm for the diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium mixture, and 23.333 mm for the terbuthylazine + mesotrione + s-metolachlor mixture. Fungi, in particular *Penicillium* spp., were highly sensitive to the tested herbicides. Somewhat different results were reported by KODAMA et al. (2001), in whose study, only the DS6F *Penicillium steckii* strain was capable of degrading simazine, and the rate of decomposition increased after glucose was introduced to the substrate as a source of carbon. Simazine, which was added to the substrate in the amount of 25 mg dm^{-3} and 50 mg dm^{-3}, was degraded in 53% after 5 days.

The dispersal of objects in a system of two principal components is presented in Figure 2. The horizontal axis explains 61.15% of total variance, the vertical axis – 21.24% of total variance, and the two explain 82.39% of variance in

primary variables. An analysis of the first principal component revealed two homogeneous groups. The first group is represented by *Rhizobium leguminosarum* bv. v*icie*, *Aspergillus* spp., *Rhizopus* spp., *Arthrobacter* spp., *Streptomyces odorifer* and *Streptomyces longisporoflavus,* and the second group – by *Fusarium* spp., *Penicillium* spp., *Streptomyces intermedius* and *Streptomyces viridis*. A homogeneous group comprising *Bradyrhizobium* spp. (*lupini*) and *Azotobacter* spp. was formed around the second principal component. The location of vectors along the axes of the coordinate system indicates that the

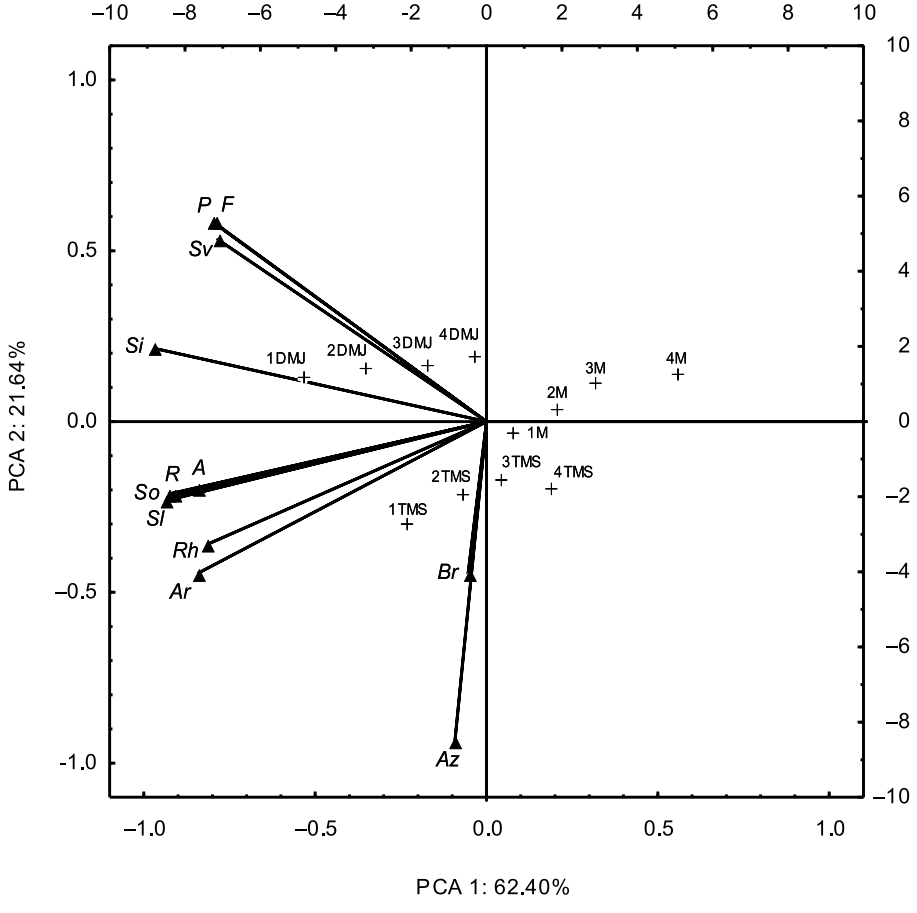

Fig. 2. A comparison of the sensitivity of microorganisms cultured on solid media to metazachlor, a mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium, and a mixture of terbuthylazine + mesotrione + s-metolachlor, determined by PCA

Explanation: 1–4 – herbicide dose, M – metazachlor, DMJ – diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium, TMS – terbuthylazine + mesotrione + s-metolachlor, *Az – Azotobacter* spp., *Ar – Arthrobacter* spp., *Br – Bradyrhizobium* spp. *(lupine), Rh – Rhizobium leguminosarum* bv. *vicie, Sl – Streptomyces longisporoflavus, Si – Streptomyces intermedius, Sv – Streptomyces viridis, So – Streptomyces odorifer, R – Rhizopus spp., A – Aspergillus* spp., *P – Penicillium* spp., *F – Fusarium* spp.

tested herbicides influenced microbial growth. The distribution of cases in the four quarters also suggests that the analyzed substances had a varied effect on the growth and development of microorganisms.

The responses of the tested microbes to herbicides were confirmed by cluster analysis involving Ward's method. The results are presented in a dendrogram in Figure 3. The analysis led to the identification of five clusters. A high degree of similarity was observed between *Azotobacter* spp. and *Aspergillus* spp., between *Arthrobacter* spp., *Rhizobium leguminosarum* bv. *viceae* and *Rhizopus* spp., between *Bradyrhizobium* spp. (lupini) and *Fusarium* spp., between *Streptomyces odorifer* and *Streptomyces longisporoflavus,* and between *Streptomyces intermedium* and *Streptomyces viridis*. *Penicillium* spp. fungi were most sensitive to the analyzed herbicides, and they differed most significantly from the remaining microorganisms.

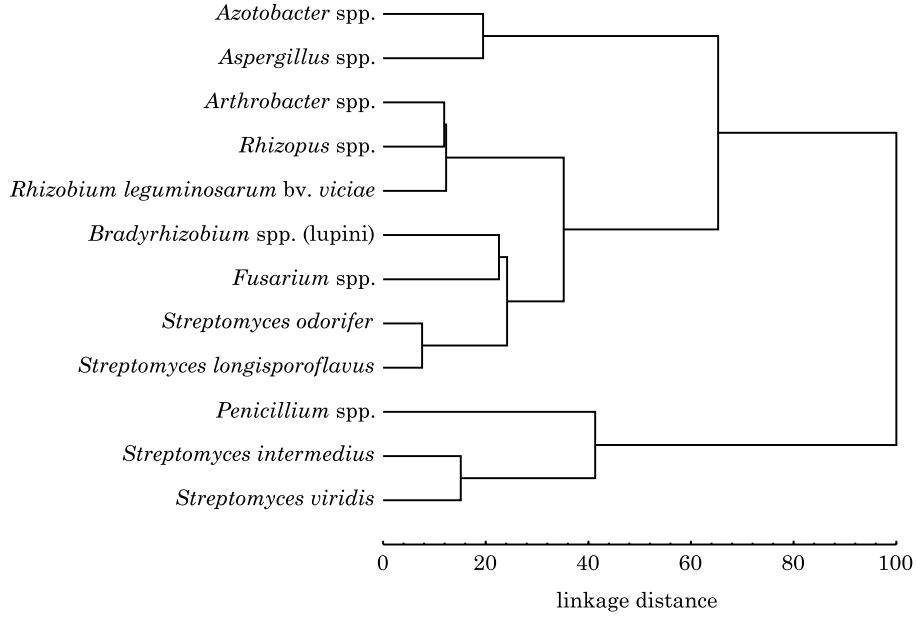

Fig. 3. Similarities in the responses of microorganisms cultured on solid media to the tested herbicides

Herbicide resistance of soil-dwelling microorganisms

Organic compounds, including herbicides, can pose a serious threat to the soil environment by causing a long-term disruption of the soil's biological balance (BAĆMAGA et al. 2014a, BAĆMAGA et al. 2014b, CHOWDHURY et al. 2008, CYCOŃ and PIOTROWSKA-SEGET 2007, JASTRZĘBSKA and KUCHARSKI 2007). The

herbicides tested in this study had a significant impact on soil-dwelling microorganisms (Table 6). The herbicide resistance of microorganisms was determined by the type and dose of the applied weed control agent. In metazachlor treatments, the lowest values of the RS index for actinomycetes (0.224) and fungi (0.281) were observed after the application of a dose that was 160-higher than the dose recommended by the manufacturer. Organotrophic bacteria were most resistant to the same dose of metazachlor, and their RS index reached 0.708. The mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium contributed to an increase in the value of the RS index. The only exception were organotrophic bacteria whose RS index decreased after the application of doses that were 20- and 40-fold higher than the recommended dose. The tested microbes were characterized by varied resistance to soil contamination with a mixture of terbuthylazine + mesotrione + s-metolachlor. When introduced to the soil at high doses, the mixture increased the RS index of actinomycetes and fungi, and lowered the RS index of organotrophic bacteria. The highest value of the RS index for organotrophic bacteria was noted in treatments with the optimal herbicide dose (RS = 0.915), for actinomycetes – in treatments were the herbicide dose was 80-times higher than the recommended dose (RS = 0.558), and for fungi – in treatments where the herbicide dose was 20- and 40-times higher than the recommended dose (RS = 0.696 and RS = 0.697, respectively). Regardless of herbicide dose, metazachlor had the most inhibitory influence on microbial development. The RS index assumed the lowest average values in metazachlor treatments: 0.537 for organotrophic bacteria, 0.288 for actinomycetes and 0.469 for fungi. The mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium had the least significant impact on the values of the RS index. The average values of the RS index indicate that organotrophic bacteria and fungi were most resistant to the above mixture. Microbial resistance to the examined herbicides, determined by PCA, is presented in Figure 4. The first two principal components explained 82.44% of total variance. The length of primary variable vectors determines their influence on the distribution of principal components. The first principal component was negatively correlated with actinomycetes and fungi, and the second principal component – with organotrophic bacteria. The distribution of cases in the chart indicates that herbicide doses exerted a varied effect on soil-dwelling microorganisms. The influence of weed control products on soil microbes could be attributed to microbial tolerance of active ingredients in the analyzed preparations. Those compounds could be an excellent source of nutrients for selected microorganisms, but they could be toxic and lethal for other microbial groups (CROUZET et al. 2010, ZABALOY et al. 2010). According to GRIFFITHS and PHILIPPOT (2013), and ORWIN and WARDLE (2004), soil resistance and resilience values can be used to determine the analyzed ecosystem's sensitivity to various

stressors. In this study, the values of the RS index indicate that metazachlor had the most inhibitory effect on the proliferation of soil microbes. Soil resistance and resilience indicators provide information about the status of soil environments contaminated with organic compounds, including pesticides (BAĆMAGA et al. 2015, ORWIN and WARDLE 2004). There is a general scarcity of published data about the influence of herbicides on soil resistance, and the results of this study can expand our knowledge about the resistance of soil contaminated with metazachlor, a mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium and a mixture of terbuthylazine + mesotrione + s-metolachlor.

Table 6

Microbial resistance (RS) to soil contamination with herbicides

Dose of herbicide	Microorganisms		
	B_{org}	Act	Fun
Metazachlor (M)			
1	0.452^{c}	0.335^{def}	0.578^{abc}
20	0.398^{c}	0.324^{def}	0.227^{c}
40	0.543^{abc}	0.296^{efg}	0.850^{ab}
80	0.586^{abc}	0.263^{fg}	0.406^{abc}
160	0.708^{abc}	0.224^{g}	0.281^{bc}
Average	0.537	0.288	0.468
r	0.938*	-0.982*	-0.384
Diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium (DMJ)			
1	0.709^{abc}	0.360^{de}	0.762^{abc}
20	0.599^{abc}	0.365^{de}	0.948^{a}
40	0.620^{abc}	0.366^{de}	0.877^{a}
80	0.862^{ab}	0.483^{ab}	0.863^{ab}
160	0.904^{a}	0.386^{cde}	0.882^{a}
Average	0.739	0.392	0.866
r	0.821	0.357	0.250
Terbuthylazine + mesotrione + s-metolachlor (TMS)			
1	0.915^{a}	0.360^{de}	0.550^{abc}
20	0.574^{abc}	0.374^{cde}	0.696^{abc}
40	0.549^{bc}	0.406^{bcd}	0.697^{abc}
80	0.546^{bc}	0.558^{a}	0.673^{abc}
160	0.542^{bc}	0.463^{bc}	0.514^{abc}
Average	0.625	0.432	0.626
r	-0.572	0.625	-0.456

Explanation: Homogeneous microbial groups are marked with the same letters in columns: B_{org} – organotrophic bacteria, Act – actinomycetes, Fun – fungi; 1 – dose recommended by the manufacturer, doses 20-, 40-, 80- and 160-higher than recommended by the manufacturer; *r* – coefficient of correlation significant at $*p=0.01$

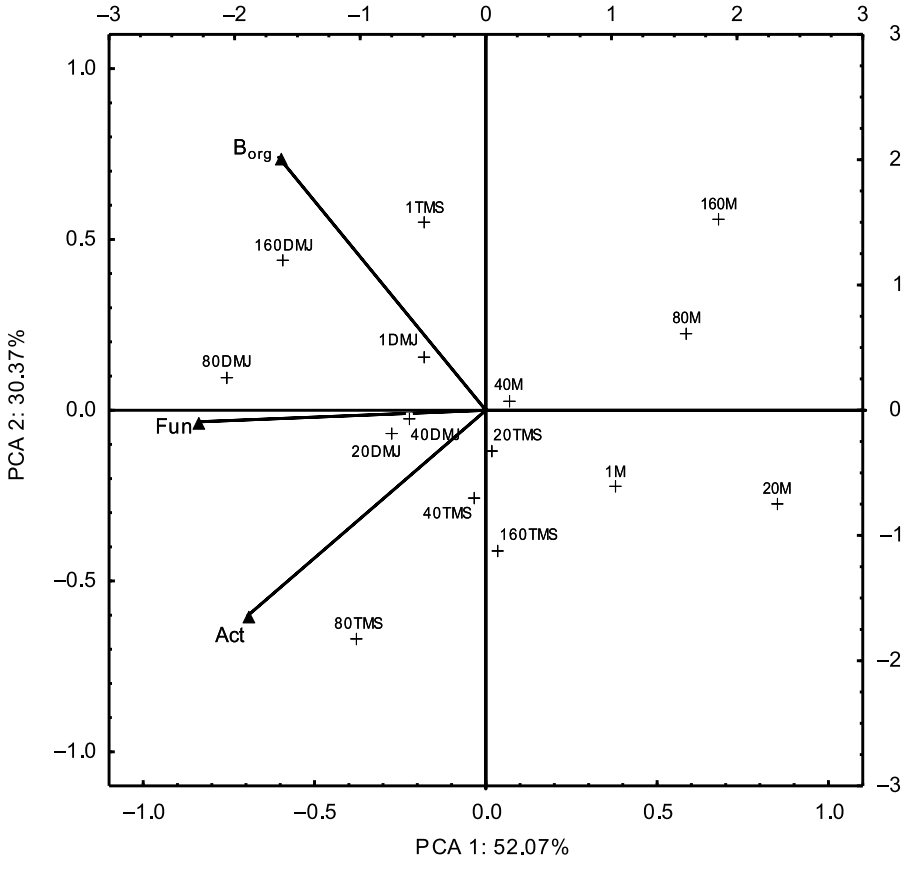

Fig. 4. Microbial resistance to soil contamination with metazachlor, a mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium, and a mixture of terbuthylazine + mesotrione + s-metolachlor, determined by PCA

Explanation: B_{org} – organotrophic bacteria, Act – actinomycetes, Fun – fungi; 1 – dose recommended by the manufacturer, doses 20-, 40-, 80- and 160-higher than recommended by the manufacturer, M – metazachlor, DMJ – diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium, TMS – terbuthylazine + mesotrione + s-metolachlor

Conclusions

In this experiment, microorganisms responded differently to the tested herbicides, subject to the type and dose of the applied product. *Penicillium* spp. fungi cultured on soil media *in vitro* were most sensitive to herbicides, in particular to the mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium. The mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium had the most inhibitory effect on actinomycetes

and fungi, whereas the mixture of terbuthylazine + mesotrione + s-metolachlor was most toxic for *Azotobacter* spp. and *Arthrobacter* spp. Metazachlor diffusing from filter paper discs into culture media had the least inhibitory effect on microorganisms, excluding *Bradyrhizobium* spp. (*lupini*) bacteria whose growth was most severely impaired by the above compound. The application of metazachlor to soil exerted the most negative influence on organotrophic bacteria, actinomycetes and fungi. The tested microbes were most resistant to increased doses of the mixture of diflufenican + mesosulfuron-methyl + iodosulfuron-methyl-sodium. Predicted environmental concentrations (PEC) calculated on day 160 indicate that increased doses of metazachlor posed the greatest threat for soil-dwelling microorganisms. The applied doses of metazachlor resulted in the highest PEC values, which points to a high risk of soil contamination with this weed control agent.

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EFFECT OF STORAGE OF ROKPOL CHESSE ON VOLATILE COMPOUNDS PROFILES

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K e y w o r d s: mold cheese, HS-SPME, volatile compounds, principal component analysis, cluster analysis.

A b s t r a c t

The aim of this study was to investigate changes of volatile compounds in Rokpol blue cheese during storage under different temperature conditions (4°C, 25°C, 35°C). Headspace solid-phase microextraction (HS-SPME) was used to isolate volatile compounds from the matrix and GC/MS was used for compounds separation and identification. Received aroma profiles were showed in the analyzed cheese and statistical analysis were done based on the identified groups of compounds. Results were interpreted on the basis of principal component analysis and cluster analysis. The dominant group of compounds represented ketones. The largest decrease in quality in the profile of volatile compounds was observed during storage at 25°C. Profile of volatile compounds remains similar during the week in 4°C and two days at 25°C.

WPŁYW WARUNKÓW PRZECHOWYWANIA NA ZMIANY W PROFILU ZWIĄZKÓW LOTNYCH SERA TYPU ROKPOL

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S ł o w a k l u c z o w e: ser pleśniowy, HS-SPME, związki lotne, analiza składowych głównych, analiza skupień.

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Abstrakt

Celem pracy było zbadanie zmian profili związków lotnych w serze pleśniowym Rokpol w czasie przechowywania w różnej temperaturze (4°C, 25°C, 35°C). W celu izolacji związków lotnych z matrycy sera wykorzystano technikę mikroekstrakcji z fazy nadpowierzchniowej (HS-SPME), natomiast do rozdziału i identyfikacji zastosowano chromatografie gazową sprzężoną ze spektrometrią mas (GC/MS). Przedstawiono otrzymane profile związków lotnych w badanym serze oraz dokonano analizy w oparciu o grupy zidentyfikowanych związków, a wyniki zinterpretowano w oparciu o analizę składowych głównych oraz analizę skupień. Dominującą grupę związków stanowiły ketony. Największy spadek jakościowy w profilu związków lotnych odnotowano podczas przechowywania w temperaturze 25°C. Profil związków lotnych pozostaje podobny podczas tygodniowego przechowywania w lodówce i dwóch dni w temperaturze 25°C.

Introduction

The formation of unique characteristics of cheese is conditioned on the production process, including the type of milk used, humidity, NaCl content, pH of the product, a kind of used starter cultures and secondary microorganism as well as production stages. All these components have a significant impact on the biochemical changes occurring during the ripening process and future storage of cheese (FOX and MCSWEENEY 2004).

Milk fat is essential for proper formation of cheese flavor. Furthermore, the fat content affects microstructure, biochemical changes, production efficiency, rheological and textural properties of the cheese. The last properties influence how fast are the flavor compounds from the matrix of the cheese released (GUINEE and MCSWEENEY 2006).

Studying the formation of flavor compounds is extremely important from the point of view of cheese products production, ripening inspection and ways of accelerating the ripening time of cheese. Moreover it helps to avoid the appearance of any foreign smell in the cheese. The main biochemical changes occurring during maturation is glycolysis, lipolysis and proteolysis. Subsequent changes include the metabolism of compounds that arose as the result of major transformations (FOX et al. 1995).

Volatile compounds of food constitute a complicated system of analytes that often occur in trace amounts (ng/kg of product). However, from the point of view of the product flavor, not only the content of individual compounds is important, but also their sensory detection threshold. It is defined as the lowest concentration level, which allows the consumer to smell a compound (JELEŃ 2004, SURBURG and JOHANNES 2006).

The aroma of cheese is affected by the variety of groups of compounds including e.g. alcohols, aldehydes, ketones, esters and lactones. The alcohols may be generated during cheese maturation in the metabolism of lactose and amino acids, the reduction of ketones or acids degradation (eg. linoleic and

linolenic acids). In the case of mildew cheeses the presence of 1-octen-3-ol is related to the metabolism of the mold *Penicillium*. Alcohols that constitue from changes of branched and aromatic amino acids and that influence cheeses aroma are e.g.: 3-methyl-butanol, 2-methyl-butanol, 2-methylpropanol, phenylethanol and tryptofol (MOLIMARD and SPINNLER 1996, YVON and RIJNEN 2001, CURIONI and BOSSET 2002, MARILLEY and CASEY 2004, VITOVA et al. 2006).

The substrate for the formation of aldehydes are amino acids produced during proteolysis. They are considered as transitional compounds because they are rapidly reduced to primary alcohols or oxidized to the suitable acids. The compounds that are often found in cheese and affecting their odor are: 2-methylpropanal, 2-metylobutanal and 3-metylobutanal (YVON and RIJNEN 2001, CURIONI and BOSSET 2002, MARILLEY and CASEY 2004).

The aim of the study was to research qualitative and quantitative changes in the profile of volatile compounds in Rokpol cheese, caused by different storage temperature and time.

Material and Methods

The research material was Rokpol cheese – Polish cheese veined throughout with the blue mold. It is patterned on the French cheese Roquefort, in which the characteristic taste and appearance is achieved by fungi *Penicillium roqueforti*. The cheese was purchased in one of Warsaw's supermarkets immediately after delivery. Then it was cut into pieces of the same size and vacuum packed. The cheese samples that was the control samples were frozen at -18°C immediately after packaging (samples marked as „0"). Further samples were placed in a refrigerator at a temperature of 4°C ± 2°C (labeled as „L"). Some were incubated at 25°C ± 2°C and the last were incubated at 35°C ± 1°C (denoted by the letters „P" and „C"). Samples were taken from the refrigerator every 7 days, while samples stored in 25°C and 35°C were collected every 24 hours. The figure accompanying the letter of the associated conditions meant another day or a week of storage. Immediately after taking each sample, it was frozed and stored at -18°C until analysis.

The procedure of sample preparation for analysis involved cheese grating with a fine mesh. Than 3 g of cheese were weighted to 20 ml vial and 1fl of internal standard solution (trans-2-decanal 0.067 μl/ml) was added. After sealing the vial, the sample was incubated at 40°C for 20 minutes. The volatiles were then extracted by headspace microextraction using SPME fiber type CAR/PDMS/DVB at 40°C for 20 minutes. The conditions for conditioning and extraction were determined experimentally. Volatiles were desorbed from the fiber in the injector chamber for 3 minutes at 220°C.

Chromatographic analysis was performed by gas chromatography coupled with mass spectrometer GCMS-QP2010S (Schimadzu). The column used was non-polar ZB-5ms (phase 5%-phenyl-95%-dimetylopolisiloksan arylene) with dimensions of 30 m x 0.25 mm x 0.25 mm. The temperature of the chromatography oven was programmed as follows: isothermal 40°C for 10 minutes, then the ramp rate of 4°C/min to 220°C isothermal for 5 minutes. The carrier gas was helium with a flow rate of 1.1 cm^3/min and a constant linear velocity. Data collection was performed in sweep 40-300 m/z, using the ionization energy of 70eV.

In order to calculate the retention indexes of the volatile compounds the mixture of n-alkanes C7 ÷ C30 was used (Sigma-Aldrich). Identification of volatiles was carried out on the basis of the mass spectra library WILEY7N2, NIST147 and NIST2008 and Kovats retention indexes which are available online (The Pherobase).

Statistical analysis was performed using Statistica 10.0. One-way analysis of variance (ANOVA) at the significance level $p \leq 0.05$. To evaluate the differences between mean values Tukey HSD test was used. In order to illustrate the differences in the volatile profiles of tested samples and because of the amount of compounds identified, obtained data were statistically analyzed by PCA (Principal Components Analysis). The results have been supplemented by CA (Cluster Analysis).

Results and Discussion

In the tested Rokpol samples a total of 37 of volatile compounds belonging to six chemical groups were identified: aldehydes (1), alcohols (9), fatty acids (5), esters (13), ketones (7), hydrocarbons (2).

Ketones, esters, alcohols and acids had the largest share in the volatile fraction of the control sample. Hydrocarbons were present at a low level and aldehydes were not detected at all. Additionally there were identified compounds commonly present in the cheese mold, such as 2-pentanol, 2,3-butanediol, 2-heptanol, 1-octen-3-ol, 2-pentanone, 3-hydroxy-2-butanone, 2-heptanone, 8-nonene-2-one and 2-nonanone. The volatile compounds typical for mould cheeses are fatty acids containing up to 10 carbon atoms and their methyl and ethyl esters. Those were also identified in the tested samples.

The largest share in the headspace phase constituted ketones such as 2-nonanone (with floral, fruity scents), and 2-heptanone (with mold, sweet and musty scents). Moreover, a large share in volatile fraction had also other compounds such as 2-heptanol, butanoic acid and hexanoic acid, as well as the ethyl esters of these acids. The group of compounds present in all samples at

a constant level irrespective of the conditions and storage time were: 2-pentanol, 2-nonanol, ethyl esters of butanoic acid and hexanoic acid and 2-undecan. The obtained volatile compounds profile of Rokpol cheese was consistent with the results obtained by other authors (MOLIMARD and SPINNLER 1996, CURIONI and BOSSET 2002, FRANK et al. 2004, TRIHAAS et al. 2005, BZDUCHA and OBIEDZIŃSKI 2006).

In the samples stored for one week under refrigerated conditions there was noticeable significant decrease in the intensity of all detected compounds as compared to the control sample. During following weeks of storage there was observed an increase in the intensity of synthesis of esters and acids, which finally after 4 weeks of storage had the largest share in the volatile fraction (Figure 1).

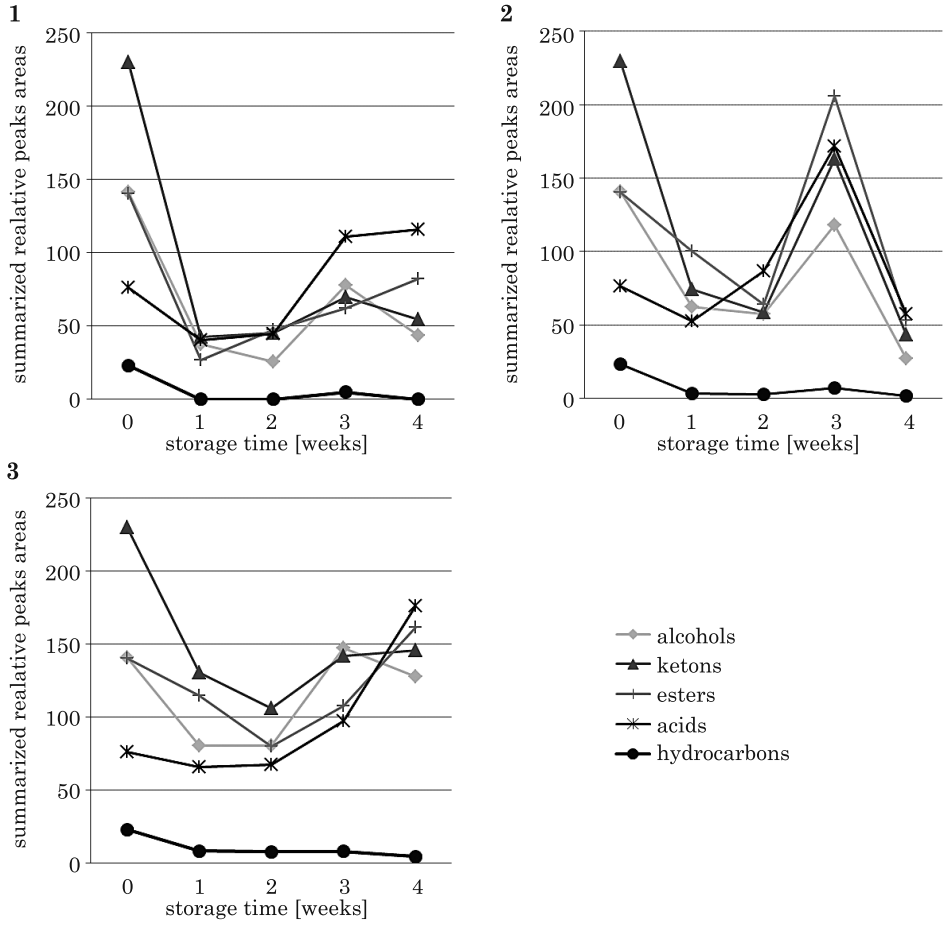

Fig. 1., 2. and 3. Relative peak areas changes of particular compound groups during different storage conditions: refrigerated (1), 25°C (2), 35°C (3)

Table 1

Content of volatile compounds in investigated cheese samples [average relative peaks areas in relation to the standard relative peak area (trans-2-decenal)]

Compound name		RI	Control	Refrigerated (4°C)				Temperature 25°C				Temperature 35°C			
			after purchase	1 week	2 week	3 week	4 week	1 day	2 day	3 day	4 day	1 day	2 day	3 day	4 day
1		2	3	4	5	6	7	8	9	10	11	12	13	14	15
Alcohols	2-pentanol	734	$10{,}1^{a}$ +4,9	$2{,}1^{a}$ +0,1	$1{,}3^{a}$ +0,2	$5{,}0^{a}$ +0,6	$3{,}0^{a}$ +0,2	$5{,}2^{a}$ +1,2	$4{,}3^{a}$ +1,4	$10{,}6^{a}$ +8,7	$2{,}3^{a}$ +0,3	$6{,}5^{a}$ +0,6	$5{,}0^{a}$ +0,6	$11{,}3^{a}$ +4,5	$9{,}0^{a}$ +0,4
	3-methyl-1-butanol	755	$11{,}6^{a}$ +4,8	$1{,}7^{b}$ +0,1	$0{,}8^{b}$ +0,1	$3{,}1^{b}$ +0,5	nd	$3{,}8^{b}$ +0,9	$1{,}9^{b}$ +0,5	$4{,}6^{a,b}$ +3,6	$1{,}0^{b}$ +0,1	$4{,}1^{b}$ +0,7	$3{,}8^{b}$ +0,2	$5{,}9^{a,b}$ +2,0	$5{,}1^{a,b}$ +0,1
	2,3-butandiol	788	$2{,}4^{a,b}$ +0,8	$0{,}8^{b,c}$ +0,1	nd	$1{,}7^{a,b,c}$ +0,5	$2{,}0^{a,b,c}$ +0,1	nd	nd	nd	nd	nd	nd	$3{,}6^{a}$ +1,7	$3{,}3^{a}$ +0,1
	1,3-butandiol	793	$5{,}8^{a,b}$ +1,0	$2{,}5^{b}$ +0,4	$4{,}5^{a,b}$ +1,1	$4{,}8^{a,b}$ +2,1	$9{,}9^{a}$ +0,4	$4{,}0^{a,b}$ +2,0	$2{,}1^{b}$ +0,7	$5{,}3^{a,b}$ +4,6	$1{,}5^{b}$ +0,7	$4{,}4^{a,b}$ +0,9	$4{,}2^{a,b}$ +0,5	$7{,}5^{a,b}$ +2,9	$7{,}1^{a,b}$ +0,9
	1,3-propandiol	831	nd	$0{,}8^{a,b}$ +0,1	nd	$2{,}0^{a,b}$ +0,6	nd	$2{,}2^{a,b}$ +1,4	$2{,}2^{a,b}$ +0,5	$3{,}2^{a}$ +1,2	$1{,}0^{b}$ +0,5^{a}	$2{,}2^{a,b}$ +0,6	$1{,}8^{a,b}$ +0,3	$2{,}2^{a,b}$ +1,5	nd
	2-heptanol	902	$73{,}2^{a}$ +17,2	$13{,}5^{c,d}$ +0,1	$7{,}5^{d}$ +0,5	$25{,}6^{b,c,d}$ +7,7	$10{,}8^{c,d}$ +0,3	$26{,}1^{b,c,d}$ +7,3	$20{,}2^{c,d}$ +4,0	$39{,}1^{a,b,c,d}$ +26,1	$12{,}0^{c,d}$ +3,0	$37{,}8^{a,b,c,d}$ +0,7	$32{,}0^{a,b,c,d}$ +1,6	$64{,}1^{a,b}$ +15,3	$50{,}7^{a,b,c}$ +11,5
	1-octen-3-ol	980	nd	nd	nd	nd	nd	0,8 +0,3^{a}	nd	nd	nd	nd	nd	nd	nd
	2-octanol	1002	nd	$6{,}8^{b}$ +0,1	nd	$12{,}2^{a,b}$ +1,5	nd	nd	$8{,}4^{a,}$ +0,3	$26{,}3^{a}$ +16,2	nd	nd	$11{,}3^{a,b}$ +1,8	$17{,}2^{a,b}$ +3,9	$15{,}4^{a,b}$ +4,9
	2-nonanol	1101	$37{,}9^{a}$ +8,0	$9{,}3^{a}$ +1,5	$11{,}2^{a}$ +0,2	$23{,}5^{a}$ +6,6	$17{,}6^{a}$ +1,3	$20{,}1^{a}$ +5,7	$18{,}2^{a}$ +1,3	$29{,}2^{a}$ +18,3	$9{,}1^{a}$ +2,5	$25{,}6^{a}$ +2,5	$22{,}2^{a}$ +0,6	$35{,}4^{a}$ +10,3	$36{,}9^{a}$ +13,7
Aldehydes	nonanal	1104	nd	nd	$0{,}7^{a}$ +0,4	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

cont. Table 1

1		2	3	4	5	6	7	8	9	10	11	12	13	14	15
Esters	methylbutanoate	747	$11{,}5^{a}$ +3,9	nd	nd	nd	nd	$9{,}2^{a,b}$ +1,6	$2{,}7^{a,b}$ +0,3	$8{,}7^{a,b}$ +7,2	$1{,}6^{a,b}$ +0,5	$5{,}0^{a,b}$ +3,9	$2{,}1^{a,b}$ +1,3	$2{,}17^{a,b}$ +0,77	nd
	ethylbutanoate	802	$33{,}1^{a}$ +6,2	$6{,}5^{a}$ +0,1	$11{,}4^{a}$ +1,4	$15{,}6^{a}$ +1,8	$23{,}4^{a}$ +0,4	$19{,}4^{a}$ +5,9	$17{,}1^{a}$ +4,4	$43{,}5^{a}$ +37,5	$14{,}8^{a}$ +4,6	$25{,}5^{a}$ +0,9	$18{,}9^{a}$ +3,7	$36{,}4^{a}$ +11,9	$36{,}8^{a}$ +2,2
	propylbutanoate	899	$10{,}7^{a,b}$ +4,4	$1{,}8^{b}$ +0,1	$1{,}3^{b}$ +0,1	$7{,}0^{a,b}$ +0,9	$4{,}5^{a,b}$ +0,2	$6{,}4^{a,b}$ +1,6	$9{,}6^{a,b}$ +2,7	$24{,}5^{a}$ +18,2	$8{,}0^{a,b}$ +1,9	$5{,}3^{a,b}$ +0,9	$4{,}4^{a,b}$ +1,0	$8{,}6^{a,b}$ +3,3	$13{,}3^{a,b}$ +0,9
	methylhexanoate	924	$25{,}2^{a}$ +2,9	$0{,}8^{a}$ +0,1	nd	$7{,}8^{a}$ +3,4	$4{,}6^{a}$ +0,9	$28{,}0^{a}$ +13,3	$6{,}1^{a}$ +0,3	$35{,}7^{a}$ +26,6	$5{,}1^{a}$ +2,9	$30{,}5^{a}$ +28,9	$17{,}4^{a}$ +10,5	$11{,}3^{a}$ +10,4	$13{,}7^{a}$ +1,3
	1-methylpropyl-butanoate	996	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	$13{,}0^{a}$ +1,5
	ethylhexanoate	999	$30{,}7^{a}$ +11,3	$13{,}1^{a}$ +0,9	$27{,}2^{a}$ +3,9	$16{,}5^{a}$ +6,0	$27{,}7^{a}$ +3,0	$18{,}5^{a}$ +6,6	$15{,}0^{a}$ +2,2	$52{,}5^{a}$ +35,7	$14{,}7^{a}$ +3,9	$26{,}6^{a}$ +3,5	$21{,}9^{a}$ +2,3	$36{,}2^{a}$ +10,2	$52{,}9^{a}$ +9,9
	1-methylbuthyl-butanoate	1056	$6{,}3^{a}$ +2,3	$0{,}9^{b}$ +0,2	nd	$2{,}2^{a,b}$ +0,7	nd	$2{,}3^{a,b}$ +0,6	$1{,}7^{a,b}$ +0,2	$4{,}9^{a,b}$ +3,3	$1{,}0^{b}$ +0,3	$2{,}2^{a,b}$ +0,4	$2{,}4^{a,b}$ +0,4	$2{,}7^{a,b}$ +1,0	$4{,}7^{a,b}$ +1,6
	propylhexanoate	1094	$13{,}1^{a,b}$ +2,7	$2{,}6^{b}$ +0,6	$3{,}1^{a,b}$ +0,1	$7{,}4^{a,b}$ +2,2	$7{,}2^{a,b}$ +0,4	$5{,}8^{a,b}$ +1,4	$6{,}1^{a,b}$ +0,2	$16{,}1^{a}$ +10,3	$4{,}4^{a,b}$ +1,1	$7{,}8^{a,b}$ +0,3	$6{,}2^{a,b}$ +0,1	$10{,}3^{a,b}$ +2,1	$13{,}5^{a,b}$ +4,8
	methyloctanoate	1123	$3{,}4^{a}$ +0,8	nd	nd	nd	nd	$4{,}2^{a}$ +2,2	nd	$4{,}6^{a}$ +3,2	nd	$4{,}1^{a}$ +3,5	$25{,}0^{a}$ +1,1	nd	nd
	1-methylbuthyl-hexanoate	1137	$3{,}2^{a,b}$ +1,1	$1{,}4^{a,b}$ +0,4	$2{,}7^{a,b}$ +0,1	$3{,}7^{a,b}$ +1,2	$3{,}5^{a,b}$ +0,6	$2{,}8^{a,b}$ +0,8	$4{,}4^{a}$ +0,2	$4{,}6^{a}$ +2,7	$1{,}6^{a,b}$ +0,5	$2{,}0^{a,b}$ +0,2	nd	nd	nd
	ethyloctanoate	1196	nd	nd	$0{,}8^{b,c}$ +0,1	nd	$3{,}9^{a,b,c}$ +0,1	nd	nd	$4{,}3^{a,b}$ +3,0	$1{,}2^{a,b,c}$ +0,2	nd	nd	nd	$5{,}1^{a}$ +2,0
	methyldecanoate	1323	$3{,}2^{a}$ +0,7	nw	nw	$2{,}0^{a}$ +0,8	$3{,}3^{a}$ +0,6	$3{,}9^{a}$ +1,7	$1{,}4^{a}$ +0,1	$6{,}2^{a}$ +3,9	$0{,}9^{a}$ +0,5	$5{,}6^{a}$ +5,5	$3{,}9^{a}$ +3,4	nd	$2{,}8^{a}$ +0,4
	ethyldecanoate	1394	nd	nd	$0{,}9^{b}$ +0,1	nd	$4{,}1^{a}$ +0,5	nd	nd	nd	nd	nd	nd	nd	$5{,}5^{a}$ +2,1

cont. Table 1

1		2	3	4	5	6	7	8	9	10	11	12	13	14	15
Ketones	2-pentanone	728	nd	nd	0,6^{a} +0,3	1,7^{a} +0,3	2,3^{a} +0,3	2,4^{a} +0,2	2,0^{a} +0,6	7,5^{a} +8,7	0,9^{a} +0,1	3,5^{a} +0,5	3,3^{a} +1,8	nd	9,5^{a} +8,5
	3-hydroxy- -2-butanone	739	3,24^{b} +0,5	nd	nd	nd	nd	nd	nd	nd	0,5^{b} +0,01	nd	1,4^{b} +0,2	10,1^{a} +3,1	nd
	2-heptanone	880	86,4^{a} +28,5	13,9^{b} +0,4	10,8^{b} +0,1	19,8^{b} +6,1	11,7^{b} +0,6	24,6^{b} +6,2	18,7^{b} +3,1	54,3^{a,b} +36,6	14,8^{b} +3,9	52,9^{a,b} +4,2	35,7^{a,b} +5,3	45,6^{a,b} +12,3	50,4^{a,b} +3,7
	2-octanone	989	13,6^{a} +3,7	3,7^{b} +0,6	6,3^{b} +1,5	3,1^{b} +0,9	nd	3,2^{b} +0,8	2,1^{b} +0,3	5,6^{b} +3,9	1,5^{b} +0,3	5,0^{b} +1,1	3,6^{b} +0,3	5,7^{b} +1,7	4,7^{b} +0,8
	8-nonen-2-one	1081	18,4^{a} +4,7	4,1^{b} +0,4	3,9^{b} +0,4	6,9^{b} +3,4	4,8^{b} +0,4	5,8^{b} +1,3	5,1^{b} +0,2	12,7^{a,b} +7,2	3,7^{b} +0,7	10,8^{a,b} +0,1	8,7^{a,b} +0,1	10,6^{a,b} +0,2	11,5^{a,b} +1,9
	2-nonanone	1090	98,9^{a} +21,9	18,6^{b} +1,8	19,9^{b} +1,3	33,9^{b} +14,2	29,1^{b} +2,2	33,5^{b} +10,0	26,7^{b} +1,5	72,7^{a,b} +12,2	19,3^{b} +4,6	52,9^{a,b} +0,9	47,7^{a,b} +1,3	63,2^{a,b} +7,6	61,0^{a,b} +17,1
	2-undecanone	1291	9,0^{a} +1,8	2,1^{a} +0,4	3,3^{a} +0,1	3,8^{a} +1,2	6,3^{a} +0,7	4,2^{a} +1,1	3,8^{a} +0,1	9,9^{a} +5,9	2,4^{a} +0,7	4,9^{a} +0,1	5,4^{a} +0,1	6,8^{a} +1,5	8,4^{a} +3,3
Acids	butyric acid	795	59,9^{a} +2,8	14,4^{a} +1,9	18,8^{a} +4,6	29,9^{a} +9,4	33,7^{a} +0,3	25,2^{a} +15,8	23,9^{a} +5,4	58,4^{a} +55,5	19,9^{a} +5,9	41,3^{a} +4,9	36,4^{a} +2,2	nd	60,7^{a} +7,1
	pentanoic acid	810	nd	8,3^{b} +5,7	nd	22,4^{b} +7,5	nd	6,6^{b} +5,8	22,1^{b} +2,7	37,7^{a,b} +15,0	5,7^{b} +0,7	4,5^{b} +1,9	7,9^{b} +6,9	76,5^{a} +33,2	27,2^{b} +9,7
	hexanoic acid	992	nd	10,6^{a,b} +8,1	12,5^{a,b} +0,2	40,2^{a,b} +15,4	45,2^{a,b} +1,1	13,7^{a,b} +4,2	28,9^{a,b} +5,2	51,7^{a,b} +42,8	24,6^{a,b} +5,5	7,2^{a,b} +4,7	11,5^{a,b} +9,1	7,4^{a,b} +2,3	61,6^{a} +14,5
	octanoic acid	1174	12,3^{a,b} +3,6	5,4^{b} +2,1	10,5^{a,b} +1,2	14,3^{a,b} +5,1	27,7^{a} +2,5	6,6^{b} +0,5	9,6^{a,b} +0,5	19,3^{a,b} +14,3	5,8^{b} +1,2	10,3^{a,b} +0,1	9,5^{a,b} +2,3	13,2^{a,b} +2,9	22,3^{a,b} +4,2
	decanoic acid	1364	3,9^{b,c} +1,2	1,2^{b,c} +0,6	2,8^{b,c} +0,5	3,8^{b,c} +1,5	8,9^{a} +1,5	nd	1,7^{b,c} +0,2	4,4^{b} +2,6	1,1^{b,c} +0,1	2,6^{b,c} +0,2	2,1^{b,c} +0,6	nd	4,3^{b,c} +1,1
Hydro- carbons	2,2,4- -trimethylopentane	729	23,0^{a} +5,7	nd	nd	nd	nd	nd	nd	nd	nd	4,6^{b} +5,3	3,9^{b} +4,0	2,8^{b} +1,3	nd
	2,2,4,6,6-pentam- ethyloheptane	988	nd	nd	nd	4,7^{a,b} +2,3	nd	3,1^{a,b} +1,4	2,4^{a,b} +0,6	7,2^{a} +5,2	1,3^{a,b} +0,5	3,8^{a,b} +0,6	3,9^{a,b} +0,1	5,4^{a,b} +0,8	4,6^{a,b} +0,7

Explanatory notes: RI – retention index; nd – not detected; Table shows mean values and standard deviation; n = 3; a–d – mean values determined in lines and denoted by different letters differ statistically significantly at $p < 0.05$

Noticeable is the high turnover that of most classes of compounds in the fragrance profile of the samples stored at 25°C. After an initial sharp decline of the shares of most classes of compounds in the first day of storage, there was also a sharp increase in their participation at day third and another sharp decline in the fourth day (Figure 2).

The Rokpol cheese samples stored for one day at 35°C were characterized by a decrease in the intensity of most groups of compounds, as in the case of other storage conditions, but the level of decrease was smaller. In the following days there has been a regular increase in the proportion primarily of acids, esters and ketones in the general volatile profile (Figure 3).

Esters and ketones had the highest share in the volatiles profile of all the group of compounds in all samples after four days of storage at ambient conditions and elevated temperature, and after four weeks of storage in refrigerator with the predominant compounds: 2-nonanone and 2-heptanone, which is confirmed in studies of other scientists (MOLIMARD and SPINNLER 1996).

Both aldehydes and hydrocarbons had the smallest share in the volatile fraction of Rokpol cheese regardless of the conditions and storage time and similar results were noted by YVON and RIJNEN L 2001. Already in the first tested samples significantly decreased of the share of hydrocarbons and in the later stages remained without statistically significant changes. In contrast, nonanal as the only compound belonging to the aldehydes was detected in only one of all the tested samples (Table. 1).

Principal Components Analysis

Appointed 12 principal components, of which the first four explaining total of 83.72% of the variation were chosen to describe the phenomenon.

Table 2

Percent rate of total variability explained by principal components obtained in PCA analysis

Principal component	% of total variation	Cumulative % of variability
1	42.13	42.13
2	18.51	60.64
3	12.35	72.99
4	10.73	83.72
5–12	16.28	100

The first principal component explained 42.13% of the total variability. It showed a strong negative correlation (0.895 – 0.974) from 2-pentanol, propyl ester, hexanoic acid, 2-heptanone, 2-nonanone, 2-nonanol, 2-undecanone and 8-nonene-2-one.

The second principal component explained 18.51% of the total variability. The same as the first principal component it clearly differentiated control sample form those which were stored. Particularly large differentiation related to the control and cheese samples stored for four days in the incubator and 4 weeks in the refrigerator (Figure 1). The stored cheese samples had larger share of hexanoic acid, decanoic acid ethyl ester and octanoic acid. In contrast, the control sample had the most of 2-oktanone and 2-butanoic acid methyl ester.

The third principal component explained 12.35% of the total variability and significantly separated the samples taken at day three of storage under 25°C and 35°C (Figure 2). Differentiation of the samples was based on decanoic and octanoic acid methyl esters and and 1,3-propanediol, which high content were determined in a sample at 25°C. In contrast, the sample taken at 35°C distinguished by a high content of 3-hydroxy-2-butanone, and 2,3-butanediol.

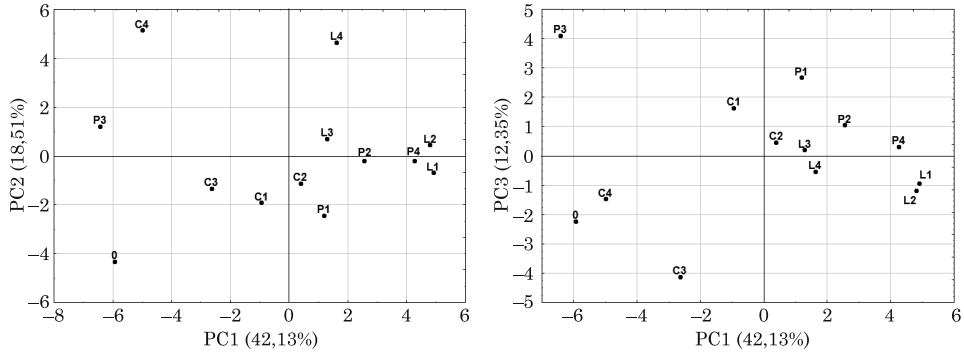

Fig. 4 and 5. PCA analysis results – projection of cases: PC1/PC2 (1), PC1/PC3 (2)

The fourth principal component explained 10.73% of the total variability and, as in the case of the third main component, the sample taken after three days of storage at 35°C was clearly separated from the other (Figure 6). The effect on the differentiation had a high pentanoic acid and 1,3-propanediol in the mentioned sample. The compounds were highly and moderately positively correlated (0.836 and 0.684 respectively) with the discussed main component. In contrast, the separation control sample from other samples was caused by decanoic acid and butanoic acid, and 2,2,4-trimethylpentane. The fourth principal component of showed a moderate negative correlation with these compounds (0.511 – 0.560).

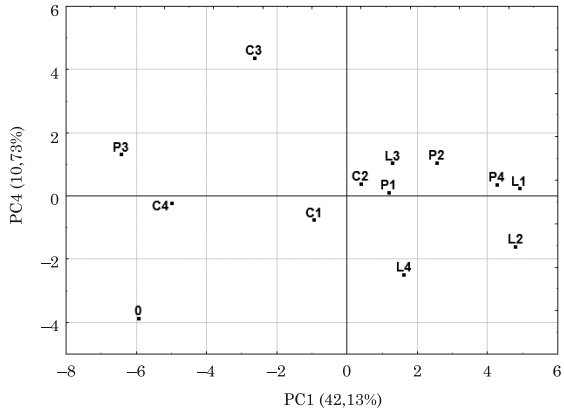

Fig. 6. PCA analysis results – projection of cases: PC1/PC4

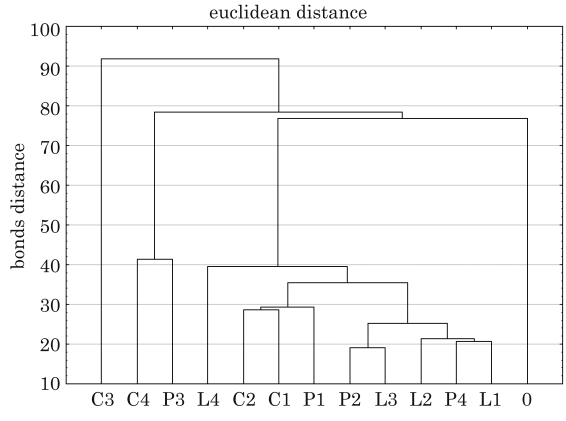

Fig. 7. Results of cluster analysis based on volatile profiles of investigated cheese samples – Ward's method (euclidean distance)

Cluster Analysis

Cluster analysis allowed to group Rokpol cheese stored samples with similarities in the composition of the volatile fractions. The similarities between established groups were reflected in the PCA test presented earlier. The dendrogram shows four groups of tested samples and three samples not classified to any group. The first two groups of samples were stored in the refrigerator for 3 weeks and samples from the second and fourth day of storage at 25°C (Figure 7). Another group of samples were stored for one day at 25°C and samples from the first and second day of storage at elevated temperature. The last group formed flavoring profiles of samples from the third day of

storage at 25°C and the fourth day at the elevated temperature. The control sample and the one from the third day of storage at elevated temperature differed from others mostly. The profile of volatile compounds of the sample from the fourth week of refrigerated storage also significantly differed. However, the chemical composition of the volatile fraction showed an affinity of that sample with the three previously described groups.

Conclusions

1. Based on cluster analysis it is visible that cheese storage for one week in the refrigerator or two days in 25°C has the same sensory effect. This is an evidence for the fact that refrigerator prolongs aroma stability.

2. The storage of cheese at 25°C and 35°C gives similar aroma result.

3. The analysis of volatile compounds profiles in different temperature conditions of cheese storage show the direction and intensity of sensory changes. It also allows for evaluation of fragrance stability and for selection proper storage parameters.

4. Obtained results show statistically insignificant slight qualitative differences in volatile fractions of tested cheese.

5. Application of headspace solid-phase microextraction (HS-SPME) in connection with statistic tools (PCA, CA) can be an useful tool in analysis of volatile compound profiles in cheese, as well as study changes of these profiles during storage or ripening.

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VISUALIZATION OF AMARANTH OIL PRESENCE ON THE SURFACE AND INSIDE SPRAY DRIED MICROCAPSULES

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K e y w o r d s: oil microcapsules, confocal Raman imaging, k-means clustering method.

A b s t r a c t

In order to increase amaranth oil availability to the consumers an encapsulation process by spray drying was applied. Based on the confocal Raman microscopy imaging technique supported by computer image analysis an algorithm for detailed visualization of oil presence on the surface or inside of aggregated microcapsules was performed. Those information are crucial in terms of oil bioavailability as well as storage properties of microcapsules.

WIZUALIZACJA OBECNOŚCI OLEJU Z AMARANTUSA NA POWIERZCHNI I WEWNĄTRZ MIKROKAPSUŁEK OTRZYMANYCH METODĄ SUSZENIA ROZPYŁOWEGO

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S ł o w a k l u c z o w e: mikrokapsułki oleju, konfokalna wizualizacja Ramanowska, klasteryzacja metodą k-średnich.

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Abstrakt

W celu podwyższenia dostępności oleju z amarantusa dla jego konsumentów zastosowano proces mikrokapsułkowania poprzez suszenie rozpyłowe. Następnie za pomocą mikroskopu konfokalnego z przystawką do spektrometrii Ramanowskiej oraz komputerowej analizy obrazu przeprowadzono wizualizację oleju znajdującego się zarówno na powierzchni, jak i wewnątrz otrzymanych mikrokapsułek. Informacje te mogą mieć wpływ na biodostępność oleju, jak również na właściwości przechowalnicze mikrokapsułek.

Introduction

The food industry aims at developing new products with functional food properties. This increases the quantity of functional and health promoting ingredients which are delivered to consumers. In order to increase availability of those ingredients to consumers encapsulation process has to be implemented. This process restricts both oxygen migration into the active substances and loss of aromatic compounds and increases resistance to various process-related factors (GOUIN 2004).

Spray drying is a commonly used method of microencapsulation of oil containing materials. The main factors that affect the encapsulation efficiency of microencapsulated oils by spray drying are: type of wall material, properties of the core materials, viscosity, droplet size and process conditions of the spray drying process such as atomization type, inlet air temperature, air flow and humidity (SOLVAL et al. 2012). One of the spray drying limitation is the quantity of material that can be used as a capsule for active ingredients. The compounds used in the process of microencapsulation must be soluble in water. Acacia gum, maltodextrins, modified starch and mixtures thereof, polysaccharides (alginate, carboxymethylcellulose, guar gum) and proteins (whey proteins, soy proteins, sodium caseinate) are widely applied in spray drying technology (FUCHS et al. 2006, GOUIN 2004).

Several techniques can be used to investigate the external and internal structures of microcapsules. Whereas the external size of food microcapsules is in the micrometer range the internal structures of the microcapsules are often in the nanometer range, where objects like pores dimensions and walls thicknesses are smaller than 100 nm, which makes their investigation extremely difficult. A wide range of techniques are available for characterizing food structure, such as: light, fluorescent, transmission and scanning electron microscopy (TIEDE et al. 2008). In order to analyze the outer and inner structure of microcapsules a proper visualization method has to be selected. One of the most popular techniques is the Scanning Electron Microscopy (SEM) (ROSENBERG and SHEU 1996, SOOTTITANTAWAT et al. 2003). The use of an electron microscope gives the opportunity for accurate measurements of the

area and shape of microcapsules and after proper preparation of the sample also the internal structure of microcapsules can be visualized and analyzed (SOOTTITANTAWAT et al. 2003). Despite the huge amount of information which can be collected from the electron microscope image about the size and even size distribution of the pores inside the microcapsule there is still lack of information about the content of microcapsules (DAJNOWIEC et al. 2011). Microcapsules can be empty, or filled with an oil droplet – which is the objective of the encapsulation process. This requires developing a proper methodology of microcapsule assessment, especially with respect to their internal structure.

Raman spectroscopy is an appropriate tool for analysing micro- and macronutrients content in food (LI-CHAN 1996). It allows for a quantitative and qualitative evaluation of tested products based on an analysis of specific spectra which permit the identification of selected bonds between the molecules. Raman spectroscopy has been so far used to determine the composition of products, to control their quality and to detect their eventual adulterations (BATSOULIS et al. 2005).

The aim of this research was evaluation of the applicability of confocal Raman imaging for visualisation of the presence in the internal structure and on the surface of amaranth oil microcapsules.

Material and Methods

The studies were carried out with microcapsules produced under laboratory conditions by spray drying of amaranth oil emulsion. The composition of microencapsulated emulsions were composed of milk protein concentrate MPC with 75% of dry basis 50 g kg^{-1} (ZPM Wolsztyn Ltd. Poland), maltodextrin (DE 7-13%) 100 g kg^{-1}, amaranth oil 200 g kg^{-1} (Szarlat Co. Poland) and water 650 g kg^{-1}. After initial mixing of all ingredients with a mechanical mixer (Zelmer Ltd. Poland), the emulsions were homogenized using a two-stage pressure homogenizer (Nirvo Soavi Panda, Italy). The process was performed at 25/5 MPa on the 1st and the 2nd stage respectively. The spray drying was carried out using a laboratory spray dryer (Anhydro, Denmark). The temperature of the inlet air was 230 ± 6°C and the outlet air was 70 ± 4°C.

Next microcapsules, after cooling in natural conditions, were analyzed in terms of oil presence on its surface and inside. In order to elicit the internal structure microcapsules were deposited on microscopy glass slides or silicon wafers and chopped with a surgical scalpel. Suitable areas of amaranth oil microcapsules were first identified with the optical microscope and then the spectral imaging was performed with a confocal Raman microscope. The instrument used was a Witec alpha 300R with objective 100x, air, NA 0.95;

laser: Spectra Physics Excelsior TEM_{00} single mode Nd:YAG 532 nm and resolution in x-y coordinates (parallel to the sample surface) around 300 nm, in z axis (perpendicular to the sample surface) around 500 nm. Great care was applied to keep the laser power low enough to prevent degradation of the sample over the complete confocal imaging cycle. Raman spectra in the x-y plane as well as along the z-axis were recorded for further analysis of the internal structure of the microcapsules. After identification of the regions characteristic for the main ingredients of investigated microcapsules the Raman spectra were evaluated using the k-means cluster analysis. The goal of the k-means algorithm was to find the best division of n entities (Raman spectra taken for each image pixel) in k=3 groups (the oil droplet, microcapsule's wall and the background), so that the total distance between the group's members x_i^j and its corresponding centroid c_j (average Raman spectrum) representative of the group (or a specific material), is minimized using the following equation:

$$\mathrm{WCSS} = \sum_{j=1}^{k} \sum_{i=1}^{n} \left\| x_i^j - c_j \right\|^2$$

where:
"WCSS" is a within-cluster sum of squares.

Results and Discussion

After inspection of images from an optical microscope two types of structure were identified: smooth oil droplet (Figure 1A) and the microcapsule (Figure 1B). From both of them Raman spectra were obtained. The Raman spectrum of the oil droplet (marked by the cross in the microscope image (Figure 1A) is presented in Figure 2. It can be distinguished from the other structures by a peak at around 1751 cm^{-1} and a distinguishable high intensity peak in the C-H regime at around 2852 cm^{-1}, which is well separated from the other C-H bands. These droplets were the amaranth oil. Figure 3 presents spectra obtained from the surface of the microcapsule marked by the cross on the microscope image Figure 1B.

Thanks to the confocal setup it was possible to obtain distinct Raman spectra of the surface of a microcapsule and from the inside of the microcapsule. The example of the spectrum from inside of the microcapsule (Figure 3B) shows only a very weak peak at 1751 cm^{-1} and a not very well distinguished peak at 2852 cm^{-1}. There is the more pronounced peak at around 482 cm^{-1},

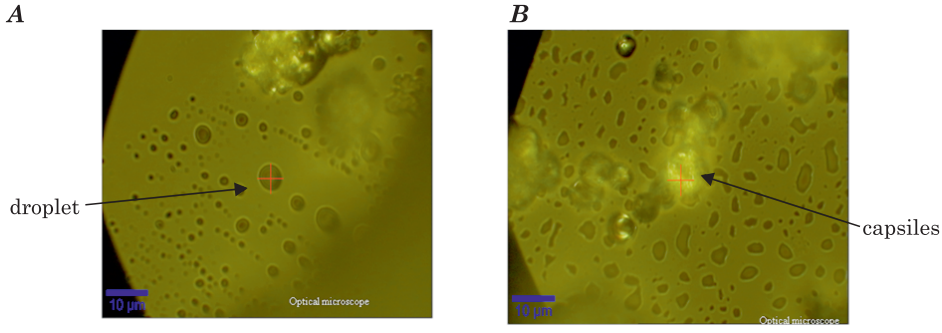

Fig. 1. Oil droplet A) and microcapsule B) visible under confocal microscope

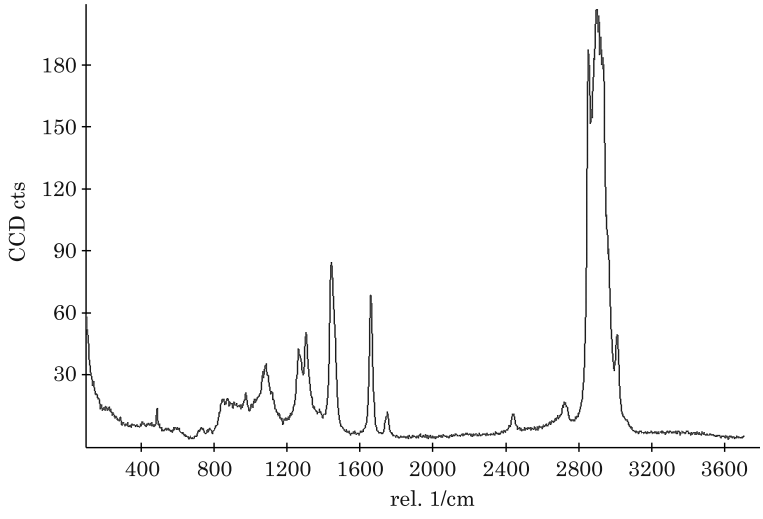

Fig. 2. Raman spectrum of the oil droplet

which is characteristic for the maltodextrin as well as the peaks around 856 cm^{-1} and 940 cm^{-1}.

These three peaks are very weak in the spectrum of the oil droplet (Figure 2). The spectrum obtained from the surface of the microcapsule (Figure 3A) shows also peaks characteristic for maltodextrin and oil. The other peaks cannot be clearly assigned, because they are present in all spectra (however, with different intensity), and probably they are the result of an overlap of the spectra of all three substances (oil, milk protein, maltodextrin). An optical microscope image of another microcapsule is presented in Figure 4A. In order to identify the presence of oil a complete Raman spectrum was made at every pixel in the area marked by a square in the microscope image (60 pixels x 60 pixels=3600 spectra, Figure 4A), and a bandpass filter over the peak at 1751 cm^{-1}

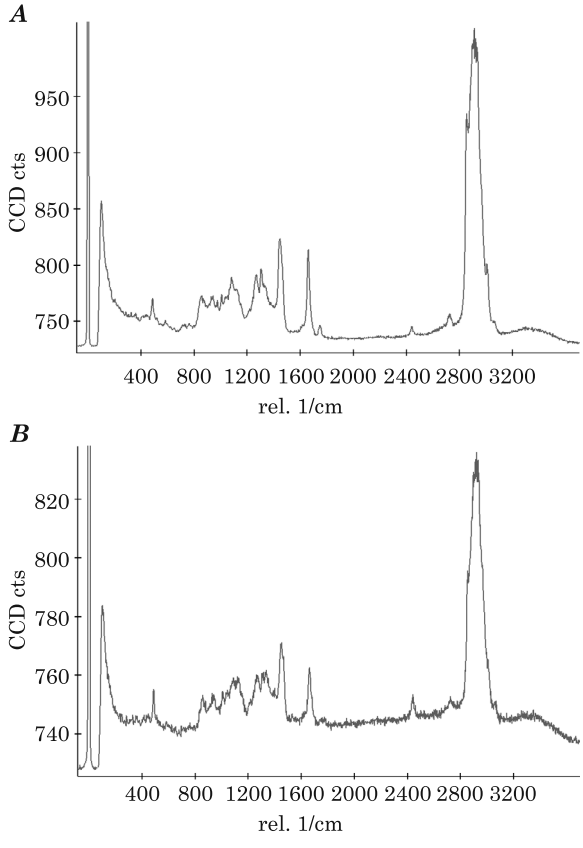

Fig. 3. Raman spectra: A) on the surface of a microcapsule and B) inside a microcapsule

(characteristic for the oil presence Figure 4B) was applied. The result – the intensity of the peak as a function of the position – is displayed in Figure 4C as spectral intensity map. The bright areas on this map show where the oil is present. In this case the oil is visible at the border of the capsule, and there are also two little oil droplets visible on the spectral map – at the top. There are also microcapsules, where oil can be identified inside the microcapsule and such an example is presented in Figure 5. The image under the optical microscope shows a capsule which has a crack probably caused by the scalpel (Figure 5A). Spectral Raman imaging was done within the square marked area and the focal plane was set in the middle of the capsule. A bandpass filter was implemented first for the C-H region between 2828 and 3014 cm^{-1}, and the result is shown in Figure 5B. This image shows all compounds of the capsule i.e., oil, milk protein and maltodextrin, because all these substances have-

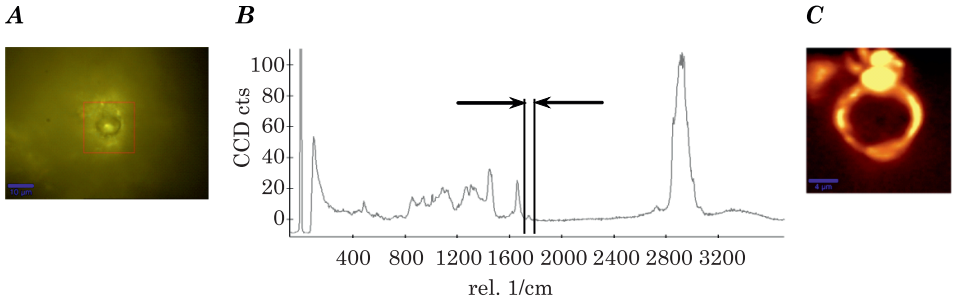

Fig. 4. Single capsule optical microscope image: A) light microscope image with marked analysed square area and the scale bar 10 μm; B) the Raman spectrum with marked region of the bandpass filter implementation; and C) spectral intensity map over the peak at 1751 cm^{-1} within the red square and with the scale bar 4 μm

vibrations in the C-H regime. However, when applying a bandpass filter over the region 1751 cm^{-1} the clear concentration of intensity was visible, which are assigned to the presence of oil.

To gain more information on this issue, a base analysis of the components was made. For this, two regions were identified:

– first region, where the peaks associated with oil at 1751 cm^{-1} and at 2852 cm^{-1} are clearly visible;

– second region, where the 1751 cm^{-1} peak is not visible or is very weak and where the 2852 cm^{-1} peak is not clearly separated, whereas the peak at 482 cm^{-1} demonstrates a high intensit.

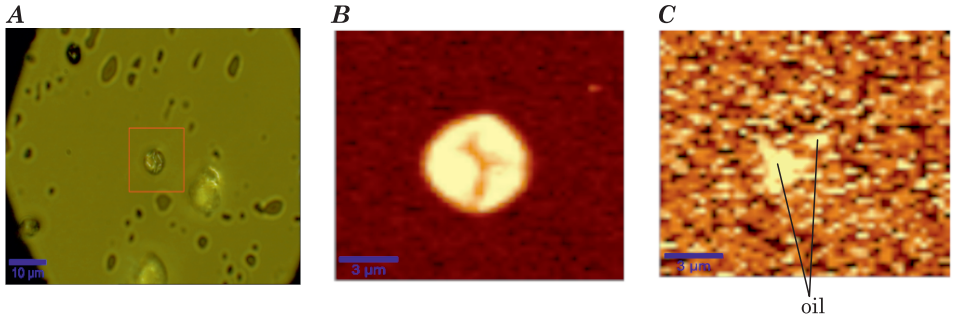

Fig. 5. An image of a single capsule under the optical microscope with marked analysed square area and the scale bar 10 μm (A) and two spectral images after bandpass filtering on the C-H region from 2828 to 3014 cm^{-1} (B) and (C) over the region around the peak 1751 cm^{-1} with a scale bar 3 μm

Average spectra collected in these two regions are displayed in Figure 6A and Figure 6B. As third base spectrum, an average spectrum from the substrate surface (glass) was also collected Figure 6C. These spectra were used as base spectra to fit the measured spectral dataset at every pixel by linear

superposition of the base spectra. As a result, the measured spectra can be very well fitted by this linear superposition, and the obtained weighing factors are displayed as a function of the position in the oil (Region A) and capsule (Region B) of the image presented as a region D in Figure 6D. They are also colour coded and superimposed to obtain the composite image in the region D of Figure 6 together with the substrate spectra region C. The yellow areas in the superposition are characteristic for the presence of oil and the blue for the presence of the capsule material without oil. The substrate is displayed in brown. The location of the oil as obtained from this analysis is in agreement with the result presented on Figure 5C (1751 cm^{-1} peak), although the base analysis shows the distribution of oil in much greater detail.

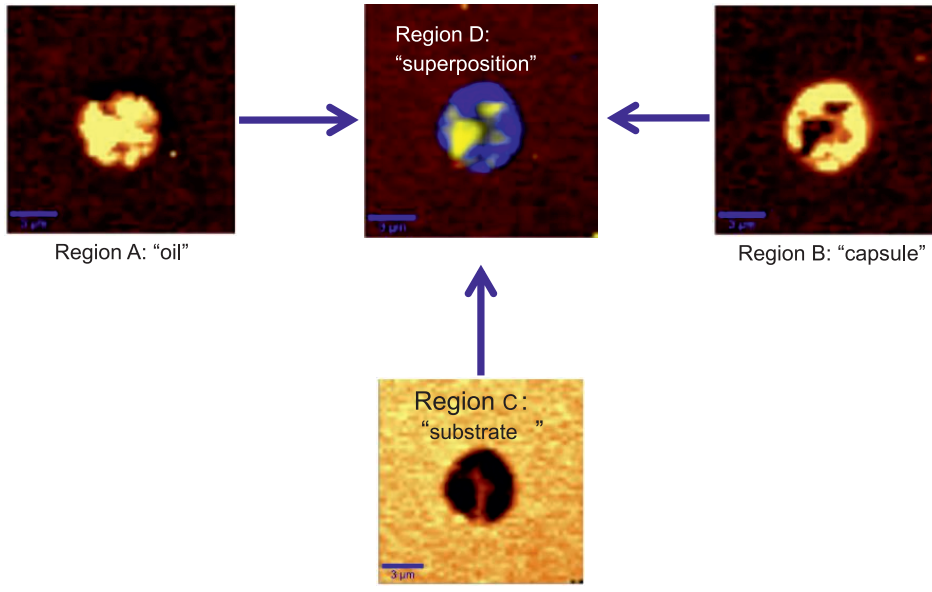

Fig. 6. Single capsule spectral images with the scale bars of 3 μm (description in the text)

For this purpose another aggregated microcapsule was analyzed for which forty complete spectral images were collected at z-distances of 500 nm (i.e., 100 pixels x100 pixels x40=400000 spectra in total). These spectral images were collected using an EMCCD detector, with 10 ms integration time per spectrum. Figure 7 shows selected of these images of the C-H region, obtained at different z-positions, to visualize the overall structure of this microcapsule.

The same microcapsule was used for a more in-depth stochastic analysis of two of these spectral images, obtained at z-positions which are around 1.5 μm apart and shown in Figure 8. In this case, a k-means cluster analysis was performed on the spectral images.

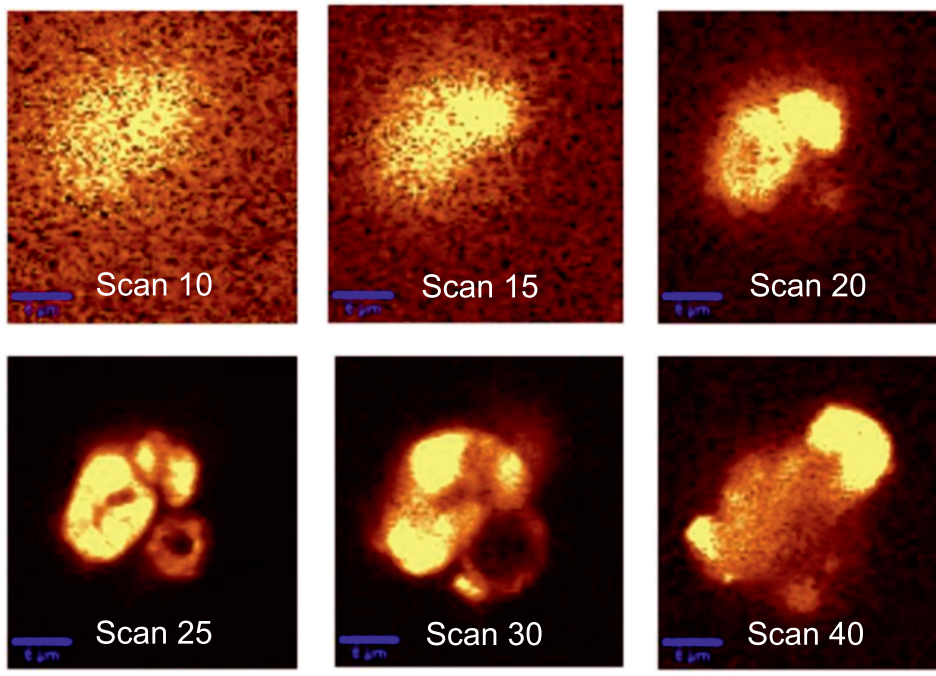

Fig. 7. Example of spectral images of the C-H region obtained at different z-positions for a single capsule (scale bar 6 μm)

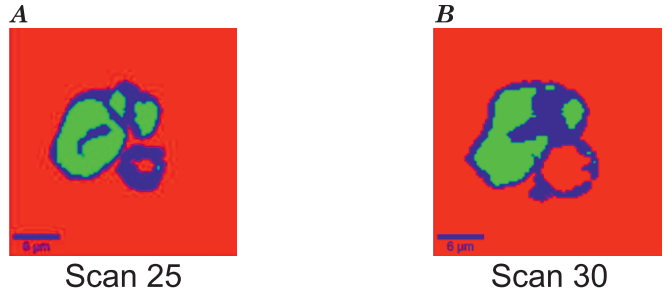

Fig. 8. Examples of images of internal structure of a microcapsule created base on the k-means cluster analysis: B) scan 25 and C) scan 30 (scale bar 6 μm)

Three colour clusters were used to visualize the internal structure of the microcapsule based on the selected Raman spectrums. The red cluster corresponds to the substrate, whereas green and blue clusters are assigned to the capsule. The green cluster corresponds to the oil (see Figure 2) and blue cluster (see Figure 3) corresponds to the capsule materials.

In this case the green areas correspond to a high concentration of oil, i. e., two capsules contain oil, whereas a third one is empty. In the cluster analysis, the cluster spectra oil and the matrix/protein are not as clearly demixed as in the base analysis of the results presented in Figure 6, but nevertheless the two results are compatible with each other and point towards the same conclusion that the oil inside the capsule can be also visualized.

Conclusions

Detailed analysis of the microstructure of the powder particles, based on confocal Raman microscopy imaging supported by computer image analysis, shows that their internal structure can be visualized. Moreover it was revealed that the confocal Raman imaging technique enables identification of particular ingredients and their distribution within the capsules, thus the presence of the surface oil could be detected. The measurements and the analysis are time consuming for routinely microcapsules analysis in the laboratory.

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IMPROVEMENT OF MANAGEMENT PROCESS BY USING LEAN SIX SIGMA TOOLS IN SOME BIG ORGANISATION OF FOOD INDUSTRY

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K e y w o r d s: Lean Management, Six Sigma, Lean Six Sigma, continuous improvement, food industry.

A b s t r a c t

The research had been conducted in some polish production companies that are using Lean Six Sigma tools on daily basis. The aim was to evaluate the impact of Lean Six Sigma tools on the certain management process to eliminate or reduce wastage. According to the results it can be stated that Lean Six Sigma tools have positive impact on the management process by controlling internal costs. Cost reduction influences the increase in profit margin what can result in achieving the competitive edge.

DOSKONALENIE ZARZĄDZANIA W BRANŻY SPOŻYWCZEJ Z WYKORZYSTANIEM NARZĘDZI LEAN SIX SIGMA

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S ł o w a k l u c z o w e: Lean Management, zarządzanie wyszczuplające, Lean Six Sigma, ciągłe doskonalenie, przemysł spożywczy.

A b s t r a k t

Badanie zostało przeprowadzone w przedsiębiorstwach produkcyjnych usytuowanych na terenie Polski, które w codziennej praktyce stosują narzędzia Lean Six Sigma. Celem była ocena wpływu narzędzi na doskonalenie procesu zarządzania. Wyniki badań potwierdzają, że narzędzia Lean Six Sigma pomagają świadomie kontrolować koszty wewnętrzne w firmie, co pozwala na ciągłe ulepszanie procesu zarządzania. Ponadto, zmniejszenie kosztów własnych jest jednym z czynników wpływających na zwiększenie marży, która może zapewnić przewagę konkurencyjną przedsiębiorstwu na dzisiejszym, bardzo turbulentnym rynku.

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Introduction

A constant price increase of raw materials and decrease of profit margin led production companies in food industry into crisis (FORKUN and KINAST and ORZEŁ 2011). Profit margin is directly influenced by price as well as direct and indirect costs of a company. A company has no influence on the price of raw materials needed for production which form an indirect cost. Therefore, if a company wants to stay on the market it needs to control its own costs, which were defined by Ohno as „7 Muda" (LISIŃSKI and OSTROWSKI 2006). Wastage is defined as all activities which generate costs and do not add value: overproduction, inventory, waiting, motion, transportation, defects and over processing. The antidote for muda is to implement Lean Six Sigma tools, which aim to identify, reduce and eliminate all wastage.

Aim of the study was to verify and evaluate Lean Six Sigma tools and their influence on improvement of management in chosen production companies in food industry. Leading and operating an organization successfully requires managing it in systematic and visible manner using „a tool to allow an institution, regardless if it is a company, church, university or hospital, achieve their goals in an outside area in which it operates" (DRUCKER 2000)

The essence of Lean Six Sigma

Japanese Lean Management philosophy and American Six Sigma concept were evolving separately until it has been decided that Lean Management on its own is not able to provide stable processes and sole Six Sigma will not eliminate all waste. Only then was a new integrated approach developed called Lean Six Sigma (GEORGE 2002). The Lean Management tools focus on speed and efficiency of a process, while those of Six Sigma on its precision and accuracy (LAUREANI and ANTONY 2009). It can be stated, therefore, that Lean Six Sigma is focused to increase quality, reduce variability and eliminate any wastage from company (FURTERER and ELSHENNAWY 2005).

The main aim of the philosophy is to please a customer, i.e. to provide reasonably priced, high quality product/service at short notice (GOERGE 2004). To do that, process needs to be enhanced. All defects i.e. everything which does not satisfy a customer must be removed from the process.

Moreover, material and information flow must also be leaned which will eliminate buffers/lines. Employees who cooperate on every stage of the process are an important aspect of Lean Six Sigma. Group work is about mutual assistance, sharing ideas, collective problem solving and making thoughtful decisions. According to Lean Six Sigma every decision is made on the basis of

real data and facts. Data is being gathered, measured and analyzed. In agreement with Lean Six Sigma companies should gather and analyze data on customer's satisfaction, company's financial status, speed of the process and defects.

Chosen Lean Six Sigma tools

Lean Six Sigma uses a set of tools of Lean Management philosophy and Six Sigma concept. In every stage of a process based on DMAIC model (Define, Measure, Analyze, Improve, Control) companies use various tools. The choice of tools is not forced by the concept, therefore, companies depending on their character (structure, size, branch) choose their own set. Because of the fact that Lean Six Sigma tools are numerous, the article will describe only the most popular ones in the authors' opinion. Methodical approach to Lean Six Sigma implementation, however, allows the use of an ordered chart from ISO

Table 1

Typical Six Sigma tools according to ISO 13053-1: 2012 Quantitative methods in process improvement – Six Sigma – Part 1: DMAIC methodology

Tool	Define	Measure	Analyse	Improve	Control
CTQ (Critical to Quality)	O	O		O	O
Financial justafication	O				
Project review	O	O	O	O	O
Chat review	O				
Six Sigma indicators	O			O	
Data colection plan		O			
MSA (Measurement System Analysis)		O	O		O
Probability distribution		O	O		
Defining sample size		O	O	O	
FMEA (Failure Mode and Effects Analysis)				O	
Control plan					O
5S				S	S
Poka-Yoke				R	R
SIPOC (Supplier, Input, Process, Output, Control)	R			S	
Pareto chart	S	S	S	S	
Value stream mapping	R				
Regression and correlation			R	R	
Hypothesis test			R	R	
TPM (Total Productive Maintenance)				S	S

O – Obligatory, R – Recommended, S – Suggested

Source: ISO 13053-1: 2012 Quantitative methods in process improvement – Six Sigma – Part 1: DMAIC methodology

standard document 13053-1: 2012 *Quantitative methods in process improvement – Six Sigma – Part 1: DMAIC methodology,* where a set of tools have been aligned with 5 stages of DMAIC concept (Table 1). The table contains only those tools from the Standard, which should obligatorily be used in DMAIC implementation and tools which according to the authors are most commonly used in enterprises, and are recommended or suggested to use by the Standard.

DMAIC model (Define, Measure, Analyze, Improve, Control)

DMAIC concept is a methodical approach to Six Sigma implementation, yet in a survey poll made in enterprises it has been treated by the questioned personnel as one of the tools. The aim was to determine if the concept is used in practice in connection with the set of 31 recommended tools regarded as indispensable, recommended or suggested in the ISO Standard 13053-1: 2012.

DMAIC model is used to improve organizational processes and remove problems. DMAIC model helps to identify a problem, determine key measurements, implement solutions, set procedures and finally control implementation process and allow continuous improvement. DMAIC cycle is focused on continuous improvement of the process to meet the needs of a customer. The model consists of 5 stages: Define, Measure, Analyze, Improve, Control. Define stage determines key characteristics of a product from a customer's point of view by the use of e.g.: „voice of the customer". On the basis of results project team decides which process shall be improved, what the problem and the target are. A visual effect of the define stage is a map of temporary process which can be created with the use of e.g. SIPOC tool (Supplier, Input, Process, Output, Customer). The second stage – measure – aims to determine key measures of the process and gather necessary data. Next stage – analyze – aims to determine a source of variability basing on data gathered in the previous stage. All the bottlenecks and limitations in the process are identified. During the fourth stage – improve – all possible solutions are determined and the best option is chosen. The solution is being implemented as a pilot project. Moreover, a map of a future, improved process is prepared. The last fifth stage – control – aims to determine if the assumed target has been achieved. If the implemented changes are efficient, standards and procedures must be determined. During control stage control charts are often used.

Value Stream Mapping

Value stream mapping is a method of presentation material and information flow. It allows identification of all activities from the moment an order had

been made by a customer until it is delivered. It also presents both activities which add value and those which don't. Activities which do not add any value and are not indispensable for company to operate on the market must be eliminated.

Value stream mapping can be divided into two key stages (SOBCZYK and OLEKSY 2011):

– creation of a map of current processes for a chosen group of products, which will be amended to include all necessary information on the current condition,

– creation of a map of a future condition, which is a vision of desirable condition.

Visualizations

Application of visual control allows employees to act immediately to any problems, distractions, production delays and any deficiencies that occur. The condition of a production system must be clear and simple and understandable for every employee, therefore, all necessary tools and parts should be stored in a visible place. Visual control requires also a simple work manual to be present at every work station. 5S is the most important method of visual control. 5S is a tool used to build and maintain a well-organized, clean, efficient and high quality work station. The use of 5S is not limited to production, but works just as well in other departments e.g. administration. 5S method is often accompanied with other Lean Management tools such as: TPM, SMED, Heijunka, Kanban, standard working conditions or Jidoka.

The name 5S comes from five Japanese words:

1S Seiri – Sort i.e. sorting out the required or not required items, removal of non required items.

2S Seiton – Storage i.e. work station arrangement, assignment a proper place and logical organization to every object in order to make their use easier.

3S Seiso – Shine i.e. maintaining a clean work station on daily basis to improve work safety and detect process distractions.

4S Seketsu – Standardize i.e. creation of work station standards to maintain the conditions created by the first three rules.

5S Shitsuke – Sustain i.e. to adhere to 5S principles by means of audits, work inspections or visualizations of the results of 5S teams.

Poka-yoke (Mistake Proofing)

Poka-yoke technique is used to avoid mistakes. It works in all processes where human error may occur. It is assumed that every person makes errors, therefore, attention must be drawn to minimize and eliminate them. An efficient Poka-yoke mechanism should be simple (simple control and measuring devices situated in the vicinity of an error) and cheap to install.

TPM (Total Productive Maintenance)

TPM is defined as „a method for improved machine maintenance performed throughout whole company by operators and employees responsible for traffic control" (CZERSKA 2006). The major target of the method is „zero breakdowns and zero defects" caused by machines. An efficient TPM will improve equipment efficiency, prolong machine life expectancy and increase employees' involvement.

TPM has nine stages of activity (CZERSKA 2006):

1. Evaluation of the current condition using OEE (Overall Equipment Effectiveness). Machine efficiency is measured basing on its performance in: availability (active work of a machine), use (planned percentage usage) and quality (of produced goods).
2. Determination of initial problems.
3. Analysis of physical conditions of actions, based on observation of work station in the aspect of identified problem.
4. Identify variable conditions which influence the work of a machine.
5. Possibility to regulate and deregulate machine basing on conclusions gathered through earlier stages.
6. To determine vital elements in a machine, their control and means of repair.
7. Results control.
8. To determine rules of control and machine repair, as well as the range of machine operator responsibilities.
9. Improvement.

SMED (Single Minute Exchange of Dies)

SMED is a method for reducing tool changeover time to a single-digit minute. A key factor in this method is to separate operations into (CZERSKA 2006);

internal – not possible to perform when a machine is running
external – possible to perform when a machine is running.

SMED main stages (MARTYNIAK 1996):

1. Preliminary stadium, a very detailed analysis of changeover process and developing ideas how to improve them.
2. Separate external from internal changeover is made in order to reduce time of internal changeover.
3. Convert internal changeover into external one by additional analysis of activities and their belonging to a particular type, and also search for new means of conversion internal changeover into external one.
4. Streamline all aspects of changeover operations. Another attempt to find additional ways to reduce changeover time after analysis of implemented changes, separation and conversion internal changeover into external one.

Methods

Research has been conducted with use of a poll questionnaire sent by electronic mail to Lean Six Sigma specialists. Specialists are representatives of food industry production companies, who were found on Goldenline portal. The respondents had been grouped as follows: Lean Management, Six Sigma, Lean Six Sigma. 30 questionnaires were sent to 30 representatives of different food industry production companies.18 out of 30 were replied. 100% replies came from large food industry production companies (over 250 employees). Additionally, individual face to face interviews were run with 4 Lean Six Sigma specialists and trainers who deal with implementation, maintenance and improvement of the philosophy in 4 different production companies on daily basis.

Results

The research indicates that the tools most often used by companies are: 5S, Kaizen, Pareto Diagram and Ishikawa Diagram (Figure 1, 2). 5S and Kaizen are the tools which companies use to start to implement the philosophy, because they give visible results fast at relatively low implementation costs. Moreover, they do not require specialist knowledge to implement, as oppose to e.g. implementation tools for statistical adjustment of data, such as: hypothesis test ANOVA analysis or DOE (design of experiments). Three quarters of the respondents uses TPM, SMED, VSM and DMAIC, more than half uses standardized work, voice of customer and process mapping. Half of the

questioned companies uses control charts, FMEA, histogram and control charts on daily basis. A surprising fact is that few companies use JIT and Jidoka), which are a basis of Toyota production system. JIT aims to eliminate stock, while Jidoka aims to eliminate production flaws. Experts say JIT and Kanban should be supported by Heijunke, and Kaizen shall go together with standards. Typical Six Sigma tools (SIPOC analysis, FMEA, DMAIC, VOC) are less popular in companies, therefore, it may be concluded that companies' first priority is to increase speed and efficiency of a process, while elimination of process variability is of secondary importance. This may be a result of the specific character of production processes in food industry, where products' best before date is very short, therefore speed and throughput are vital and both are guaranteed by Lean Management tools.

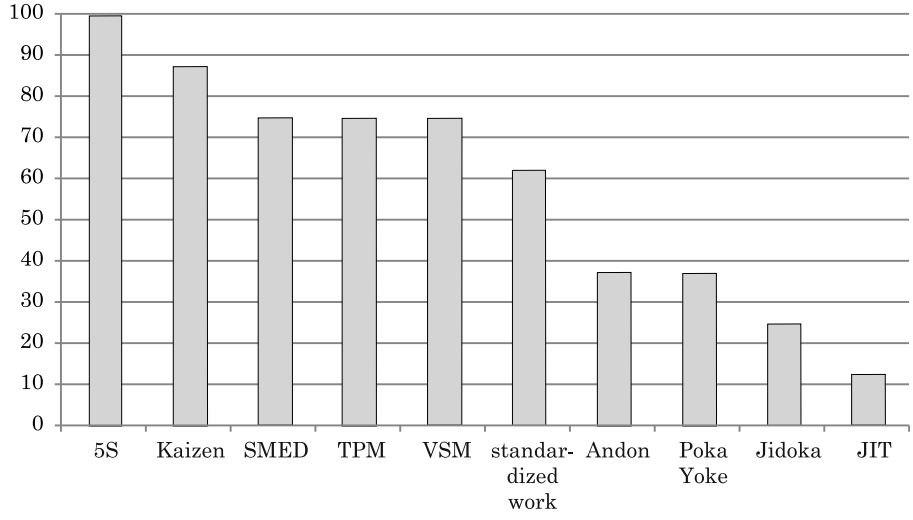

Fig. 1 The most commonly used Lean Management tools by production companies in food industry
Source: Own source

Most companies, (Figure 3) seem to follow the words of dr Mikel J. Harry: "If we do not measure, we do not know anything. If we don't know, we cannot act. If we do not act, we are prone to lose" and Lean Six Sigma methodology, and measure efficiency and productivity of their activities. Financial factor is the most frequently measured factor which seems to confirm that profit is what companies' owners care for the most. Less than half of the questioned companies do measure customer's satisfaction, which is not only a reason to worry but also against Lean Six Sigma assumption that a customer is most important. Only less than half focus their actions on customers' needs analysis

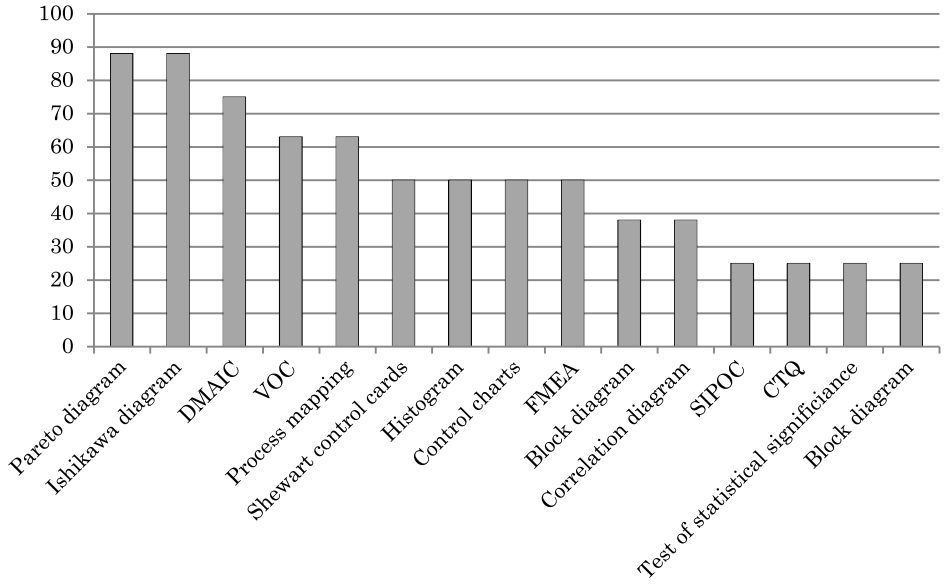

Fig. 2 The most commonly used Six Sigma tools by chosen production companies in food industry
Source: Own source

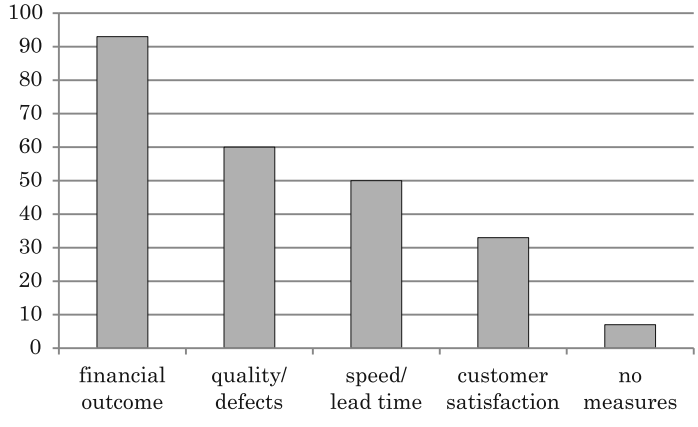

Fig. 3 Efficiency and effectiveness indicators of Lean Sic Sigma tools

Source: Own source

(Figure 3). More than half respondents do measure defects, 50% the speed of a process i.e. factors such as Lead Time or Takt Time. Only 7% of companies do not use any indicators).

The benefits from Lean Six Sigma implementation have also been analyzed. Every interviewed company has noticed positive effects of Lean Management and Six Sigma tools implementation. None of the companies denied positive effects, which confirms usefulness of Lean Six Sigma tools implemen-

tation, despite the fact that not every company fully adheres to Six Sigma rules – only three quarters respondents confirmed their actions are based on DMAIC. Companies which use chosen tools identify a great improvement in group work activities which is one of Lean Six Sigma philosophy pillars. Most of the companies claim their employees cooperate better in a team and see benefit as an improvement in problem solving process and decision making process. 5S has been implemented by all of the companies, as a result most of them noticed improvement in working conditions; increased efficiency and improved safety. Yet other benefits are strictly connected with control and reduction of personal costs. Three quarter respondents claim that their costs decreased, reduced process wastage and eliminated stock which generates additional costs. Half of the companies shortened process realization time, and only less than one quarter cut down time of delivery. For companies from food market, where speed is significant, shortening time of vital processes may result in achieving competitive edge. A surprising fact is that only 37% companies noticed an increase in customers' satisfaction as benefit, while it is a major purpose to implement Lean Six Sigma. It may be attributed to the fact that companies are more focused on processes than on customers directly. In their opinion customers will be satisfied with the quality and speed of delivery, therefore are unwilling to spend additional resource for expanded study on the increase of consumer's satisfaction.

Conclusions

The above mentioned analyses suggest that food producers search for solutions to achieve competitive edge. They take actions on various levels such as: to optimize chain of delivery, eliminate errors caused by processes and machines, eliminate any wastage and increase employees' involvement. For this purpose they most frequently use Lean Management tools: 5S, Kazein, SMED, VSM, standardized work, TPM and Six Sigma tools: process mapping, control charts, histogram, Ishikawa and Pareto diagrams. Lean Management tools are chosen more frequently which may be attributed to a specific character of the business where speed is vital. Companies declare that the use of tools brings positive results such as: cost reduction, stock reduction, wastage elimination, processes time reduction, team work improvement and improvement of problem solving and decision taking processes. The above mentioned benefits confirm usefulness of Lean Six Sigma tools implementation in food industry companies as a tool to improve management.

By the analysis of Lean Six Sigma tools implementation, maintenance and development it may be concluded that the biggest problem is to treat Lean Six

Sigma as a „toolbox" to reduce costs in a company. Companies implement tools without considering the most important factor – change the way of thinking. Companies still first look for the guilty party and only then will they think on how to solve the problem. According to Lean Six Sigma philosophy first priority is to visit the place where a problem occurred (Gemba), then gather employees and think together on a solution. 96% of problems lie inside a process and only 4% are caused by human error (Deming). Employees; fear of changes (POTWORA 2012), based on intellectual, socio-cultural and emotional backgrounds, is an enormous barrier. Employees, on one hand, fear to lose their job, on the other hand, fear additional responsibilities, schemes and structures. The reason why Lean Six Sigma tools are less efficient than they might have been may be caused by: leader's lack of leadership abilities and/or competence, lack of funds for necessary trainings for employees when it comes to competence and qualification increase, not outsourcing a Sensei.

Despite many barriers and difficulties hampering Lean Management and Six Sigma rules from full implementation, it may be stated that particular tools may indeed influence growth of competitive edge in food industry improving the management process. Growth is achieved by elimination of activities which add no value, 7 Muda reduction i.e. conscious cost management. The division of costs into indirect, the costs a company has no influence on, and direct, which are costs that company manages itself by using set of Lean Six Sigma tools, is an obvious sign that a company is improving their management. Moreover, a proper use of Lean Six Sigma tools may increase a profit margin (cost reduction) and improve products' quality which may help company stay on the market during crisis period.

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EVALUATION OF THE MICROBIOLOGICAL QUALITY OF DAIRY PRODUCTS USING TEMPO SYSTEM

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K e y w o r d s: dairy products, microbiological quality, TEMPO.

A b s t r a c t

In order to ensure microbiological safety of foodstuffs a comprehensive and integrated approach needs to be applied to production process, comply with all obligatory quality standards at each step. In present work a microbiological quality of selected ripened cheeses (5) and liquid dairy products (5) was analyzed using following tests available in TEMPO system: YM – yeasts and molds, STA – the number of *Staphylococcus*, LAB – lactic acid bacteria, EC – *Escherichia coli*, CC – the number of coliforms, TC – total number of coliforms, EB – the number of bacteria belonging to *Enterobacteriaceae*family, TVC – total number of mesophilic microorganisms. Performed analysis using TEMPO system indicated that microbiological quality of selected ripened cheeses and liquid dairy products is satisfactory. TEMPO system turned out to be an useful tool in establishing parameters that define microbiological purity of analyzed food products.

OCENA JAKOŚCI MIKROBIOLOGICZNEJ PRODUKTÓW MLECZARSKICH Z WYKORZYSTANIEM URZĄDZENIA TEMPO

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S ł o w a k l u c z o w e: produkty mleczarskie, jakość mikrobiologiczna, TEMPO.

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Abstrakt

W celu zapewnienia bezpieczeństwa zdrowotnego produktów spożywczych należy zastosować wszechstronne i zintegrowane podejście do procesu produkcji, spełniając wymagane normy jakości na każdym jego etapie. W ramach pracy przeanalizowano jakość mikrobiologiczną wybranych serów dojrzewających (5) oraz płynnych produktów mleczarskich (5) z wykorzystaniem testów dostępnych w systemie TEMPO: YM – drożdże i pleśnie, STA – liczba bakterii *Staphylococcus*, LAB – liczba bakterii mlekowych, EC – liczba *Escherichia coli*, CC – liczba bakterii z grupy *coli*, TC – ogólna liczba bakterii z grupy *coli*, EB – liczba bakterii z rodziny *Enterobacteriaceae*, TVC – ogólna liczba drobnoustrojów mezofilnych. Analizy wykonane za pomocą urządzenia TEMPO wykazały, iż jakość mikrobiologiczna wybranych serów dojrzewających oraz płynnych produktów mleczarskich jest zadowalająca. System TEMPO okazał się przydatnym urządzeniem do określenia wskaźników stanowiących o czystości mikrobiologicznej badanych produktów.

Introduction

Assurance of microbiological safety of food is one of the aim of the food policy which has particular meaning in public health protection. According to Food Hygiene Basic Texts food safety denotes that food will not cause harm to the consumer when it is prepared and/or eaten according to its intended use (CODEX ALIMENTARIUS 2009). In order to guarantee high microbiological quality of the final product, one needs to comply with the safety standards at raw material production step, assuring appropriate quality of fodder and conditions for animal husbandry, through production process of foodstuffs control, as well as their distribution and storage. Each step has significant meaning to obtain high quality of food product, influencing also their commercial quality.

Requirements regarding microbiological quality of food products is defined by Regulation EC 2073/2005, together with later modifications (1441/2007 and 365/2010). This Regulation introduces following definitions:

– **microbiological criterion**: means a criterion allowing the acceptability of a product, a batch of foodstuffs or a process, based on the absence, presence or number of microorganisms and/or their toxins or metabolites, per unit of mass, volume, area or batch,

– **food safety criterion**: means a criterion defining the acceptability of a product or a batch of foodstuffs applicable to products placed on the market,

– **process hygiene criterion**: a criterion indicating the acceptable functioning of the production process; it sets an indicative contamination value above which corrective actions are required in order to maintain the hygiene of the process in compliance with food law (Regulation EC 2073/2005).

Above regulations sets microbiological criteria for dairy products present in Tables 1 and 2. Regulation 2073/2005 additionally defines safety criteria for ready-to-eat foods for infants and ready-to-eat foods for special medical purposes.

Table 1

Food safety criteria for dairy products

Food category	Micro-organisms/their toxins, metabolites	Sampling plan		Limits	
		n	c	m	M
1.2. Ready-to-eat foods able to support the growth of L. monocytogenes, other than those intended for infants and foe special medical purposes	*Listeria monocytogenes*	5	0	100 cfu/g	
		5	0	Absence in 25 g	
1.3. Ready-to-eat foods unable to support the growth of L. monocytogenes, other than those intended for infants and foe special medical purposes	*Listeria monocytogenes*	5	0	100 cfu/g	
1.11. Cheeses, butter and cream made from raw milk or milk that has undergone a lower heat treatment than pasteurisation	*Salmonella*	5	0	Absence in 25 g	
1.12. Milk powder and whey powder	*Salmonella*	5	0	Absence in 25 g	
1.13 Ice cream, excluding products where the manufacturing process or the composition of the product will eliminate the salmonella risk	*Salmonella*	5	0	Absence in 25 g	
1.21. Cheeses, milk powder and whey powder, as referred to in the coagulase-positive staphylococci criteria	Staphylococcal enterotoxins	5	0	Not detected in 25 g	

Explanations to Table 1: n – number of units comprising the sample, c – number of sample units giving values between m and M , m=M, cfu – colonies forming units. References: Regulations 2073 (2005), 1441 (2007), 365 (2010)

Evaluation of microbiological quality of food products based on traditional plate counts method is time-consuming, labor- and material-intensive, and in consequencemuch higher costs of analysis are generated. Another essential issue is waiting time for the results of analysis, especially where one deals with perishable foods. Food safety management systems demand fast evaluation of microbiological quality in order to make a decision about actions which eliminate hazard to the health of consumers. One of the methods which significantly shortens the waiting time for the results of analysis, and uses much less materials and work, is a method based on fluorescence phenomena used in TEMPO device (Biomerieux). The mechanism of reading is based on the measurements of fluorescence signal as a result of fluorescent compound light induction which is obtained through reaction performed by microorganisms present in analyzed product (NOWAK and CHARLIŃSKI 2012). TEMPO is an automated system that enables the quantitative analysis of such microbiological indicators as total number of microorganisms, *Enterobacteriaceae*, the

Table 2

Process hygiene criteria for dairy products

Food category	Micro-organisms/their toxins, metabolites	Sampling plan		Limits (cfu/g or ml)	
		n	c	m	M
2.2.1. Pasteurised milk and other pasteurized liquid dairy products	*Enterobacteriaceae*	5	0	10 cfu/ml	
2.2.2. Cheesed made from milk or whey that has undergone heat treatment	*E. coli*	5	2	100 cfu/g	1000 cfu/g
2.2.3. Cheeses made from raw milk	Coagulase-positive staphylococci	5	2	10^4 cfu/g	10^5 cfu/g
2.2.4. Cheeses made from milk that has undergone a lower heat treatment than pasteurisation and ripened cheeses made from milk or whey that has undergone pasteurisation or a stronger heat treatment	Coagulase-positive staphylococci	5	2	100 cfu/g	1000 cfu/g
2.2.5. Unripened soft cheeses made from milk or whey that has undergone pasteurization or a stronger heat treatment	Coagulase-positive staphylococci	5	2	10 cfu/g	100 cfu/g
2.2.6. Butter and cream made from raw milk or milk that has undergone a lower heat treatment than pasteurisation	*E. coli*	5	2	10 cfu/g	100 cfu/g
2.2.7. Milk powder and whey powder	*Enterobacteriaceae*	5	0	10 cfu/g	
	Coagulase-positive staphylococci	5	2	10 cfu/g	100 cfu/g
2.2.8. Ice cream and frozen dairy desserts	*Enterobacteriaceae*	5	2	10 cfu/g	100 cfu/g

Explanations to Table 2: n – number of units comprising the sample, c – number of sample units giving values between m and M , m=M, cfu – colonies forming units. References: Regulations 2073 (2005), 1441 (2007), 365 (2010)

number of *coliforms*, *E. coli*, coagulase positive *S. aureus*, yeasts and molds, lactic acid bacteria (CHARLIŃSKI 2012).

The aim of present work was to (i) evaluate the microbiological quality of selected dairy products using TEMPO system, as well as (ii) to analyze the possibilities of using TEMPO system to check the compliance with the hygienic and safety criteria of tested food products according to Regulation EC 2073/2005 with further modifications.

Materials and Method

Analyzed dairy products

Following dairy products were bought in a local supermarket and tested microbiologically:

– ripened cheeses: Gouda, Ementaler, Edam, Brie, Camembert,

– liquid products: pasteurized milk, buttermilk, kefir, cranberry yoghurt, plain yoghurt.

Microbiological experiment was performed in three replicates (three items of the same product from different batches were purchased).

Determination of the safety and hygiene criteria for analyzed dairy products according to Regulation EC 2073/2005 with further modifications

Table 3

Food safety and process hygiene criteria for analyzed dairy products according to Regulation 2073/2005 with later modifications

Product	Food safety criteria		Process hygiene criteria		
	Listeria monocytogenes	Staphylococcal enterotoxins (*St. aureus*)	*Enterobactericeae*	*E. coli*	Coagulase-positive staphylococci
Gouda	–	+	+	+	+
Ementaler	–	+	+	+	+
Edam	–	+	+	+	+
Brie	–	+	+	+	+
Camembert	–	+	+	+	+
Pasteurised milk	–	+	+	+	+
Buttermilk	–	+	+	+	+
Kefir	–	+	+	+	+
Cranberry yoghurt	–	+	+	+	+
Plain yoghurt	–	+	+	+	+

Explanations to Table 3: – no possibility to detect in Tempo system, + possibility to detect in Tempo system

Tested dairy products can be qualified to following food categories by analyzing the Regulation 2073/2005:

– ripened cheeses:
  - food safety criteria: 1.2, 1.2.1
  - process hygiene criteria: 2.2.2, 2.2.4
– pasteurised milk:
  - food safety criteria: 1.2

- process hygiene criteria: 2.2.1

– fermented dairy products:
  - food safety criteria: 1.3

Above qualification enabled to define particular microorganisms for analyzed dairy products on the basis of food safety and process hygiene criteria (Table 3).

Microbiological analyses using Tempo system

Depending on analyzed dairy product a 1/4, 1/40 or 1/400 dilution was prepared. TEMPO test requires to hydrate the selective media by adding 3 ml of sterile water. After adding a 1 ml of appropriately diluted product, a scan was made. Prepared samples were placed in the filler. After reading the data and closing the cards, a stands containing cards were transferred to incubators and stored in the following conditions:

– TEMPO EC (*Eschierichia coli)*: 37°C /24h
– TEMPO EB (*Enterobacteriaceae):* 35°C/24h
– TEMPO TC (total number of *coliforms*): 30°C/24h
– TEMPO LAB (lactic acid bacteria): 30°C/48h
– TEMPO TVC (total number of mesophilic microorganisms): 30°C/48h
– TEMPO STA (the number of *Staphylococcus aureus)*: 37°C/24h
– TEMPO YM (yeasts and molds): 25°C/72h.

From the cards filler station data were sent to reading station. After incubation period the cards were placed in TEMPO Reader station, where the data was saved. The last stage of work was validation and printing.

Results and Discussion

In present paper the microbiological quality of selected ripened cheeses and liquid dairy products using TEMPO system is analyzed. The main disadvantage of the device is lack of microbiological selective tests against *Listeria monocytogenes*, which makes unable to check the accordance with food safety criteria for this pathogen. Table 4 presents the results of microbiological analysis performed in soft, semi-hard and hard cheeses.

Tests performed in order to determine the number of lactic acid bacteria (LAB) indicated their level ca. > 4.9×10^4 cfu/g in each of the analyzed cheese. This bacteria ferment lactose to lactic acid and they are essential in dairy products production processes, e.g. they are responsible for cheese maturation. The evaluation of total number of mesophilic microorganisms (TVC) revealed their level > 4.9×10^4 cfu/g. This number is in accordance with the polish

Table 4

Results of microbiological analysis performed in selected ripened cheeses (cfu/g)

Test/Product	Gouda	Ementaler	Edam	Brie	Camembert
LAB	> $4.9x10^4$	> $4.9x10^4$	> $4.9x10^4$	> $4.9x10^4$	> $4.9x10^4$
TVC	> $4.9x10^4$	> $4.9x10^4$	> $4.9x10^4$	> $4.9x10^4$	> $4.9x10^4$
STA	< 10	< 10	< 10	< 10	< 10
EB	< 10	< 10	< 10	< 10	< 10
EC	< 10	< 10	< 10	< 10	< 10
TC	< 10	< 10	< 10	< 10	< 10
YM	< 100	< 100	< 100	> $4.9x10^4$	> $4.9x10^4$

Explanations to Table 4: LAB – lactic acid bacteria, TVC – total number of mesophilic microorganisms, STA – *Staphylococcus aureus,* EB – *Enterobacteriaceae*, EC – *Escherichia coli*, TC – total number of coliforms ISO30°C, YM – yeasts and molds

Ministry of Health Regulation from 13th of January year where the maximum number of microorganisms should not exceed $5x10^5$ cfu/g. Obtained results for *S. aureus* (STA), *Enterobacteriaceae* (EB) and *E. coli* (EC) in ripened cheeses showed their level < 10 cfu/g. It suggests that analyzed cheeses do not contain pathogenic microorganisms that can be harmful for consumers. Similar results were obtained for coliforms (TC) – their level did not exceed 10 cfu/g. In case of mold cheeses Brie and Camembert, the number of molds and yeasts was > $4.9x10^4$cfu/g.

According to the Regulation 2073/2005 the process hygiene criteria are determined by such microorganisms like *E. coli* and coagulase-positive *S. aureus*. Mentioned Regulation demands that in the case of cheeses made from milk or whey that has undergone heat treatment, the level of *E. coli* does not exceed 100 cfu/g. Performed analyses using TEMPO system confirmed that the cheese samples contain less than 10 cfu/g. The source of *E. coli* in ripened cheeses can be the raw material, as well as the reinfection of final product. Studies performed by BERTHOLD and STACHURA (2009) showed that *E. coli* and enterohemorrhagicstrain O157:H7 grow well in soft cheeses. Moreover, in cited paper it was stated that the microflora of hard cheese creates appropriate conditions for *E. coli* growth.

The Regulation 2073/2005 with later modifications says that cheeses made from milk that has undergone a heat treatment should contain < 100 cfu/g of coagulase-positive *S. aureus* (STA). Microbiological analyses performed in present paper showed that cheeses available on the market contain approximately >10 cfu/g of *S. aureus*. Total viable count (TVC) in each kind of cheese was around > $4.9x10^4$ cfu/g. Total number of microorganisms in cheese depends firstly on the microflora of raw material, production and ripening conditions, as well as treatment of cheeses after production (BERTHOLD 2009). Contamina-

tion of all samples of cheese by yeasts and molds varied between 10^2 – $4.9x10^4$ cfu/g. According to literature the number of yeasts and molds that has negative impact on cheese quality is around $>10^5$ cfu/g (URARTE 1999). The higher number can deteriorate the sensory properties of cheese, and can influence the taste defect – yeast and foreign off-flavour (BERTHOLD 2009).

Table 5 presents the results of microbiological analysis performed in liquid dairy products.

Table 5

Results of microbiological analysis performed in liquid dairy products (cfu/ml)

Test/Product	Pasteurised milk	Butter milk	Kefir	Cranberry yoghurt	Plain yoghurt
LAB	< 1	> $4.9x10^4$	> $4.9x10^4$	> $4.9x10^4$	> $4.9x10^4$
TVC	< 1	> $4.9x10^4$	> $4.9x10^4$	> $4.9x10^4$	> $4.9x10^4$
STA	< 1	< 10	< 10	< 10	< 10
EB	< 1	< 1	< 1	< 1	< 1
EC	< 1	< 1	< 1	< 1	< 1
TC	< 1	< 1	< 10	< 10	< 1
YM	< 1	< 100	< 100	< 100	<100

Explanations to Table 5: LAB – lactic acid bacteria, TVC – total number of mesophilic microorganisms, STA – *Staphylococcus aureus,* EB – *Enterobacteriaceae*, EC – *Escherichia coli*, TC – total number of coliforms ISO30°C, YM – yeasts and molds

Total number of coliforms (TC) in analyzed liquid dairy products was from <1.0 cfu/ml to <10.0 cfu/ml, whereas *E. coli* (EC) for each product was <1.0 cfu/ml. According to the polish Ministry of Health Regulation from 13th of January 2003 coliforms cannot be present in foodstuffs. In studies performed by PLUTA et al. (2001) the presence of coliforms was determined in all samples of yoghurt and bio-yoghurt tested in 1998–2001. The level of *Enterobacteriaceae* (EB) and *E. coli* was less than 1.0 cfu/ml. The number of *S. aureus* (STA) was less than 1.0 cfu/ml in case of pasteurized milk, and less than 10 cfu/ml for other fermented dairy products. Microbiological tests performed in order to assess the number of spoilage microflora, such as STA, EB and EC determined their level in a range 1.0 – 10.0 cfu/ml. It suggests that analyzed dairy products does not contain foodborne pathogens that can be harmful to consumers. In performed studies an appropriate level of lactic acid bacteria (LAB) was obtained: for pasteurized milk – <1.0 cfu/ml, for buttermilk, kefir, cranberry yoghurt, plain yoghurt the number of LAB was approximately $>4.9x10^4$ cfu/ml. Lactic acid bacteria are typical microorganisms found in fermented dairy products. Total viable counts of mesophilic microorganisms (TVC) for milk was <1.0 cfu/ml, and for fermented dairy products $>4.9x10^4$

cfu/ml. It is a proper level because maximum number of microorganisms is $5x10^5$ cfu/ml and above this number food product becomes disqualified and cannot be placed on the market (BERTHOLD 2009). The last microbiological test which was performed in order to determine the number of yeasts and molds (YM) revealed their level below 1 cfu/ml in case of pasteurized milk, and below 100 cfu/ml for analyzed fermented dairy products. PLUTA et al. (2001) studied the prevalence of yeasts and molds in 1995–1998 and 2000–2001 in food products. The researchers found the number of yeasts and molds above 10^4 cfu/g in yoghurts and bio-yoghurts in 10% of analyzed samples, whereas in 2000–2001 this level of microorganisms was in 7% of tested yoghurts and bio-yoghurts. In another studies conducted also in 2000–2001 by ORZECHOWSKA et al. (2001) yeasts and molds were absent in 0.1g of product in 85% of analyzed samples, and in a range of 10^1–10^3 cfu in 1g of product in 15% of analyzed products.

Conclusions

On the basis on performed studies it was stated that TEMPO system used in the following studies increased the cohesion of obtained results and facilitated their interpretation. Nevertheless the main disadvantage of this device is lack of test in order to determine the number of *Listeria monocytogenes*, which makes unable to check the accordance with obligatory Regulation 2073/2005 with later modifications. Performed analysis confirmed that tested dairy products were produced from raw material with appropriate microbiological quality; each step of dairy products production was made in adequate manner, in order to achieve a satisfactory quality of food product placed on the market.

Translated by AUTHOR

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COMPOSITION AND SEASONAL CHANGES OF LITTER ALONG THE SHORELINES OF SELECTED WATER BODIES IN WARMIA AND MAZURY REGION (NORTH-EASTERN POLAND)

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K e y w o r d s: rubbish, plastic, lake, tourism, recreational fisheries.

A b s t r a c t

Pollution of the shoreline of aquatic ecosystems, including inland water bodies, can pose a serious threat to the natural environment. The aim of this study were to quantify the spatial and temporal variation in anthropogenic litter abundance along 5 water bodies shore. This study examines the number, composition and seasonal changes in the litter found along the shores of selected water bodies in north-eastern Poland (the region of Warmia and Mazury). Water bodies and the shoreline fragments were selected for the study which represents different types of waters, lotic and lentic waters as well as those built by humans and natural ones. These water bodies are easily accessible to anglers from the shore and to hiking tourists and cyclists. Litter items were counted 3 times in 2013, before the tourist season, during the season and thereafter. The amount of litter along the shores of different water bodies differed, with the largest amounts being found along the Wadąg River (from 1172 to 1756 items ha^{-1}). However, the amount of litter found along the shorelines of the water bodies under study was not found to be season-dependent. The largest group of litter found along the shorelines was what can be included in the „other" category (from 23.1% to 38.1%), as well as plastic bags (from 15.1 to 24.0%), which can be particularly harmful to aquatic organisms. This study indicates that litter accumulated along the shores of water bodies in Warmia and Mazury is a considerable problem, which could create a barrier for further sustainable development of tourism and recreational fisheries in the region.

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SKŁAD I SEZONOWE ZMIANY ZAŚMIECENIA LINII BRZEGOWEJ WYBRANYCH ZBIORNIKÓW WODNYCH W REGIONIE WARMII I MAZUR (PÓŁNOCNO-WSCHODNIA POLSKA)

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S ł o w a k l u c z o w e: śmieci, plastik, jezioro, turystyka, rybactwo rekreacyjne.

A b s t r a k t

Zanieczyszczenie linii brzegowej ekosystemów wodnych, w tym akwenów śródlądowych, może stanowić realne zagrożenie dla środowiska naturalnego. Celem niniejszej pracy było określenie przestrzennej i czasowej zmienności obfitości antropogenicznych śmieci wzdłuż brzegu 5 zbiorników wodnych. W pracy zbadano liczebność, skład, a także zmiany sezonowe śmieci występujących wzdłuż linii brzegowej wybranych zbiorników i cieków w północno-wschodniej Polsce (region Warmia i Mazury). Akweny i fragmenty linii brzegowej zostały tak dobrane żeby z jednej strony reprezentowały różne typy wód: płynące, stojące, sztuczne kanały oraz naturalne zbiorniki, z drugiej zaś, były łatwo dostępne dla wędkarzy łowiących z brzegu oraz turystów pieszych i rowerzystów. Śmieci liczono trzykrotnie w roku 2013, przed sezonem turystycznym, w środku sezonu oraz po sezonie. Ilość śmieci w linii brzegowej poszczególnych akwenów różniła się, a największą ich ilość znajdowano nad rzeką Wadąg (od 1172 do 1756 szt. ha^{-1}). Nie stwierdzono natomiast istotnego wpływu pory roku na ilość śmieci znajdującą się nad brzegami badanych zbiorników i cieków. Najliczniejszą grupę odpadów znajdowanych w linii brzegowej stanowiły śmieci zaliczone do kategorii „inne" (od 23.1% do 38.1%) oraz wykonane z tworzyw sztucznych torby foliowe (od 15.1 do 24.0%), które mogą być szczególnie niebezpieczne dla organizmów wodnych. Wyniki niniejszych badań sugerują, że zaśmiecenie linii brzegowej zbiorników i cieków na Warmii i Mazurach jest sporym problemem, który nierozwiązany może stanowić barierę dla dalszego zrównoważonego rozwoju turystyki i rybołówstwa rekreacyjnego w regionie.

Introduction

The problem of litter and the littering of waters and shore zones has been increasing since the 1950s, when plastics entered common use (CARPENTER et al. 1972, BARNES et al. 2009). Currently, the accumulation of litter in aquatic ecosystems is a growing problem around the globe (WETZEL et al. 2004). Litter in waters has an obvious effect on nature; microplastic is particularly harmful because it is eaten by many aquatic organisms, such as bivalves (CANESI et al. 2012), crustaceans (MURRAY and COWIE 2011), and fish (BOERGER et al. 2010).

Plastic pellets are worldwide contaminants that accumulate in the ocean, especially in sandy beaches (TURRA et al. 2014). The issue of littering waters and the coastal areas of seas and oceans has been explored more thoroughly than freshwater ecosystems (KORDELLA et al. 2013, HOELLEIN et al. 2014, NGUPULA et al. 2014). Most of the research studies have dealt directly with litter on the bottom, the water and the surface of seas and oceans (MOORE et al. 2001, KOUTSODENDRIS et al. 2008, BOERGER et al. 2010). There have been many studies on littering coastlines and beaches of salt water (CLAEREBOUDT 2004, MARTINEZ-RIBES et al. 2007, TOPCU et al. 2013) and relatively scarce reports on litter accumulated along the shorelines of inland waters (HOELLEIN et al. 2014, DRIEDGER et al. 2015).

Warmia and Mazury is a geographic and cultural region in the north-east of Poland, mainly in the lakeland; administratively, it lies within the province of Warmia and Mazury. The city of Olsztyn is the capital of the region and is the largest (population of about 180 thousand) city. Warmia and Mazury is a specific region of Poland, mainly because of its natural features. Of particular importance to the region are surface waters which occupy 138 566 ha, which is 5.7% of the area of the province of Warmia and Mazury and represents the highest proportion of water surface in the entire country (SZCZERBOWSKI 1995). The littering of beaches and coastal areas is one of the most visible manifestations of human activities in various aquatic ecosystems. For this reason, areas attractive to tourists may be particularly susceptible to such pollution (TURRA et al. 2014) and pollution caused by tourism appears to be increasing (GILBERT 2008). The touristic and recreational pressure in the area of Warmia and Mazury seems to be increasing; the number of guests in accommodation facilities in the years 2005–2011 increased from 760 to nearly 960 thousand (Statistical Office in Olsztyn 2012). The latest marketing activities aimed at promoting the region, including the „Mazury Cud Natury" („Mazury – a wonder of nature") campaign, have raised interest in the region. However, this also affects the deteriorating condition of the environment, especially the shoreline areas of lakes, rivers and other water bodies, which are major tourist attractions in the region (CZARKOWSKI et al. 2012, 2014). According to MAMCARZ and SKRZYPCZAK (2011), the development of tourism and recreation on waters in the north-east of Poland does not entail analysis of the pressure on the environment, which – together with uncontrolled promotion of the region and improper fisheries management – may bring negative effects by reducing regional biodiversity.

Surface water monitoring in Warmia and Mazury has been conducted for some time. This has included examination of the chemical condition, level of eutrophication, vascular plant condition, zoo- and phytoplankton, benthos, ichthyofauna, as well as physical and chemical properties of water. However,

no studies have been conducted on the amount and composition of litter left behind by people on the shores of inland water bodies in Warmia and Mazury. It seems that the litter left behind in the shore areas is becoming an increasingly serious problem in the lake districts, including Warmia and Mazury. It is well known that littered shoreline and beaches can repel tourists, thereby having a negative effect on tourism (KAI 2005). To deal with the problem effectively, it is necessary to determine the scale, components and the nature of the phenomenon, as well as its seasonal fluctuations. Therefore, it was decided to examine the extent of littering of the shoreline of selected water bodies in Warmia and Mazury over three study periods.

Materials and Methods

The study was conducted along fragments of the shoreline of 5 water bodies in Warmia and Mazury in the north-east of Poland, in the Province of Warmia and Mazury. These included the following bodies: Lakes Giławy, Łowne Duże and Ustrych, the Wadąg River and Kanał Węgorzewski (Węgorzewski Canal) (Figure 1, Table 1). Water bodies and the shoreline fragments were selected for the study which represent different types of waters, lotic and lentic waters as well as those built by humans and natural ones. These water bodies are easily accessible to anglers from the shore and to hiking tourists and cyclists. Moreover, it is noteworthy that Lake Ustrych is in the „Las Warmiński" Nature Reserve. A total of 4.73 km of shoreline was examined with an area of 4.70 ha (Table 1).

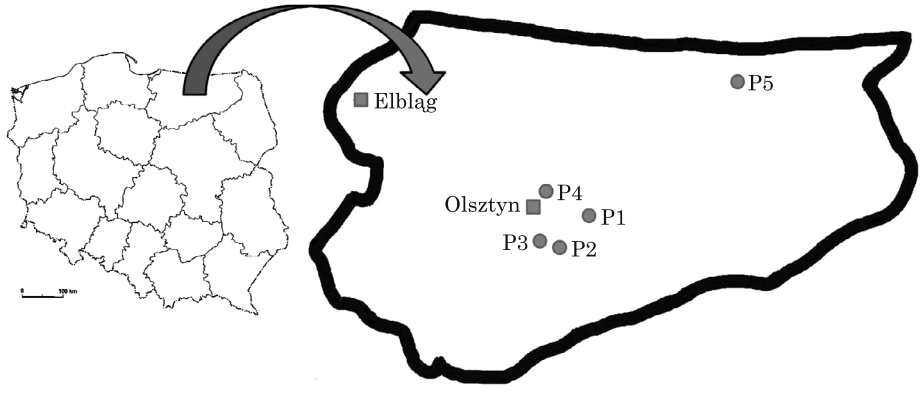

Fig. 1. Localization of studied water bodies in the Province of Warmia and Mazury: Lake Giławy (P1), Lake Łowne Duże (P2), Lake Ustrych (P3), Wądąg River (P4), Węgorzewski Canal (P5)

Table 1

Localization and characteristics of studied water bodies in Warmia and Mazury

Parameters	Lake Giławy	Lake Łowne Duże	Lake Ustrych	Wadąg River	Węgorzewski Canal
Latitude (°N)	53°42'44.04"	53°35'59.4"	53°38'12.89"	53°48'52.04"	54°12'21.35"
Longitude (°E)	20°49'2.44"	20°38'20.12"	20°29'46.53"	20°31'38.79"	21°43'45.8"
Shoreline length (m)	950	780	690	490	1820
Shoreline area (ha)	0.95	0.92	1.51	0.25	1.07
Distance from the city center (km)	26.2	22.5	15.5	3.7	0.8
Fished sites	16	16	7	28	59
Litter bins	0	0	0	0	9

Litter items were counted 3 times in 2013, before the tourist season (01–25.05; spring), during the season (11.07–02.08; summer) and thereafter (01–09.11; autumn). The litter was not collected to enable season-to-season comparisons, assuming that it was not only left behind, but it could also be collected by people for various reasons. Solid items were counted on the land while walking along the shore (the water/land junction) of the water bodies. The study zone was limited by a dirt road running in the immediate vicinity of the water. Since the belt in which litter items were counted was 4–12 m wide along the majority of the shoreline under study, the amount of litter was referred to as the area of the shore zone. The litter was classified into 10 groups: 1) empty packages of artificial lures; 2) empty packages and boxes of live and artificial bait; 3) small items of angling equipment and its packages; 4) plastic buckets; 5) empty cans of food and drinks; 6) plastic PET bottles; 7) empty glass alcohol bottles and other glass containers; 8) foil and plastic bags; 9) empty cigarette packets; 10) other (small paper pieces, food packages, tyres, air tubes and other rubber items). Piers and footbridges for anglers as well as other fishing sites and litter bins placed in the shore zone of the water bodies were also counted. The percentage of different categories (%) and the amount of different types of litter, expressed in absolute and relative numbers for different water bodies and between seasons, was also compared.

Statistical analysis

The statistical analysis was preceded by data transformation ($\log n+1$) and subsequent verification of homogeneity of variance with Levene's test. Subsequently, a two-way ANOVA was used to compare the amounts of litter in these categories across the aquatic ecosystems and seasons. *Post-hoc* analyses were conducted after statistically significant values of the F-test were achieved.

Tukey's test for a different number of variables was used to find statistically significant differences between the variables under study. The water bodies being examined were grouped by classification analysis. Data agglomeration was performed by the Ward method, using Euclidean distances as a measure of similarity. All statistical analyses were conducted with Statistica software (Statsoft Polska, Kraków).

Results

A total of 710 items of different kinds of litter were recorded before the tourist season along the shoreline of the water bodies under study. Debris densities not varied significantly between season ($P > 0.05$, Figure 2) whereas sites were significantly different from each other ($P < 0.05$, Table 2). A higher number of litter was observed in autumn. The highest amount was found along the Wadąg River and the lowest amount was along Węgorzewski Canal, both shoreline fragments had the largest number of identified angling sites, but the number of litter bins was completely different (Table 1). The number of litter items after the tourist season increased, reaching a total of 860. As in the previous periods, the highest amount of litter items was found on the Wadąg River and the lowest amount was found along Lake Łowne Duże.

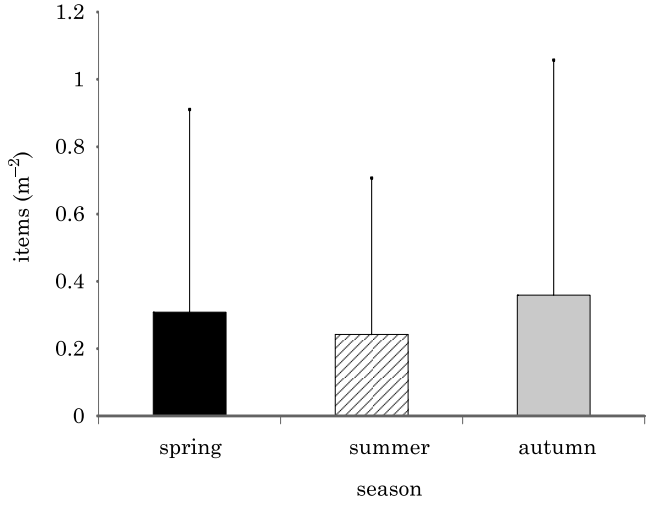

Fig. 2. Mean litter densities (± SD) in five water bodies in Warmia and Mazury during three seasons. SD – standard deviation

Table 2

Number (mean ± SD) of litter items per ha along the shorelines of selected water bodies. The mean values with the same letter index are not significantly different (ANOVA, $P<0.05$). SD – standard deviation

Litter category	Lake Giławy	Lake Łowne Duże	Lake Ustrych	Wadąg River	Węgorzewski Canal
Groundbait packages	$8.4^{b}\pm1.05$	$0.4^{b}\pm0.63$	$1.1^{b}\pm1.38$	$29.3^{a}\pm18.04$	$0.6^{b}\pm0.54$
Empty packages and boxes of lures	$13.0^{b}\pm2.19$	$4.0^{b}\pm3.32$	$5.5^{b}\pm0.76$	$122.7^{a}\pm34.02$	$1.6^{b}\pm1.94$
Small angling equipment	$1.4^{b}\pm0.61$	$0.7^{b}\pm0.63$	$0.2^{b}\pm0.38$	$25.3^{a}\pm23.44$	$0.6^{b}\pm1.08$
Plastic buckets	$1.1^{b}\pm0.01$	$3.3^{b}\pm1.09$	0.00	$8.0^{a}\pm8.00$	$0.3^{b}\pm0.54$
Cans	$11.2^{b}\pm2.65$	$13.4^{b}\pm8.44$	$7.7^{b}\pm1.91$	$108.0^{a}\pm63.50$	$9.7^{b}\pm3.89$
PET bottles	$12.3^{b}\pm0.61$	$5.1^{b}\pm2.26$	$2.6^{b}\pm1.15$	$244.0^{a}\pm97.73$	$5.0^{b}\pm3.78$
Glass bottles	$9.1^{b}\pm3.38$	$15.2^{b}\pm13.18$	$15.0^{b}\pm9.28$	$78.7^{a}\pm37.17$	$10.0^{b}\pm10.25$
Plastic bags	$27.0^{b}\pm20.37$	$9.1^{b}\pm3.49$	$13.0^{b}\pm3.40$	$350.7^{a}\pm46.88$	$12.8^{b}\pm6.76$
Cigarette packets	$3.9^{b}\pm1.61$	$2.5^{b}\pm1.25$	$2.4^{b}\pm1.38$	$48.0^{a}\pm0.01$	$4.7^{b}\pm1.87$
Other	$58.6^{b}\pm29.20$	$26.4^{c}\pm5.36$	$24.5^{b}\pm15.17$	$465.3^{a}\pm94.85$	$20.2^{c}\pm13.09$

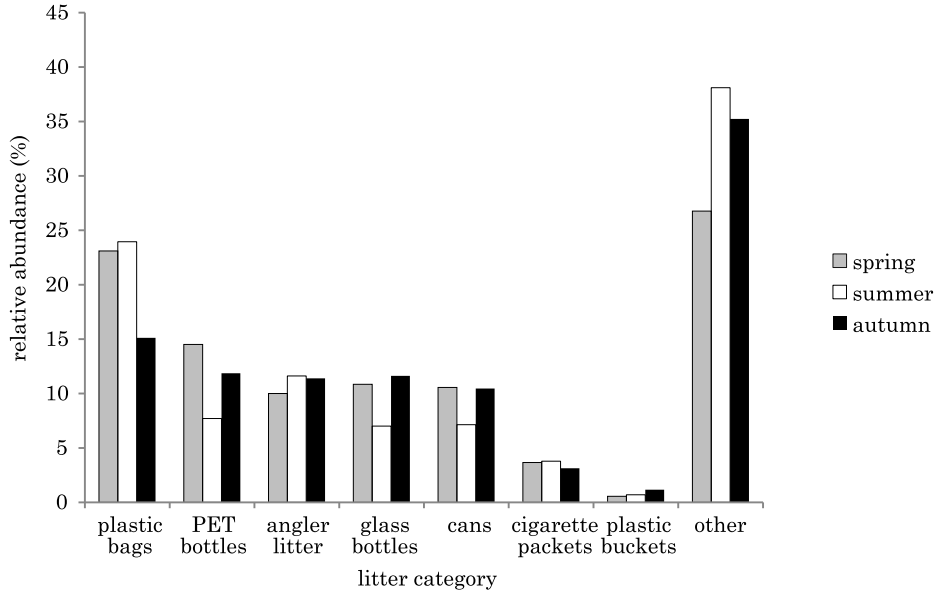

Fig. 3. Seasonal relative abundance of litter found on five water bodies in Warmia and Mazury

The number of litter items along the shoreline of different water bodies was significantly different ($P < 0.05$, Table 2). The highest amount of litter items was found on the Wadąg River (from 1172 to 1756 items ha^{-1}, Figure 2) and the lowest amount was along the Węgorzewski Canal (48.6–83.2 items ha^{-1}). The number of litter items at other sites was similar, except the „other" category ($P > 0.05$). The amount of litter found along the shorelines of the water bodies under study was not found to be season-dependent ($P > 0.05$).

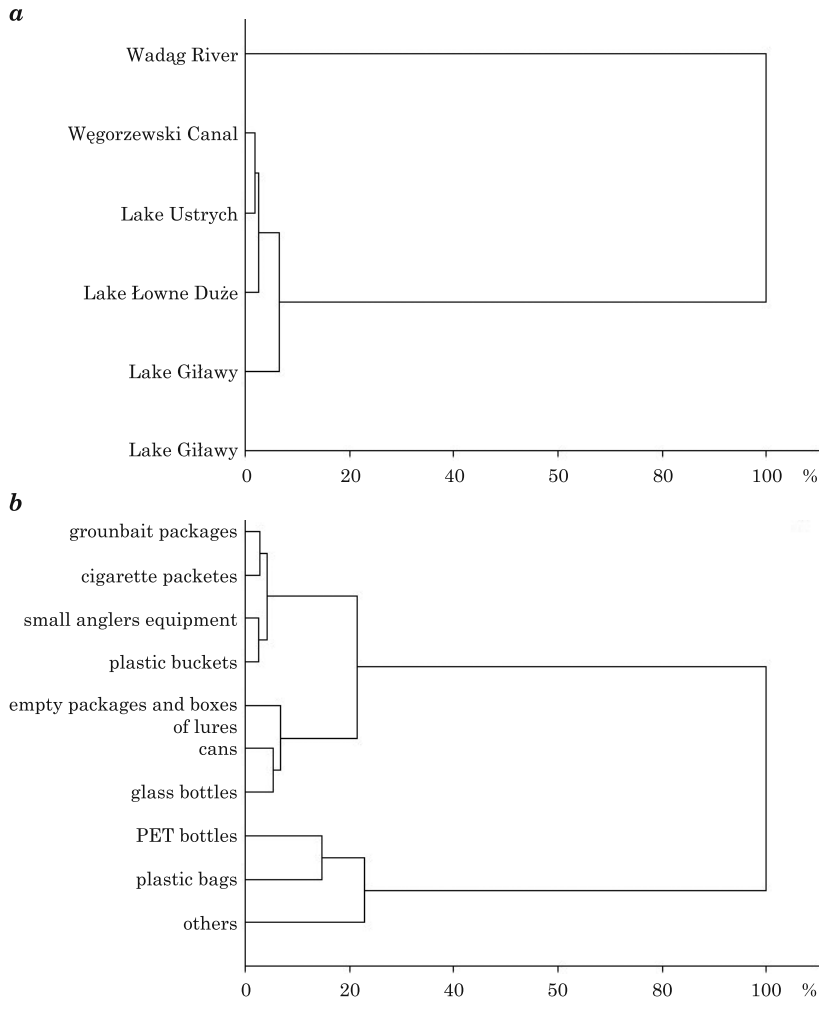

Fig. 4. The classification analysis for the sites (a) and categories of litter under study (b)

Litter classified as „other" accounted for the majority of waste at all the sites, before, during and after the tourist season; it accounted for: 23.1, 38.1 and 35.2% of all the litter (Figure 2). Moreover, high percentages of foil bags were recorded before and during the tourist season (23.1 and 24.0%, respectively). Angler litter accounted for a considerable portion of the rubbish during the tourist season (Figure 3). On the other hand, after the season the number of foil bags decreased (15.1%) and the number of plastic bottles increased (11.9%, Figure 3).

The analysis identified two clusters of the classification tree (Figure 4a). The first branch, considerably different than the other sites, was created by

the Wadąg River, whereas Lake Giławy stands out in the other part of the tree diagram. A comparative analysis of the waste (Figure 4b) shows that a separate branch of the tree diagram was made up of the litter classified as „other" as well as foil bags and PET bottles. These categories of waste accounted for between 50.7–71.6% of the litter. The smallest amount of litter in these categories was found along Lake Łowne and the largest amount was along the Wadąg River. In the other part of the dendrogram, the remaining categories of litter were grouped into two branches. One of them comprised bait boxes, cans and glass bottles, which accounted for from 20.9% of all the litter on the Wadąg River to 40.7% on Lake Łowne Duże. The other branch comprised the litter categories with the fewest items found along the shoreline of the water bodies. These included buckets, cigarette packets, fishing equipment and empty lure boxes. They accounted for 5.1% along the shoreline of Lake Ustrych and up to 10.1% on Lake Giławy.

Discussion

Depending on the season, the number of litter items found on the water bodies under study ranged from 710 to 860, along the total distance of 4.73 km of the shoreline, which is equivalent to 150.1 to 181.8 items km^{-1}. These findings, especially compared to the data obtained for the coastline of salt water bodies visited by tourists, can be regarded as positive because, for example, as many as over 130 items were found per one metre of the coastline in the Balearic Islands (MARTINEZ-RIBES et al. 2007). For comparison, the number of litter items along the coastline of the Baltic (the province of Pomerania) did not exceed 100 per km (GILBERT 2008), whereas 213.7 items per km were found along the shoreline of inland waters in the province of Saskatchewan, Canada (Great Canadian Shoreline Clean-up 2012). When the amount of litter is referred to a unit area, the results of this study are considerably different than those obtained on the beaches of Ghana by TSAGBEY et al. (2009), where much more litter was found.

Regardless of the season, the largest amount of litter was found on the Wadąg River, whose fragment under study lies within the boundaries of Olsztyn, a mere 4 km away from the city centre. Moreover, the number of accessible angling sites per 1 km of the shoreline (57.1) was larger than elsewhere. It appears that this may contribute to the higher level of shoreline littering. Although the area lies within the city boundaries, there are no litter bins. On the other hand, the amount of litter items found on the Węgorzewski Canal, which also lies within the town boundaries and is popular with both tourists and town inhabitants, was the smallest. Although the number of litter

bins along Węgorzewski Canal ranged from 7 to 9. No statistical differences were found between the amounts of litter during different seasons, but TOPCU et al. (2013) indicated distinct seasonal variability, because much more litter was found in autumn. Since the statistical analysis did not reveal any significant differences across seasons, litter appears to have been collected, not only left behind. Regular, coordinated cleaning of the shoreline was probably only done on the Węgorzewski Canal. Litter on other water bodies may have been collected, on a more or less individual basis, both by people collecting recyclable materials, as well as by more environmentally-aware fishermen, tourists or people living nearby.

During this study, different items were found along the shorelines which had been brought there by water. Most of them were packages which were left in the water after their contents had been used. As the analysis shows, the largest group of items included plastic objects (bags and bottles) and waste included in the „other" category, which were more difficult to classify specifically. These two categories of litter also dominated in the study conducted by MARTINEZ-RIBES (2007). In a study conducted recently on the Thames, plastic items, along with empty food and tobacco packages, also accounted for a considerable portion of the intercepted litter (MORRITT et al. 2014). The plastic waste, mainly bags and bottles, accounted for 61% of the litter along the Baltic coastline (Gilbert 2008). Plastic is regarded as one of the major agents polluting aquatic ecosystems, especially in salt waters (DERRAIK 2002, BARNES et al. 2009). Recently, it has been regarded as a potential threat to fresh waters; this applies mainly to the smallest plastic items. Plastic is a problem both in European lakes (FAURE et al. 2012) and the large American lakes (ZBYSZEWSKI and CORCORAN 2011, ERIKSEN et al. 2013). Plastic may have a negative impact on aquatic animals (fish, birds, mammals) because they may mistake plastic items for food and swallow them, which frequently ends up clogging their alimentary tract or poisoning them with toxins transmitted on plastic objects.

Inland recreational fisheries is becoming the main form of exploitation of wild freshwater fauna, both around the world (COWX et al. 2010) and in Poland (WOŁOS and DRASZKIEWICZ-MIODUSZEWSKA 2012, MICKIEWICZ 2013, LIRSKI and HRYSZKO 2014). Unfortunately, its negative effect on aquatic ecosystems, including the shore area, is becoming noticeable (COOKE and COWX 2006, ARLINGHAUS and COOKE 2009). One of the problems associated with recreational fisheries is the littering of the shoreline by anglers. The amount of litter left behind at sites of increased activity of anglers may be exceedingly large. For example, the amount of litter left behind by anglers during one season in a small marina (0.22 ha) situated about 20 km away from Olsztyn, on Lake Kośno, was 1311 items, with 313 empty bait and lure packages (CZARKOWSKI and KOZŁOWSKI unpublished data). This is why items of litter directly

associated with recreational fisheries (empty lure and bait packages, small items of fishing equipment and its packages, plastic buckets) were counted separately. Such typically angling-related litter accounted for up to 24.7% of all the litter found, depending on the water body and the season. In total, along all the fragments of the shoreline under study, litter accounted for from 10.6% before the season to 12.6% after the season, with a considerable contribution of bait and lure boxes and packages (up to 8.1% of the total amount). The total number of bait boxes per unit length of the shoreline under study ranged from 10.4 before the season to 13.3 items per km after the season. Compared with the Saskatchewan data, where the number of bait packages was close to 2.9 items per km (Great Canadian Shoreline Clean-up, 2012), the numbers recorded in Warmia and Mazury were much higher. In general, litter originating from angling activities may contribute considerably to the pollution of shores around the world (CLAEREBOUDT 2004, TOURINHO and FILLMANN 2011).

The amount of litter along the shores of different water bodies differed and the differences were statistically different. The largest amounts of litter were found on the Wadąg River. However, the amount of litter found along the shorelines of the water bodies under study was not found to be season-dependent. The largest group of litter found along the shorelines was what can be included in the „other" category, as well as plastic bags and PET bottles, which can be particularly harmful to aquatic organisms. This study indicates that littering along the shorelines of water bodies in the region of Warmia and Mazury is a considerable problem, which if left unsolved, could create a barrier to further sustainable development of tourism and recreational fisheries. However, more resources need to be allocated for research into the problem. It is necessary to develop appropriate solutions to prevent further degradation of the shore and the aquatic environment without entailing considerable financial outlays. This approach will be consistent with a sustainable lakeland development strategy.

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PATHOMORPHOLOGICAL EXAMINATION OF THE LIVER IN CATTLE IN THE COURSE OF A NATURAL INFESTATION WITH *FASCIOLA HEPATICA* (L.)

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K e y w o r d s: *Fasciola hepatica,* cows, liver, pathomorphology, ACP, SDH.

A b s t r a c t

An evaluation of lesions in the livers of Black and White cattle, aged 4 to 7, naturally infested with *Fasciola hepatica* was performed. The studies included a comprehensive analysis of histological lesions and liver enzyme activities determined with histochemical methods depending on the degree of natural *Fasciola hepatica* infestation. The studies revealed increased number of reticular fibres, an increase of acid phosphatase (ACP) activity, a reduced activity of succinic acid dehydrogenase (SDH) and a decrease in the content of glycogen in the hepatocytes. The damage of the hepatocytes was potentiated together with increasing severity of fluke infestation, which caused mononuclear cell and eosinophilic granulocyte infiltrates, abscess formation and connective tissue proliferation. The presented lesions in liver were determined by duration of the pathogenic effects of flukes infestation, the number of fluke parasites and the age of the animals.

BADANIE PATOMORFOLOGICZNE WĄTRÓB BYDŁA W PRZEBIEGU NATURALNEJ INWAZJI *FASCIOLA HEPATICA* (L.)

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S ł o w a k l u c z o w e: *Fasciola hepatica,* krowy, wątroba, patomorfologia, ACP, SDH.

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Abstrakt

Przeprowadzono ocenę zmian patologicznych w wątrobach bydła rasy czarno-białej, w wieku od 4 do 7 lat naturalnie zarażonego *Fasciola hepatica*. Badania obejmowały kompleksową analizę zmian histologicznych i aktywności enzymów wątrobowych oznaczanych metodami histochemicznymi w zależności od stopnia naturalnego zarażenia *Fasciola hepatica*. Badania wykazały rozplem włókien retikulinowych, wzrost aktywności fosfatazy kwaśnej (ACP), zmniejszoną aktywność dehydrogenazy kwasu bursztynowego (SDH) i spadek zawartości glikogenu w komórkach wątrobowych. Wraz z wzrostem intensywności inwazji przywr uszkodzenia komórek wątrobowych nasilały się, co powodowało nacieki komórek jednojądrzastych, granulocytów kwasochłonnych, tworzenie się ropni i rozplem tkanki łącznej. Opisane zmiany w wątrobie były zależne od czasu chorobotwórczego oddziaływania przywr, liczby pasożytów i wieku zwierząt.

Introduction

Fasciolosis (liver fluke disease) in cattle is relatively common in Poland and still presents a significant economic issue, as it does in many other countries (CARRADA-BRAVO 2003, GAJEWSKA et al. 2005, KOZŁOWSKA-ŁÓJ and ŁÓJ-MACZULSKA 2013, MAGE et al. 2002, NOVOBILSKY et al. 2014). Animals infested with *Fasciola hepatica* are much less efficient in feed conversion, weight gains and milk production, and their resistance to infection is reduced. Apart from economic losses, these flukes cause disturbances in a number of organs and systems, e.g. the reproductive system (ROMANIUK 1977). Hepatic dysfunctions mainly involve the protein system and liver enzymes. During fluke infestation, the α- and β-globulin fractions in the blood significantly increase and their level is correlated with liver damage. In livers infested with flukes, a significant reduction in glycogen and reductive sugars is detected (NANSEN 1971, FURMAGA and GUNDŁACH 1972).

Migrating juvenile flukes form „migratory tunnels" which, over time, transform into connective tissue scars damaging the hepatic parenchyma over large areas. Bile ducts in the portal areas are significantly enlarged. Diffuse proliferation of the epithelial cells in the bile ducts, especially adjacent to blood vessels was reported (RAHKO 1971).

Flukes have a strong affinity to the hepatic tissue and bile ducts. Resultant lesions in the liver depend on the severity and stage of infestation (GAJEWSKA et al. 2005, KONRAD 1968). DOY et al. (1984) in a gross examination, found multiple red and brown or, locally, white foci under the liver capsule. The majority of these foci (approximately 80%) were situated in the left hepatic lobe, particularly under the capsule. Histology revealed inflammatory cell infiltrates consist of lymphocytes and neutrophils located under the capsule. Additionally indistinct lobular architecture of the parenchyma and haemorrhages were noted. DOY et al. (1984) on 14 days post infection, observed infiltrates of numerous eosinophilic granulocytes in the hepatic parenchyma.

After 21 days post infection, fibrous adhesions of the left hepatic lobe with the diaphragm, infiltrates of numerous eosinophilic granulocytes and regressive lesions in the hepatocytes were observed. Apart from the discussed lesions, DOW et al. (1968) also found hypertrophy and fibrosis of the intima in the hepatic blood vessels. Mechanical damage caused by migration of juvenile stages of liver flukes resulted in thrombosis and inflammatory processes in the blood vessels.

In the available literature, there is a lack of reports on a comprehensive analysis of histological lesions and liver enzyme activity determined with histochemical methods, depending on the degree of a natural infestation with *Fasciola hepatica* flukes.

The objective of the studies was to evaluate the pathomorphological lesions in the liver and to perform a histochemical assessment of the activity of selected enzymes in the hepatocytes in cows naturally infested with *Fasciola hepatica* flukes.

Materials and Methods

The studies were carried out with 22 Black and White cows, aged 4 to 7 years, slaughtered in an abattoir and divided into two groups: those with a liver fluke infestation and those without infestation ($n=9$). The group of animals with liver fluke infestation was further divided into three subgroups depending on the degree of severity and the type of lesions detected macroscopically. Subgroup I included 9 animals with slightly thickened bile ducts, whereas subgroup II consisted of 7 animals with significantly thickened bile ducts, calcification foci and parenchymatous degeneration of the liver; subgroup III included 6 animals with diffuse calcification of bile ducts, single abscesses and lipidosis of the liver.

Two samples of the left hepatic lobe of approximately 100 grams each were collected from the animals infested with *F. hepatica* ($n=22$) and without infestation ($n=9$). One sample for histopathology was fixed in 10% buffered formalin (pH = 7.4). The second was put in a vacuum flask with dry ice for testing succinic acid dehydrogenase (SDH) and acid phosphatase (ACP) activity and for detecting lipids.

The liver samples for histopathology were embedded in paraffin and 5μm-thick paraffin sections were prepared. These paraffin sections were stained with haematoxylin and eosin (HE) according to the PAS method by McManus and silvered according to the method by Gomori (BANCROFT and GAMBLE 2008).

The frozen liver sections were stained for lipids with Sudan IV according to Lillie-Ashhurn (BANCROFT and GAMBLE 2008). In addition, the cryostat liver

sections were tested for succinic acid dehydrogenase activity (BANCROFT and GAMBLE 2008) and acid phosphatase activity (BANCROFT and GAMBLE 2008).

The evaluation of reticular fibres in the liver was achieved by dividing them into three groups: 1 – normal reticular fibres; 2 – an increased number of branched reticular fibres; and 3 – an increased number of reticular fibres of different thickness and extensively branched.

The contents of polysaccharides in the hepatocytes was determined according to a established 3-degree scale: (+) – moderate content of polysaccharides (slightly more intensive pigmentation of the hepatocytes); (++) high content of polysaccharides (intensive pigmentation of the hepatocytes); (+++) – very high content of polysaccharides (very intensive pigmentation of the hepatocytes).

The content of lipids in the hepatocytes was determined microscopically by distinguishing two groups: 1 – a lack of lipid vacuoles in the hepatocytes; and 2 – the presence of lipid vacuoles in single hepatocytes.

The histochemical activity of acid phosphatase and activity of succinic acid dehydrogenase was determined according to a personal 3-degree scale: low (+); moderate (++), and high (+++).

Results

The livers of all 9 animals from the control group had a regular tawny colour and dense texture. The microscopic examination of the liver in these animals revealed hyperaemia in 3 animals and parenchymatous degeneration of the hepatocytes in 2 animals.

In the experimental group, in a majority of cases the number of liver flukes ranged from 6 to 10 (26.3%) and from 36 to 45 (22.3%). In 22 examined cows with evidence of fluke infestation, a microscopic examination showed small clusters of mononuclear cells in the lobules and mononuclear cell infiltrates with eosinophilic granulocytes situated perivascularly, with proliferation and oedema of hepatic stellate cells and hyperaemia of intralobular veins and central veins, subcapsular and interlobular vessels. Furthermore, there was proliferation of intralobular and interlobular connective tissue and connective tissue proliferation around the central veins and around migratory tunnels in the hepatic parenchyma. In addition, parenchymatous and vacuolar degeneration of single hepatocytes and necrosis of the hepatocytes were observed. The foci with adult flukes were located within the bile ducts and surrounded by connective tissue, and there was fatty degeneration of the hepatocytes. The hepatic stellate cell proliferation as well as dissociation of the hepatocytes were observed. The foci composed with neutrophils (abscessation foci) were also found in the hepatic parenchyma. Fatty degeneration of the hepatocytes in the

cows with liver fluke infestation was recorded in 27% of the specimens in subgroup I, in 18% of the specimens in subgroup II and in 22% of the specimens in subgroup III. Few foci of coagulative necrosis in the hepatic parenchyma were found in the cows from subgroup I, slightly more in subgroup III, and the most were found in subgroup II. Hepatic cell dissociation was only found in the liver from the cows in subgroup III, whereas hyperaemia was seen in all liver specimens from subgroups I, II and III in 87%, 82% and 100% of the sections, respectively. Mononuclear cell and eosinophilic granulocyte infiltrates were observed in 70% of the specimens in subgroup II, compared to 80% in subgroup I and 89% in subgroup III. Proliferation or atrophy of hepatic stellate cells was observed in approximately 27% of liver specimens in the subgroup I and in about 35% in subgroups II and III, whereas connective tissue proliferation was demonstrated in 50% of the liver samples in subgroup II.

An increase in the number of extensively-branched reticular fibres was detected in the cows from all experimental groups (Table 1). The results of polysaccharide content in the hepatocytes are shown in Table 2. In the control cows, a moderate amount of polysaccharides was demonstrated in 25% of the examined samples and a high content in 75% of the examined samples, while in subgroup I the content was 53% moderate, 33% high and 14% very high in the samples. These results were 65%, 25% and 10%, respectively, in subgroup II and 77 %, 18 % and 5 %, respectively, in subgroup III.

Table 1

The structure of reticular fibres in the cattle liver

The kind of changes	Affirmed changes in % of preparations			
	K	I	II	III
The normal reticular fibres	85	–	–	–
The increased number of branched reticular fibres	15	80	47	11
The increased number of reticular fibres of different thicknesses and extensively branched	–	20	53	89

Explanation: K – control group, I group – weak thickened bile ducts, II group – strongly thickened bile ducts, focal calcification, parenchymatous degeneration, III group – calcified bile ducts, single abscesses, fatty degeneration

Table 2

Contents of the polysaccharides in the liver hepatocytes (%)

Contents of the polysaccharides in the hepatocytes	Group of animals			
	K	I	II	III
Moderate	25	53	65	77
High	75	33	25	18
Very high	–	14	10	5

The results for lipid content in the hepatocytes demonstrate that no lipid vacuoles were observed in the hepatocytes in the control group samples, whereas single hepatocytes with lipid-containing vacuoles were found in the cows naturally infested with *F. hepatica*.

The results of histochemical assays are presented in Tables 3 and 4. The activity of acid phosphatase (ACP) in the cows from all experimental groups was significantly lower compared to the animals from the control group. The activity of succinic acid dehydrogenase (SDH) in subgroup I was average and low in the subgroup II and III in comparison to the control group, in which it was very high.

Table 3

Activity of acid phosphatase (ACP) in the cattle liver infested by *Fasciola hepatica* flukes

Acid phosphatase activity	Group of animals			
	K	I	II	III
Low	–	+	+	+
Moderate	+	+	+	+
High	–	–	+	+

Table 4

Activity of succinic acid dehydrogenase (SDH) in the cattle liver infested by *Fasciola hepatica* flukes

Succinic acid dehydrogenase activity	Group of animals			
	K	I	II	III
Low	–	–	+	+
Moderate	–	+	–	–
High	+	–	–	–

Discussion

The described lesions in the liver were found in the cows with liver fluke infestation, which was not reported in the control cows. The performed assays indicate that the least apparent lesions in the liver were found in the cows from subgroup I, slightly more severe in subgroup III and the most severe in the subgroup III. These lesions were determined by the pathogenic effects of flukes, age of the animals and the number of parasites. The results of personal studies are consistent with findings reported by other authors (DOW et al. 1968, GAJEWSKA et al. 2005, RAHKO 1971, RAHKO 1973, RAHKO 1973).

In the cows from the subgroups I and II, there was a increased number of reticular fibres with an increased number of branches fibrils surrounding hepatocytes. It was found that the reticular fibres of connective tissue in-

creased in number together with an increasing number of parasites damaging the hepatic parenchyma. These lesions were particularly evident in the subgroup III cows. A similar type and nature of the reticular fibres was reported by ROMANIUK et al. (1973). Similar results were reported by MARCOS et al. (2007), who found increasing amounts of connective tissue together with an escalating intensity of parasitic infestation, which was also found in our studies.

In the liver sections from the subgroups I and II, a moderate content of polysaccharides was detected in approximately 50% of the samples, whereas in about 70% of the samples in the subgroup III; the content was very high in 14%, 10% and 5%, respectively. The demonstrated lesions indicate that the level of polysaccharides in the cattle liver significantly decreases with increasing severity of liver fluke infestation (damage to the hepatocytes). Similar lesions in the course of *F. hepatica* infestation were reported by RAHKO (1971).

Single hepatocytes with lipid-containing vacuoles were observed. These cells were located around the bile ducts. Degenerated hepatocytes were more often found in subgroup III in comparison with the other groups of animals.

The activity of acid phosphatase (ACP) is linked to the function of lysosomes in the cells. Under pathological conditions, its activity increases, which indicates damage to the intracellular structures. In the analysed cows, ACP assay was enhanced in the central zones of hepatic lobules and near the parenchyma damaged by flukes and was intensified together with the degree of infestation. The activity of this enzyme was clearly increased in the cows from subgroups II and III. A similar acid phosphatase activity was demonstrated by THORPE (1967).

The succinic acid dehydrogenase (SDH) assay in the cattle infested with *F. hepatica* was the most evident in the central lobular zone. Reduced SDH activity was detected in the liver, adjacent to microabscesses. This assay was determined by the number of parasites, which is consistent with the findings reported by THORPE (1967).

The parasites exert a mechanical impact (by damaging with tiny spikes on the cuticle) on the host, as well as a chemical impact (mainly through their metabolic products such as toxins) and a direct impact (by introducing pathogenic microorganisms). The presence of *F. hepatica* in the host initiated protective mechanism interactions in a very complex way (OLDENBORG et al. 1976), resulting in biochemical changes (in the internal organs and body fluids), clinical symptoms and pathological changes (FURMAGA and GUNDŁACH 1972). A comprehensive analysis demonstrated that in the liver of cows in which the severity of *F. hepatica* infestation was highest, a low content of polysaccharides (reserve material and energy source), a high number of

reticular fibres (responsible for fibrosis of the organ), a high activity of acid phosphatase (ACP) and a low enzymatic activity of hepatocytes (manifested by a poor reaction of succinic acid dehydrogenase (SDH) reaction) were found.

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