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SWEET MAIZE YIELD STRUCTURE DEPENDING ON CULTIVATION TECHNOLOGY UNDER THE DRIP-IRRIGATED CONDITIONS

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Key words: mineral fertilization, irrigation, plant density, ploughing, productivity.

Abstract

The results of the study devoted to cultivation technology effects on the indices of yield structure of sweet maize are presented. Field trials with the crop were carried out during 2014–2016 at the dark-chestnut slightly saline drip-irrigated soil by using the randomized split plot design method in four replications. Three factors were studied in the trials: depth of mouldboard ploughing (20–22, 28–30 cm), nutritive background (no fertilizers, $N_{60}P_{60}$, $N_{120}P_{120}$), plant density (35 000, 50 000, 65 000, 80 000 plants ha^{-1}). Increase in the depth of ploughing and plant density to 80 000 plants ha^{-1} leads to considerable decrease of the structural indices and yields. The best yield structure was obtained under the mouldboard ploughing at the depth of 20–22 cm, nutritive background $N_{120}P_{120}$ and plant density of 35 000 plants ha^{-1}. The highest yields of sweet maize ears with husks (14.00 t ha^{-1}) were achieved under the higher plant density of 65 000 plants ha^{-1}.

Introduction

Sweet maize is a valuable vegetable crop that has high nutritive and dietary value. It is widely cultivated all over the world (EFTHIMIADOU et al. 2009). Most cultivation areas are situated in the USA where sweet maize is a national product. Hungary is the European top producer of high-quality sweet maize (SZYMANEK et al. 2006). Sweet maize becomes more and more popular in Europe from year to year. Demand for fresh and processed sweet maize products increases, and satisfaction of the growing demand requires significant increase in produced gross volumes of the

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crop. Thereby, scientific researches in the field of sweet maize cultivation technology are on the table for modern agricultural science. Previously conducted studies proved that sweet maize yields and their structure depends on a number of natural and anthropogenic factors, viz.: genetic features of the cultivated hybrids (LAZCANO et al. 2011), phytosanitary status of the cultivation area (TURSUN et al. 2016), environmental and weather conditions (GARCIA y GARCIA et al. 2009), water and nutrients availability (MAHARJAN et al. 2016), general peculiarities of cultivation technology (KWABIAH 2004, SAHOO and MAHAPATRA 2004), etc. Special attention in the researches has to be paid to the peculiarities of yield structure formation, because yield structure determines not only yield levels, but qualitative parameters of the obtained yield, such as size and mass of the marketable ears, and these parameters are very important for fresh market realization. The aim of our investigations was to determine effects of some cultivation technology treatments (depth of ploughing, nutritive background, plants density) on the sweet maize yields and their structure under the drip-irrigated conditions of the South of Ukraine.

Materials and Methods

The field experiments were carried out by using the randomized split plot design method in four replications during the period from 2014 to 2016 at the irrigated lands of the Agricultural Cooperative Farm "Radianska Zemlia" (Kherson region, Ukraine). Geographical coordinates of the experimental field are: latitude 46°43′42″N, longitude 32°17′38″E, altitude 42 m. The study envisaged research of the following treatments:

– factor *A* – primary soil tillage (mouldboard ploughing at the depth of 20–22 and 28–30 cm);

– factor *B* – nutritive background (no mineral fertilizers applied; mineral fertilizers applied at rates of $N_{60}P_{60}$ and $N_{120}P_{120}$);

– factor *C* – plants density (35 000, 50 000, 65 000 and 80 000 plants ha^{-1}).

The soil in the field experiments was represented by the dark-chestnut slightly saline soil. The humus content in the arable soil layer was 2.5%. The bulk density of the 0–100 cm soil layer was 1.35 t m^{-3}. The content of the lightly-hydrolyzed Nitrogen was 35 mg kg^{-1}, the mobile Phosphorus content was 32 mg kg^{-1}, the exchangeable Potassium content was 430 mg kg^{-1} in the arable soil layer. The weather conditions during the period of the experiments are presented in the Table 1. The hydrothermal coefficient (HTC) was calculated as a relation of the sum of precipitation to the sum of positive temperatures above 10ºC (USHKARENKO et al. 2014).

Table 1

Weather conditions in the field experiments with sweet maize

Month	Decade	Air temperature [°C]	Relative air humidity [%]	Precipitation amounts [mm]	HTC [units]
2014					
May	I	13.7	75	33.0	2.4
	II	17.8	75	5.2	0.3
	III	22.2	61	0.0	0.0
June	I	22.4	64	13.3	0.6
	II	20.0	58	28.6	1.4
	III	20.0	64	22.5	1.1
July	I	23.5	53	0.0	0.0
	II	25.5	56	9.4	0.4
	III	26.1	49	10.0	0.3
2015					
May	III	19.6	69	70.7	3.3
June	I	21.3	61	7.1	0.3
	II	21.3	67	3.4	0.2
	III	20.0	73	27.8	1.4
July	I	22.8	74	84.9	3.7
	II	21.0	66	19.7	0.9
	III	26.0	67	0.0	0.0
August	I	26.0	49	0.0	0.0
2016					
May	III	18.5	77	20.7	1.0
June	I	17.8	70	16.2	0.9
	II	21.9	75	12.8	0.6
	III	26.5	62	14.0	0.5
July	I	22.4	61	21.6	1.0
	II	25.8	59	0.0	0.0
	III	25.0	54	24.7	0.9
August	I	26.0	55	0.6	0.0
Long-term data (for the period from 1986 until 2005)					
May	I	14.1	63	15.0	1.1
	II	16.6	62	14.0	0.8
	III	17.4	66	13.0	0.7
June	I	19.2	68	13.0	0.7
	II	19.5	65	18.0	0.9
	III	21.2	67	14.0	0.7
July	I	21.3	62	22.0	1.0
	II	22.3	61	14.0	0.6
	III	22.1	61	13.0	0.5
August	I	22.4	61	7.0	0.3

We used the variety Brusnytsia (standard sweet – *su,* with duration of vegetation period of 77–79 days) in the field experiments. Sweet maize cultivation technology was based on the common recommendations for cultivation of the crop under the irrigated conditions of the South of Ukraine. The previous crop was winter wheat. Stubbling at the depth of 10–12 cm followed by the mouldboard ploughing was conducted after the harvesting of the previous crop. Mineral fertilizers (ammonium nitrate and superphosphate) were applied with accordance to the experimental design in pre-ploughing period by the means of a seed drill. Soil cultivations at the depth of 8–10, and further at the depth of 5–6 cm were carried out in the spring period. Sweet corn was sown at the depth of 5–6 cm with an interrow spacing of 70 cm. The time of sowing was: 1st of May in 2014, 22nd of May in 2015 and 21st of May in 2016, respectively. Herbicide Harnes (a.s. – acetochlor, 900 g dm^{-3}) was applied in pre-sowing period in the dose of 2.0 dm^3 ha^{-1}. Karate Zeon insecticide (a.s. – lambda-cyhalothrin, 50 g dm^{-3}) was applied at the stage of 3–5 leaves of the crop in the dose of 0.2 dm^3 ha^{-1}. Master Power herbicide (a.s. – foramsulfuron, 31.5 g dm^{-3}, iodosulfuron, 1.0 g dm^{-3}, tienecarbazon-methyl, 10 g dm^{-3}, cyprosulfamide (antidote), 15 g dm^{-3}) was applied at the stage of 7–8 leaves of the crop in the dose of 1.25 dm^3 ha^{-1}. Koragen insecticide (a.s. – chlorantraniliprole, 200 g dm^{-3}) was applied at the beginning of the flowering stage in the dose of 0.1 dm^3 ha^{-1}. Soil moisture during the crop vegetation was maintained at the level of 80% of the field water-holding capacity by the means of drip irrigation. We applied irrigation water 10 times at the rate of 5 mm before the stage of 7–8 leaves of the sweet maize, and then 12 times at the rate of 10 mm in the further period of the crop vegetation in 2014. In 2015 and 2016 we applied irrigation water in the above-mentioned volumes, but fewer times: 6 and 9 in 2015, 8 and 12 in 2016, respectively. The total volume of the irrigation water applied at the field was 170 mm in 2014, 120 mm in 2015, and 160 mm in 2016.

Sweet maize yields at the technical ripeness stage were hand-harvested and weighed on the digital weighs. Time of harvesting depended on the cultivation technology treatments, and it is given in the Table 2. The number of marketable ears per plant was counted in the pre-harvesting period. Physical sizes (such as diameter and length) of the marketable ears were assessed by the means of calliper.

The experimental data were processed by the means of the multi-factor analysis of variance (ANOVA). The least significant difference (LSD) was estimated at the reliability level of 95%. We used AgroStat add-on for the Microsoft Office Excel as a tool for the statistical evaluation of the experimental data (USHKARENKO et al. 2014).

Table 2

Time of harvesting of sweet maize ears depending on the cultivation technology

Cultivation technology treatments			Years of study		
Mouldboard ploughing depth	nutritive background	plant density [plants ha^{-1}]	2014	2015	2016
20–22 cm	no fertilizers	35 000	15.VII	31.VII	1.VIII
		50 000	15.VII	1.VIII	2.VIII
		65 000	17.VII	1.VIII	2.VIII
		80 000	18.VII	3.VIII	3.VIII
	$N_{60}P_{60}$	35 000	19.VII	3.VIII	4.VIII
		50 000	20.VII	4.VIII	4.VIII
		65 000	22.VII	7.VIII	4.VIII
		80 000	23.VII	8.VIII	4.VIII
	$N_{120}P_{120}$	35 000	21.VII	5.VIII	4.VIII
		50 000	22.VII	7.VIII	4.VIII
		65 000	23.VII	8.VIII	4.VIII
		80 000	25.VII	10.VIII	5.VIII
28–30 cm	no fertilizers	35 000	15.VII	31.VII	4.VIII
		50 000	15.VII	1.VIII	4.VIII
		65 000	17.VII	1.VIII	4.VIII
		80 000	18.VII	3.VIII	4.VIII
	$N_{60}P_{60}$	35 000	19.VII	3.VIII	5.VIII
		50 000	20.VII	4.VIII	5.VIII
		65 000	22.VII	7.VIII	5.VIII
		80 000	23.VII	8.VIII	6.VIII
	$N_{120}P_{120}$	35 000	21.VII	5.VIII	6.VIII
		50 000	22.VII	7.VIII	6.VIII
		65 000	23.VII	8.VIII	7.VIII
		80 000	25.VII	10.VIII	7.VIII

Results and Discussion

The most important yield structure indices for sweet maize are: number of rows per ear, number of kernels per row, length and diameter of ear, ear mass, quantity of marketable ears per 100 plants (Table 3).

All the studied treatments had significant effect on the yield structure indices of sweet maize. We determined that mouldboard ploughing at the depth of 28–30 cm does not have positive effect on the yield structure, unless the crop is cultivated without fertilization (Table 4). This fact could be explained by the peculiarities of nutrition absorption by plants from

Table 3

Sweet maize yield structure depending on the cultivation technology (average for 2014–2016)

Mouldboard ploughing depth	Nutritive background	Plant density [plants ha^{-1}]	Number of				Physical sizes of the marketable ears		
			rows	kernels in a row	kernels per ear	marketable ears per 100 plants	length [cm]	diameter [cm]	mass [g]
20–22 cm	no fertilizers	35 000	14.1	25.5	358	55	16.2	4.3	187
		50 000	13.8	25.2	346	42	16.0	4.2	182
		65 000	13.7	24.7	339	35	15.9	4.2	178
		80 000	13.5	24.3	327	30	15.7	4.1	166
	$N_{60}P_{60}$	35 000	14.4	28.1	405	100	16.9	4.5	203
		50 000	14.2	27.5	388	85	16.6	4.4	192
		65 000	14.1	26.9	378	82	16.4	4.4	188
		80 000	13.9	26.7	371	61	16.3	4.3	181
	$N_{120}P_{120}$	35 000	14.8	30.1	445	120	17.6	4.8	229
		50 000	14.6	28.6	419	102	17.4	4.7	218
		65 000	14.5	28.4	412	100	17.2	4.6	215
		80 000	14.2	27.3	387	75	16.6	4.4	205
28–30 cm	no fertilizers	35 000	14.1	25.5	361	61	16.3	4.3	190
		50 000	14.0	25.2	354	49	16.2	4.3	183
		65 000	13.8	24.9	345	41	16.0	4.2	179
		80 000	13.7	24.5	336	34	15.8	4.1	167
	$N_{60}P_{60}$	35 000	14.3	27.5	394	93	16.5	4.4	193
		50 000	14.1	27.2	383	78	16.3	4.3	186
		65 000	14.0	26.9	376	69	16.1	4.2	183
		80 000	13.7	26.5	363	54	15.9	4.1	169
	$N_{120}P_{120}$	35 000	14.6	28.9	421	105	17.1	4.5	222
		50 000	14.3	28.6	408	88	16.9	4.5	215
		65 000	14.2	28.2	402	80	16.6	4.4	213
		80 000	13.9	27.7	386	60	16.5	4.3	200
LSD (at $p < 0.05$)		*A*	N/*A*		9.63	1.7	0.15	0.08	1.57
		B			12.95	1.2	0.12	0.08	0.78
		C			10.84	2.1	0.19	0.14	2.46
		ABC			30.08	5.4	0.49	0.37	6.14

the soil and mineral fertilizers (USHKARENKO 1994). If we do not provide the crop with additional nutrition in the form of fertilizers it needs to develop more roots and go deeper into the soil profile to find the necessary elements for growth and development. Therefore, sweet maize cultivated

with no fertilization shows better performance in yields and their structure at the deeper ploughing, which makes easier for the roots to penetrate down into the soil profile. However, if we give the crop additional artificial nutrition, there is no need to go deeper to find nutrition. In this case, the crop has an opportunity to find all the necessary elements for growth and development in the upper layers. And deep ploughing under the condition of artificial humidification just leads to migration of mineral fertilizers down by the soil profile and makes them less usable for the crops. Increasing crop density is also unreasonable, because it leads to significant decrease of all the studied structural indices because of increasing intro-specie competition and more plants per the same unit of life factors (light, air, nutrition, etc.). Application of mineral fertilizers is a factor of the greatest improvement in the studied parameters of sweet maize yields.

Table 4

Sweet maize yields depending on the cultivation technology [t ha^{-1}] (average for 2014–2016)

Mouldboard ploughing depth	Plant density [plants ha^{-1}]	Nutritive background		
		no fertilizers	$N_{60}P_{60}$	$N_{120}P_{120}$
20–22 cm	35 000	3.60	7.09	9.62
	50 000	3.82	8.14	11.14
	65 000	4.05	10.03	14.00
	80 000	3.99	8.82	12.32
28–30 cm	35 000	4.06	6.29	8.15
	50 000	4.49	7.24	9.45
	65 000	4.78	8.20	11.07
	80 000	4.54	7.30	9.62
LSD (at $p < 0.05$)			*A*	0.07
			B	0.15
			C	0.18
			ABC	0.47

And this fact is not surprising: the better you feed plants, the better productivity they usually show. The highest outlet of the marketable ears with the best visual parameters (sizes and mass) was achieved under the combination of treatments: mouldboard ploughing at the depth of 20–22 cm, plant density 35 000 plants ha^{-1}, nutritive background $N_{120}P_{120}$. The results of our study are in agreement with ones obtained by the foreign scientists in this field. So, we proved that the higher nutritive background is, the better sweet maize yield structure is. And the structural parameters become worse under the higher plant densities (OKTEM and OKTEM 2005). Also, we were not the first who mentioned that maize yields increase due to the better structural parameters under the tillage minimization (TORBERT et al. 2001).

We established that all the studied factors significantly affected the yields of the crop according to the ANOVA results. Previous scientific investigations discovered that better sweet maize yields could be obtained under the conventional tillage system than under the conservation tillage (EDGELL et al. 2015). However, it was defined that increased depth of mouldboard ploughing led to considerable decrease in yields on the fertilized experimental treatments in our study. On average, deep ploughing decreased sweet maize yields by 13.4–14.1%. The best performance of the deep ploughing was established on the non-fertilized variants (sweet maize yields increased by 15.7%), while fertilization levelled the advantages of the deep soil loosening. It is interesting that the results of some other studies report that the best sweet maize yields could be obtained not under the mouldboard, but under the disk ploughing (SHAMS et al. 2015, SHAMSABADI et al. 2017). These differences in the crop productivity could be put on the differences in the conditions of sweet maize cultivation, and differences in used machinery and varieties. Increasing sweet maize plant density from 35 000 to 65 000 plants ha^{-1} is an effective way of productivity increase – in average, it raised by 33.9%. Of course, the ears picked from he plots with plants density of 35 000 plants ha^{-1} were larger than the ones picked from the plots with higher crop density. But a significant increase in the number of ears per area unit provided a bigger increase in total mass of ears than a decrease in the mass of every single ear. However, enormously dense crops cause a negative effect and lead to significant losses of the yields (in average by 11.4%). The same results presenting that too dense crops have lower productivity were obtained earlier (BHATT 2012). We think that this phenomenon is connected with drastically increase of intro-species competition within the crops that fatigue plants, which is reached at the certain point of plants per area unit. And this point of curve will be different for different varieties cultivated under the different environmental and technological conditions. Some researches pointed out that sweet maize yields at different plants densities depend on the genotype features of the cultivated hybrids of the crop (AL-NAGGAR et al. 2015). However, if we discuss green mass yields they are considerably higher under the higher plants density (RAGHAVENDRA et al. 2016). All in all, nutrient management and mineral fertilization seem to be the most important factor of sweet maize productivity (WADILE et al. 2016). Higher fertilization rates significantly increased crop yields: application of mineral fertilizers at rates of $N_{60}P_{60}$ – by to 96.1%, and $N_{120}P_{120}$ – by 168.4% in comparison with non-fertilized treatments. The similar results were earlier obtained by some other scientific groups (AKPAN and UDOH 2017, RIVERA-HERNÁNDEZ et al. 2010). It was determined that sweet maize

yields increased by 34%, 44%, 52%, and 54%, respectively, under the application of Nitrogen mineral fertilizers in the doses of 30, 60, 90, and 120 kg ha^{-1} (KHAN et al. 2018). As we see, the question is not of "do we need to fertilize sweet maize", the question is "how much fertilizers should be applied in this concrete conditions". However, cultivation technology of sweet maize is a complex system and needs a complex investigation approach. So, our study has a number of limitations and the question of sweet maize agrotechnology remains actual and needs further researches.

Conclusions

1. The results of the investigations showed that the yield and yield structure of sweet maize were significantly affected by the studied factors. However, the strength of the effect was uneven. The highest effect on the studied parameters of the crop productivity had mineral fertilizers, and the least – the depth of mouldboard ploughing.

2. The best parameters of the sweet maize yield structure were provided by the mouldboard ploughing at the depth of 20–22 cm, nutritive background of $N_{120}P_{120}$ and plants density of 35 000 plants ha^{-1} as follows: marketable ears length was 17.6 cm, diameter – 4.8 cm, ears mass in husks – 229.0 g, marketable ears per 100 plants – 119.8.

3. The maximum yield of marketable sweet maize ears with husks was observed under the mouldboard ploughing at the depth of 20–22 cm, nutritive background of $N_{120}P_{120}$ and plants density of 65 000 plants ha^{-1}.

4. There is an obvious tendency of significant increase in sweet maize yields and yield structure with increase of the mineral fertilizers application rate. However, increase of the depth of ploughing considerably decreased the above-mentioned parameters of the crop productivity. Regulation of the plants density is a flexible instrument of adjusting the yields and their parameters for the actual market demands.

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References

AKPAN E.A., UDOH V.S. 2017. *Effects of fertilizer levels on growth and yield attributes of three dwarf sweet corn varieties (Zea mays L. Saccharata Strut) in Itu Flood Plain, Akwa Ibom State, Nigeria.* Canadian J. Agric. Crops, 2(1): 60–67.

AL-NAGGAR A.M.M., SHABANA R.A., ATTA M.M., AL-KHALIL T.H. 2015. *Maize response to elevated plant density combined with lowered N-fertilizer rate is genotype-dependent*. Crop J., 3(2): 96–109.

BHATT S.P. 2012. *Response of sweet corn hybrid to varying plant densities and nitrogen levels*. Afr. J. Agr. Res., 7(46): 6158–6166.

EDGELL J., OSMOND D.L., LINE D.E., HOYT G.D., GROSSMAN J.M., LARSEN E.M. 2015. *Comparison of surface water quality and yields from organically and conventionally produced sweet corn plots with conservation and conventional tillage*. J. Environ. Qual., 44(6): 1861–1870.

EFTHIMIADOU A., BILALIS D., KARKANIS A., FROUD-WILLIAMS B., ELEFTHEROCHORINOS I. 2009. *Effects of cultural system (organic and conventional) on growth, photosynthesis and yield components of sweet corn (Zea mays L.) under semi-arid environment*. Not. Bot. Horti Agrobot. Cluj Napoca, 37(2): 104–111.

GARCIA y GARCIA A.G., GUERRA L.C., HOOGENBOOM G. 2009. *Water use and water use efficiency of sweet corn under different weather conditions and soil moisture regimes*. Agric. Water Manag., 96(10): 1369–1376.

KHAN A.A., HUSSAIN A., GANAI M.A., SOFI N.R., TALIB S. 2018. *Yield, nutrient uptake and quality of sweet corn as influenced by transplanting dates and nitrogen levels*. J. Pharmacogn. Phytochem., 7(2): 3567–3571.

KWABIAH A.B. 2004. *Growth and yield of sweet corn (Zea mays L.) cultivars in response to planting date and plastic mulch in a short-season environment*. Sci. Hort., 102(2): 147–166.

LAZCANO C., REVILLA P., MALVAR R.A., DOMÍNGUEZ J. 2011. *Yield and fruit quality of four sweet corn hybrids (Zea mays) under conventional and integrated fertilization with vermicompost*. J. Sci. Food Agric., 91(7): 1244–1253.

MAHARJAN B., ROSEN C.J., LAMB J.A., VENTEREA R.T. 2016. *Corn response to nitrogen management under fully-irrigated vs. water-stressed conditions*. Agron. J., 108(5): 2089–2098.

OKTEM A.G., OKTEM A. 2005. *Effect of nitrogen and intra row spaces on sweet corn (Zea mays saccharata Sturt) ear characteristics*. Asian J. Plant Sci., 4(4): 361–364.

RIVERA-HERNÁNDEZ B., CARRILLO-ÁVILA E., OBRADOR-OLÁN J.J., JUÁREZ-LÓPEZ J.F., ACEVES-NAVARRO L.A. 2010. *Morphological quality of sweet corn (Zea mays L.) ears as response to soil moisture tension and phosphate fertilization in Campeche, Mexico*. Agric. Water Manag., 97(9): 1365–1374.

RAGHAVENDRA S., DESAI B.K., RAJESH S.R., VINAYAK H., PRASHANTH K.M. 2016. *Effect of nutrient management and plant density on yield components, yield and economics of sweet corn*. Environ. Ecol., 34(3A): 1109–1112.

SAHOO S.C., MAHAPATRA P.K. 2004. *Response of sweet corn (Zea mays) to nitrogen levels and plant population*. Indian J. Agr. Sci., 74: 337–338.

SHAMS A.H., TAHERIRAD A.R., KHORRAMDEL S., NIKKHAH A. 2015. *The effect of tillage methods, plant density and planting patterns on growth characteristics, yield components and gain yield of sweet corn under Malaysia climatic conditions*. Electr. J. Crop Product., 8(1): 79–98.

SHAMSABADI H.A.T., AHMAD D., AZMI Y. 2017. *Yield components of sweet corn (Zea mays) and some soil physical properties towards different tillage methods and plant population*. Agricult. Eng. Internat.: CIGR J., 19(3): 56–63.

SZYMANEK M., DOBRZAŃSKI B., NIEDZIÓŁKA I., RYBCZYŃSKI R. 2006. *Sweet corn: harvest and technology physical properties and quality*. Institute of Agrophysics, Polish Academy of Sciences, Lublin.

TORBERT H.A., POTTER K.N., MORRISON J.E. 2001. *Tillage system, fertilizer nitrogen rate, and timing effect on corn yields in the Texas Blackland Prairie*. Agron J., 93(5): 1119–1124.

TURSUN N., DATTA A., SAKINMAZ M.S., KANTARCI Z., KNEZEVIC S.Z., CHAUHAN B.S. 2016. *The critical period for weed control in three corn (Zea mays L.) types*. Crop Prot., 90: 59–65.

USHKARENKO V.O. 1994. *Irrigated agriculture: Textbook*. Urozhai, Kyiv.

USHKARENKO V.O., KOKOVIKHIN S.V., HOLOBORODKO S.P., VOZHEHOVA R.A. 2014. *Methodology of the field experiment (Irrigated agriculture): Textbook*. Hrin DS, Kherson.

WADILE S.C., PAWAR P.P., ILHE S.S., RATHOD V.M. 2016. *Nutrient management on growth, yield and quality of sweet corn, baby corn and maize*. BIOINFOLET-A Quarterly Journal of Life Sciences, 13(1a): 67–69.

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CHANGES IN MACRO- AND MICROELEMENTS CONTENT IN SOIL AS WELL AS GRAINS OF WINTER WHEAT OF RGT KILIMANJARO CV. (*TRITICUM AESTIVUM VAR. KILIMANJARO*) UNDER THE INFLUENCE OF BIOMASS ASH AND LIME FERTILIZATION

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Key words: ash from biomass, lime, macro- and microelements, soil, wheat.

Abstract

Present paper analyzes the effect of biomass ash and lime fertilization on changes in macro- and microelements content in soil and in winter wheat grain of RGT Kilimanjaro cv. (*Triticum aestivum var. Kilimanjaro*). Fertilization with wood or straw ash had no influence on changes in the pH and content of organic carbon, nitrogen and available phosphorus in the soil from experiment. As a result of fertilization with ash from wood or straw, a significant increase in the soil abundance was achieved in relation to: available potassium and exchangeable calcium. Increasing the dose was a factor that resulted in even higher efficiency of fertilizers. Analysis of the content of microelements in the soil (copper, chromium, nickel and lead) shows that the application of fertilization in the form of wood or straw ashes and lime PROFITKALK did not exceed the threshold values for soil from the first group of ground Results concerning the impact of ashes from wood or straw and lime PROFITKALK on changes in the content of macro- and microelements indicate that when cultivating wheat of Kilimanjaro cv., it is justified to use them as a fertilizing material.

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Introduction

Ashes from biomass are increasingly treated as a fertilizer. The biomass ash is one of the oldest mineral fertilizers. They contain almost all nutrients except nitrogen (FÜZESI et al. 2015, ZAPAŁOWSKA et al. 2017). The impact of used ashes from wood and straw of crops is the subject of many studies emphasizing the beneficial effect on both the quality of soil and plants (BAKISGAN 2009, SCHIEMENZ and EICHLER-LÖBERMANN 2010, PIEKARCZYK et al. 2017, OCHECOVA et al. 2014, BRADNA et al. 2016).

The composition of ash from biomass is generally very diverse and depends on the type of biomass combusted (PIEKARCZYK et al. 2011, PIEKARCZYK et al. 2017). The reduction in the resources of biomass from forestry and the wood industry induces attempts to look for other plant materials that constitute energy, as for example straw. In Poland, it is predicted that in 2020 year straw production will amount 30.5 million tones, of which 17.4 million tones will be used for agricultural purposes, and the remaining 13.1 million tones may be used for energy purposes (MADEJ 2016). Literature data indicate higher calcium and magnesium contents in wood ash as compared to straw ash (KAJDA-SZCZEŚNIAK 2014).

As you know use of biomass ash can cause changes in soil chemical properties, especially in the upper layer. The use of ash is possible only if it does not threaten the quality of the soil and the obtained crops of plants are of good quality.

Winter wheat of RGT Kilimanjaro cv. is a cultivar recently introduced into cultivation, which justifies conducting thorough studies on the impact of using different fertilizers on changes in the chemical composition of the grain.

Present paper analyzes the effect of biomass ash from wood and ash from straw and lime fertilization on changes in macro- and microelements content in soil and in wheat grain of RGT Kilimanjaro cv. (*Triticum aestivum var. Kilimanjaro*).

Materials and Methods

Experimental characteristics

The experiment was carried out in 2016 in Wrześnica (54º40'N, 16º77'E), the Sławno County in the West Pomeranian province. The study compared two factors: wood ash and straw ash (I. factor), 4 doses of ash and lime mixture (mixture composition in the proportion of 70% ash and

30% lime) (factor II). The subsequent doses were as follows: *a* – 0; *b* – 7 + 2.1; *c* – 14 + 4.2; *d* – 21 + 6.3 Mg ha^{-1}. The fertilizer lime used is a calcium post-cellulose fertilizer, variety 07, with the trade name PROFITKALK and imported by the Polish company Agro Trade Ltd. from Scandinavia. The lime contained in the fertilizer is in the form of carbonate and its content is 28.0% Ca. The fertilizer contains phosphorus and magnesium in the amount of 0.25% P i 0.42% Mg (*Wapno PROFITKALK*… 2019). Value of pH and content of macro- and microelements in ash from wood and straw are given in Table 1.

Table 1

Value of pH and content of macro- and microelements in ash from wood and straw

Parameter	Type of ash	
	wood	straw
pH in H_2O	12.3	10.2
pH in KCl	12.5	10.0
g kg^{-1}		
Phosphorus/P	13.6	20.8
Potassium/K	6.8	80.9
Calcium/Ca	35.4	15.6
Magnesium/Mg	6.4	3.9
mg kg^{-1}		
Iron/Fe	8290	988
Maganese/Mn	9220	351
Zinc/Zn	1830	966
Copper/Cu	157	61.2
Chrome/Cr	35.2	2.64
Lead/Pb	34.5	33.0
Nickel/Ni	26.4	2.97

Fertilization was applied in autumn 2015 before wheat sowing, on 25 September. The experiment was established by means of a random block method in 4 replicates The soil was loamy sand (USDA 2006). The soil from the experiment was characterized by the following parameters: pH_{KCl} = 6.4, C_{org} = 11.0 g kg^{-1}, P_{avail} = 37.0 mg kg^{-1}, $K_{avail.}$ = 177 mg kg^{-1}, $Mg_{exch.}$ = 165 mg kg^{-1}. It was the soil contained an average level of available phosphorus and potassium and very high exchangeable magnesium (EGNER et al. 1960, *Soil quality*… ISO 13536:1995).

The area of the plot was 500 m^2. Material for analysis consisted of winter wheat of Kilimanjaro cv. grain (*Triticum aestivum var. Kilimanjaro*). In Poland, winter wheat RGT Kilimanjaro has been entered into the National Register of Varieties on 19.09.2014 and the expiration date is

31.12.2024 (Polish National List of Agricultural Plant Varieties 2017). RGT Kilimanjaro is currently one of the highest yielding winter wheat cultivars available in Poland; it is very highly evaluated for winter hardiness – the score 4 classifies it in the forefront of the most winter-resistant cultivars. Good quality results of the grain resulted in qualifying it to the quality group *A*.

Wheat was grown on a post after winter oilseed rape. Nitrogen fertilizers were sown on 2 April 4, 2016 in an amount of 120 kg of ammonium sulfate (25.5 kg of N) and 300 kg of urea, (140 kg of N) per ha and on May 28 – 150 kg of urea (70 kg of N) per hectare. Wheat was harvested on August 15, 2016. Care treatments for sowing were carried out in accordance with the principles of Good Agricultural Practice.

Methodology of chemical analyses

Soil samples were taken after winter wheat rape harvest, using Egner-Riehm's cane from a 0–20 cm layer in accordance with the standard (*Analiza chemiczno-rolnicza…* PN-R-04031:1997) from each plot. The pH of the soil was determined potentiometrically in accordance with the standard (*Soil quality…* ISO 10390:1997). The amount of organic carbon was determined using dichromate(VI) oxidation and combined with sulfuric(VI) acid (*Soil quality…* ISO 14235:1998). Nitrogen was determined in solutions after mineralization of soil samples and in sulfuric(VI) acid with H_2O_2 by means of Kjeldahl method (*Soil quality…* ISO 11261:2002). Available forms of phosphorus and potassium in the soil were determined using the Egner-Riehm method (EGNER et al. 1960). In order to determine exchangeable forms of magnesium and calcium contents in the soil, a buffered barium chloride solution was used (pH = 8.1) (*Soil quality…* ISO 13536:1995). Determining the total content of metals: potassium, calcium, magnesium, iron, manganese, zinc, sodium, nickel, lead, copper and chromium in soil samples and grains were wet digested in a mixture of nitric(V) and chloric(VII) acids at 1:1 ratio (*Animal feeding…* ISO 6869:2000). Analyses were performed using the Atomic Absorption Spectrometer Apparatus (Thermo Fisher Scientific iCE 3000 Series). After grain mineralization in sulfuric(VI) acid in combination with H_2O_2, nitrogen content was determined applying the Kjeldahl method (*Cereals and pulses…* ISO 20483:2013) and phosphorus by the colorimetric method using ammonium molybdate at 470 nm (*Animal feeding…* ISO 6491:1998).

Statistical treatment of data

The results were statistically processed using the variance analysis in a 2-factor system of random blocks. Confidence sub-intervals were calculated using Tukey's multiple test, assuming a significance level of $p = 0.05$. In addition, the analysis of variance with regression for the quantitative factor – the dose of mixture – was performed for selected soil features. The significance of regression equations was determined using the F-Fisher-Snedecor test. Regression lines are shown in diagrams. Statistical analysis of results was carried out using the Statistica 10.0 software.

Climatic conditions

The fairly high air temperature maintained in November 2015, on the one hand, created favorable conditions for emergence, growth and development of winter crops, on the other hand, deficiencies of moisture in the soil occurring in this period caused weaker growth of plants before winter. January frosts in the absence of snow cover, caused losses in winter cereal crops. The weather course in February 2016 posed a slight threat to plants, and the high temperature of air and soil that persisted during the month caused disturbances in the winter dormancy of plants. Weather in March favored drying up of fields and heating the soil, as well as vegetation. The cold rainy days occurring in April inhibited the growth and development of plants. Shortage of rainfall caused that the water needs of crops were not fully satisfied. The warm and sunny weather at the beginning of May favored the growth and development of plants. As a result of the spring shortage of rainfall, the condition of many crops has deteriorated. Rainfall recorded in June improved the condition of soil moisture (*Serwis IMGW-PIB*... 2019).

Results and Discussion

Soil acidity, organic carbon and nitrogen in soil

After the experiment was completed, the soil from the control object was characterized by slightly acidic reaction – pH in KCl = 6.36 (*Soil quality*… ISO 10390:1997P). The introduced fertilizing materials were characterized by alkaline reaction (Table 1), however, fertilization with ash from wood or straw did not cause soil alkalization (Table 2). As a result of the use of combined fertilization with ash and lime PROFITKALK, however, an increase in the soil pH to 6.75 was recorded (Table 3 and Figure 1). The alkalizing effect of lime as a fertilizer is confirmed in the literature on this subject (Eichler-Löbermann et al. 2008, Gibczyńska et al. 2014, Goulding 2016).

Table 2
Value of pH and content of macro- and microelements in the soil depending on the type of ash

Parameter	Control	Type of ash			
		wood	straw	average	$LSD_{0.05}$
pH in KCl	6.36	6.61	6.62	6.62	n.s.
g kg^{-1}					
Organic carbon	12.5	12.6	12.7	12.6	n.s.
Nitrogen/N [mg kg^{-1}]	0.94	0.97	1.01	0.99	n.s.
Iron/Fe	8.21	8.62	8.73	8.67	n.s.
mg kg^{-11}					
Available phosphorus/P_{avail}	37.2	40.8	39.4	40.1	n.s.
Available potassium/K_{avail}	173	210	225	217	18.3
Exchangeable calcium/Ca_{exch}	780	1020	946	983	105.3
Exchangeable magnesium/Mg_{exch}	188	205	177	191	n.s.
Manganese/Mn	470	458	447	452	n.s.
Zinc/Zn	40.7	40.9	39.8	40.3	n.s.
Copper/Cu	6.19	6.37	6.74	6.56	n.s.
Chrome/Cr	21.2	21.2	20.7	20.9	n.s.
Nickel/Ni	7.47	8.09	7.00	7.54	n.s.
Lead/Pb	13.1	15.0	10.7	12.8	1.729

n.s. – not significant difference

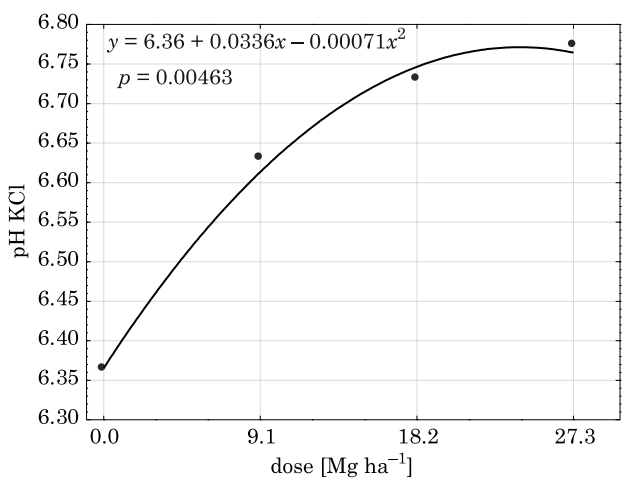

Fig. 1. Regression equation between dose of mixture and pH in the soil

The average organic carbon content in the soil from the experiment was 12.5 g C kg^{-1} while the nitrogen content was 0.94 g N kg^{-1}. Fertilization with biomass ashes and PROFITKALK lime did not affect changes in the amount of these parameters in the soil (Table 2 and Table 3).

Table 3

Value of pH and content of macro- and microelements in the soil depending on the ash doses

Parameter	Dose of ash [Mg ha^{-1}]					
	0	7 + 2.1	14+4.2	21+6.3	average	$LSD_{0.05}$
pH in KCl	6.36	6.63	6.73	6.75	6.62	0.283
g kg^{-1}						
Organic carbon	12.5	12.4	12.6	13.0	12.6	n.s.
Nitrogen/N	0.937	1.046	1.010	0.957	0.987	n.s.
Iron/Fe	8.21	9.20	8.57	8.72	8.67	n.s.
mg kg^{-1}						
Available phosphorus/P_{avail}	37.2	41.5	42.2	44.4	41.3	4.53
Available potassium/K_{avail}	173	219	232	246	217	45.3
Exchangeable calcium/Ca_{exch}	780	1009	1039	1104	983	226.4
Exchangeable magnesium/Mg_{exch}	188	191	195	193	192	n.s.
Manganese/Mn	470	440	454	447	452	n.s.
Zinc/Zn	40.7	40.6	40.1	40.0	40.3	n.s.
Copper/Cu	6.19	6.34	7.32	6.39	6.56	n.s.
Chrome/Cr	21.2	21.8	20.2	20.6	20.9	n.s.
Nickel/Ni	7.47	7.58	6.76	8.37	7.54	n.s.
Lead/Pb	13.1	12.2	12.6	13.5	12.8	n.s.

n.s. – not significant difference

Available phosphorus and potassium in soil

The content of available phosphorus in the soil from the experiment was 37.2 mg P kg^{-1}. Ashes from biomass were characterized by relatively large amount of this element (Table 1), however, there was no effect of their presence on changes in the amount of phosphorus available to plants in the soil. Soil alkalization resulting from the use of lime was a factor responsible for a dose-proportional, significant increase in available phosphorus to the level of 44.4 mg P kg^{-1} (Table 2 and Figure 2).

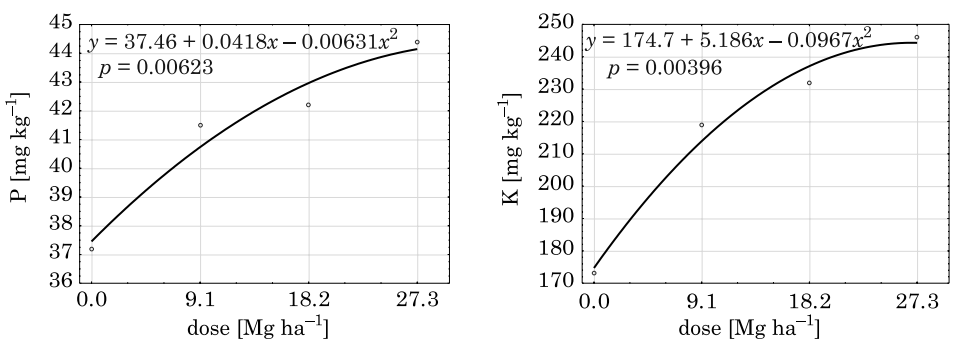

Fig. 2. Regression equation between dose of mixture and phosphorus and potassium content in the soil

In the experiment, ash from straw contained about ten times more potassium than wood ash (Table 1). For comparison, JAGUSTYN et al. (2011) show that the potassium content in ash from straw was as much as 19.9%. The abundance of ashes relative to potassium was a factor causing significant increase in the content of available potassium in the soil, after the completion of Kilimanjaro cv. wheat cultivation to 225 mg K kg^{-1}. As a result of a gradual increase in the doses of both fertilizer materials, the amount of available potassium in the soil amounted to 246 mg K kg^{-1} and the soil was characterized by very high available potassium level (Table 2 and Figure 2).

Exchangeable calcium and magnesium

Unlike potassium, wood contains more calcium than straw, and it ranges from 0.8 to 2.7 g K kg^{-1} (SZÁSZ-LEN et al. 2016) and the above dependence is reflected in the abundance of ashes (Table 1). As a result of fertilization using ash, a significant increase in the amount of exchangeable calcium in the soil was obtained: by 31 and 21%, respectively.

By using the combined fertilization with PROFITKALK ash and lime, a proportional significant increase in the content of exchangeable calcium in the soil was observed by as much as 41% at the maximum dose (Table 3 and Figure 3). FÜZESI et al. (2015) explain the above relationship that the calcium oxide present in fertilizers getting into the soil, in combination with water, transforms into calcium hydroxide. The hydroxide reacts with carbon dioxide from the air, which results in the formation of more easily soluble calcium carbonate. Higher amount of magnesium in wood ash, as a result of its use as a fertilizer, caused significant increase in the content

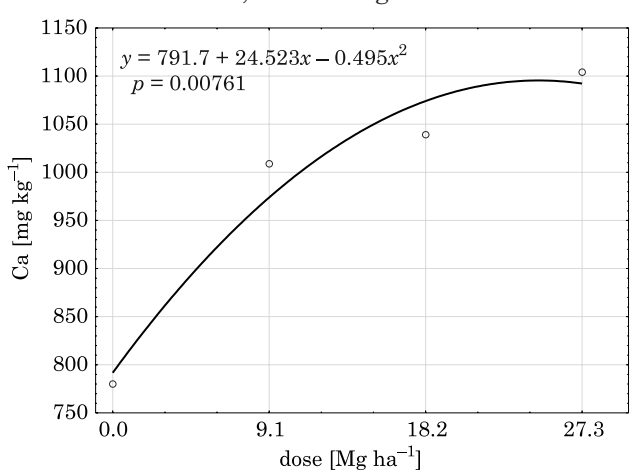

Fig. 3. Regression equation between dose of ash and calcium content in the soil

of this macroelements from 188 to 205 g Mg kg^{-1} of soil (Table 2). The increase in ash doses in combination with lime was not reflected in changes in the amount of exchangeable magnesium in the soil from experiment (Table 3). PROFITKALK fertilizer in its composition contains little magnesium (0.7% MgO) and the increase in soil pH value as a result of fertilization may be a factor that inhibits the solubility of magnesium compounds.

Iron, manganese, zinc, copper, chromium, nickel and lead

Manganese and iron undergo analogous geochemical processes. The amount of iron in wood ash was many times higher than in ash from straw. The soil from the experiment was characterized by an iron content of 8.21 g Fe kg^{-1} and manganese 470 mg Mn kg^{-1} soil.

Wood ash contained twice as much zinc as compared with straw ash, i.e. 1830 and 966 mg Zn kg^{-1}. General abundance of zinc in light soils varies between 7 and 150 mg Zn kg^{-1} soil (KABATA-PENDIAS 2011). The average content of zinc in the soil of variant without fertilization was low – about 35 mg Zn kg^{-1} soil.

The amount of total copper in wood ash was about twenty times higher than in the studied soil, whereas in ash straws – tenfold and were respectively 157 and 61.2 mg Cu kg^{-1}. Soil from the control object contained much less copper, i.e. 6.19 mg Cu kg^{-1} soil.

Wood ash is richer in terms of chromium in comparison with ash from straw (Table 1). The content of chromium in soils is generally low. The range of mean concentrations of chromium varies from 7 to 150 mg Cr kg^{-1} and its content and distribution in the soil largely depends on the type of the soil's mother rock (JANKIEWICZ and PTASZYŃSKI 2005). The amount of chromium in the soil from the experiment was 20.0 mg Cr kg^{-1} and it was lower than the average content of 60.0 mg Cr kg^{-1} soil (KABATA-PENDIAS 2011).

Nickel plays an important role in regulating the assimilation of free nitrogen in soil by bacteria; its content in the soil from the control object was 7.47 mg Ni kg^{-1} soil, and this is a level many times lower than permissible standard for the first class soil (Rozporządzenie Ministra Środowiska… DZ.U. 2016, pos. 1395).

Soil fertilization with biomass ashes, as well as increasing their doses in combination with lime did not cause significant changes in the soil fertility in relation to iron, manganese, zinc, copper, chromium and nickel.

The average amount of lead in Poland's soils is 27.0 mg Pb kg^{-1} soil (KABATA-PENDIAS 2011). Soil from the experiment was characterized by lower lead content, i.e. 13.1 mg Pb kg^{-1} soil. As a result of soil fertilization with wood ash, an increase in lead in soil by 15% was obtained. As a result of soil fertilization with straw ash, an decrease in lead in soil by 18% was

obtained. In view of the results of lead content in soil as a result of fertilization with biomass ashes, there is no indication against their use as fertilizers.

Macro and microelements in Kilimanjaro winter wheat grain

KOZLOVSKÝ et al. (2009) reported that the average amount of nitrogen in wheat grain is 2.18%. The content of nitrogen in the wheat grain from the experiment was on a similar level, i.e. 23.8 g N kg^{-1}. The lack of influence of ash fertilization from biomass and lime on changes in soil was confirmed by uniform amount of nitrogen in wheat grain cultivated in the experiment (Table 4 and Table 5).

Table 4

Content of macro- and microelements in the wheat grains depending on the type of ash from biomass

Parameter	Control	Type of ash			
		wood	straw	average	$LSD_{0.05}$
Nitrogen/N [g kg^{-1}]	23.4	23.8	23.8	23.8	n.s.
mg kg^{-1}					
Phosphorus/P	4.40	4.25	4.65	4.45	0.346
Potassium/K	3.90	3.86	4.04	3.95	n.s.
Calcium/Ca	0.467	0.539	0.535	0.537	n.s.
Magnesium/Mg	1.02	1.02	1.00	1.01	n.s.
Sodium/Na	38.1	38.5	38.1	38.3	n.s.
Iron/Fe	25.9	29.1	28.2	28.7	n.s.
Manganese/Mn	7.21	7.96	6.02	6.99	1.15
Zinc/Zn	31.6	27.7	29.2	28.4	n.s.
Lead/Pb	0.047	0.055	0.055	0.055	n.s.

n.s. – not significant difference

Table 5

Content of macro- and microelements in the wheat grain depending on the ash doses

Parameter	Dose of ash [Mg ha^{-1}]					
	0	7 + 2.1	14+4.2	21+6.3	average	$LSD_{0.05}$
Nitrogen/N [g kg^{-1}]	23.4	23.9	24.1	23.8	23.8	n.s.
mg kg^{-1}						
Phosphorus/P	4.40	4.56	4.46	4.38	4.45	n.s.
Potassium/K	3.90	4.08	3.84	3.98	3.95	n.s.
Calcium/Ca	0.467	0.551	0.559	0.571	0.537	n.s.
Magnesium /Mg	1.02	1.05	0.99	0.99	1.01	n.s.
Sodium/Na	38.1	37.5	37.4	40.3	38.3	n.s.
Iron/Fe	25.9	30.6	30.1	28.0	28.7	n.s.
Manganese/Mn	7.21	6.53	6.90	7.31	6.99	n.s.
Zinc/Zn	31.6	27.9	26.7	27.6	28.4	n.s.
Lead/Pb	0.047	0.054	0.056	0.063	0.055	0.011

n.s. – not significant difference

The standard wheat of Tonacja cv. is characterized by the content of macroelements in the dry mass of grain in the amount of: phosphorus 0.38%, potassium 0.42%, magnesium 0.13% and calcium 0.034% (RACHOŃ and SZUMIŁO 2009). Assessment of the abundance of the aforementioned macroelements in grain cultivated in the Kilimanjaro wheat cultivar shows that it was very similar to data characterizing the standard wheat, with the exception of phosphorus, the content of which was higher. Changes due to fertilization, soil content, available phosphorus and potassium, and exchangeable calcium and magnesium were not reflected in the abundance of wheat grain of Kilimanjaro cv.

The amount of sodium in Kilimanjaro wheat grain was on average 38.3 mg Na kg^{-1} which was, close to 32 mg Na kg^{-1} given by NABIPOUR et al. (2007) for wheat of Chamran cv.

Content of iron in wheat grain varies and depends on the cultivar ranging from 21.9 to 40.3 mg Fe kg^{-1} (WOŹNIAK and MAKARSKI 2012). The amount of iron determined in the wheat grain of Kilimanjaro cv. was on the level from 25.9 to 30.6 mg Fe kg^{-1} (Tables 4 and Table 5).

The average content of zinc in wheat was 28.4 mg Zn kg^{-1} which was at a lower level than in wheat grain of Tonacja cv. (34.9 mg Zn kg^{-1}) (RACHOŃ and SZUMIŁO 2009).

The lack of influence of the applied fertilization on changes in the amount of iron and zinc in the wheat grain results from an analogous dependence on this parameter in the soil (Table 2–5).

The range of manganese content for wheat cultivated in Poland according to SZTEKE et al. (2004) is 24–29 mg Mn kg^{-1}. Grain of Kilimanjaro wheat cultivated in the experiment contained one fourth of this value, on average 6.99 mg Mn kg^{-1}. Many times smaller amount of manganese in ash from the straw compared to wood ash probably caused a reduction of this element in the grain to the level of 6.02 mg Mn kg^{-1} (16%) (Table 4).

Wheat grain cultivated in the control object contained 0.047 mg Pb kg^{-1}. Increasing the ash doses in combination with lime resulted in a 34% increase in the amount of lead in grain, and its maximum content was 0.063 mg Pb kg^{-1} (Table 5). Permissible lead content in cereals and legumes determined in COMMISSION REGULATION (EU) 2015/1005 of 25 June 2015 amending Regulation (EC) No. 1881/2006 as regards maximum levels of lead in certain foodstuffs, amounts to 0.2 mg Pb kg^{-1}, therefore, there are no contraindications for introducing ashes from biomass or lime into the soil (Official Journal of the European Union L 161/9. 26.6.2015).

Conclusions

1. Fertilization with wood or straw ash had no influence on changes in the pH and content of organic carbon, nitrogen and available phosphorus in the soil from experiment.

2. As a result of fertilization with ash from wood or straw, a significant increase in the soil abundance was achieved in relation to: available potassium and exchangeable calcium. Increasing the dose was a factor that resulted in even higher efficiency of fertilizers.

3. As a result of soil fertilization with wood ash, an increase and straw ash, an decrease in lead in soil and was obtained.

4. The presence of lime fertilizer PROFITKALK was a factor causing as the dose increases proportional in the content of available phosphorus, potassium and exchangeable calcium in the soil.

5. Analysis of the content of microelements in the soil (copper, chromium, nickel and lead) shows that the application of fertilization in the form of wood or straw ashes and lime PROFITKALK did not exceed the threshold values for soil from the first group of ground defined in the Regulation of the Minister of Environment of 1 September 2016 on the way of assessing the pollution of the earth's surface.

6. Results concerning the impact of ashes from wood or straw and lime PROFITKALK on changes in the content of macro- and microelements indicate that when cultivating wheat of Kilimanjaro cv., it is justified to use them as a fertilizing material.

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References

Analiza chemiczno-rolnicza gleby. Pobieranie próbek. PN-R-04031:1997.

Animal feeding stuffs – Determination of the contents of calcium, copper, iron, magnesium, manganese, potassium, sodium and zinc – Method using atomic absorption spectrometry. ISO 6869:2000.

Animal feeding stuffs – Determination of phosphorus content – Spectrometric method. ISO 6491:1998.

Bakisgan C., Dumanli AG, Yürüm Y. 2009. *Trace elements in Turkish biomass fuels: Ashes of wheat straw, olive bagasse and hazelnut shell*. Fuel, 88: 1842–1851.

Bradna J., Malaťák J., Hájek D. 2016. *The properties of wheat straw combustion and use of fly ash as a soil amendment*. Agron. Res., 14(4): 1257–1265.

Cereals and pulses – Determination of the nitrogen content and calculation of the crude protein content – Kjeldahl method. ISO 20483:2013

Egner H., Riehm H., Domingo W. 1960. *Untersuchungen über die chemische Bodenanalyse als Grundlage für die Beurteilung des Nährstoffzustandes der Böden. II. Chemische Extraktions-*

methoden zur Phosphor- und Kalium Bestimmung. Kungliga Lantbrukshögskolans Annaler. 26: 199–215.

EICHLER-LÖBERMANN B., SCHIEMENZ K., MAKADI M., VAGO I., KOEPPEN D. 2008. *Nutrient cycling by using residues of bioenergy production-II. Effects of biomass ashes on plant and soil parameters.* Cereal Res. Commun., 36: 1259–1262.

FÜZESI I., HEIL B., KOVÁCS G. 2015. *Effects of Wood Ash on the Chemical Properties of Soil and Crop Vitality in Small Plot Experiments*. Acta Silv. Lign. Hung., 11(1): 55–64. doi: 10.1515/aslh-2015-0004.

GIBCZYŃSKA M., STANKOWSKI S., HURY G., KUGLARZ K. 2014. *Effects of limestone, Ash from biomass and compost use on chemical proprieties soil.* Soil Sci. Ann., 65(2): 59–64.

GOULDING K.W.T. 2016. *Soil acidification and the importance of liming agricultural soils with particular reference to the United Kingdom*. Soil Use Manage., 32(3): 390–399.

JAGUSTYN B., BĄTOREK-GIESA N., WILK B. 2011. *Assessment of biomass properties used for energy purposes.* Chemik, 65(6): 557–563.

JANKIEWICZ B., PTASZYŃSKI B. 2005. *Letter to editor determination of chromium in soil of Łódź Gardens.* Pol. J. Environ. Stud., 14(6): 869–875.

KABATA-PENDIAS A., 2011. *Trace elements in soil and plants*. Ed. 4. CRC Press, Taylor & Francis, pp. 1–534.

KAJDA-SZCZEŚNIAK M. 2014. *Characteristics of ashes from fireplace*. Archiwum Gospodarki Odpadami i Ochrony Środowiska, 16(3): 73–78.

KOZLOVSKÝ O., BALÍK J., ČERNÝ J., KULHÁNEK M., KOS M., PRÁŠILOVÁ M. 2009. *Influence of nitrogen fertilizer injection (CULTAN) on yield, yield components formation and quality of winter wheat grain*. Plant Soil Environ., 55(12): 536–543.

MADEJ A. 2016. *Bilans słomy w Polsce w latach 2010–2014 oraz prognoza do 2030 roku.* Rocz. Nauk., 18(1): 163–168.

NABIPOUR M., MESKARBASHEE M., FARZAD S. 2007. *Sodium and potassium accumulation in different parts of wheat under salinity levels.* Asian J. Agri. Res., 1(3): 97–104.

OCHECOVA P., TLUSTOS P., SZAKOVA J. 2014. *Wheat and soil response to wood fly ash application in contaminated soils.* Agron. J., 106(3): 995–1002.

Official Journal of the European Union L 161/9. 26.6.2015. – COMMISSION REGULATION (EU) 2015/1005 of 25 June 2015 amending Regulation (EC) No 1881/2006 as regards maximum levels of lead in certain foodstuffs.

Polish National List of Agricultural Plant Varieties. 2017. Ed. COBORU, Słupia Wielka. 54.

PIEKARCZYK M., KOTWICA K., JASKULSKI D. 2011. *The elemental composition of ash from straw and hay in the context of their agricultural utilization.* Acta Sci. Pol., Agricultura, 10(2): 97–104.

PIEKARCZYK M., KOBIERSKI M., GAŁĘZEWSKI L. 2017. *The influence of the applicat ion of barley, wheat and rape straw ash into sandy soil on the changes of soil reaction and the content of available phosphorus, potassium and magnesium*. Acta Sci. Pol. Agricultura, 16(3): 139–146.

RACHOŃ L., SZUMIŁO G. 2009. *Comparison of chemical composition of selected winter wheat species.* J. Elementol., 14(1): 135–146.

Rozporządzenie Ministra Środowiska z 1 września 2016 r. w sprawie sposobu prowadzenia oceny zanieczyszczenia powierzchni ziemi. DZ.U. 2016, pos. 1395.

SCHIEMENZ K., EICHLER-LÖBERMANN B. 2010. *Biomass ashes and their phosphorus fertilizing effect on different crops.* Nutr. Cycl. Agroecosys., 87:471–482.

Serwis IMGW-PIB. Klimat Polski. Mapy klimatu Polski, http://klimatpogodynka.pl/pl/climate-maps, access: 24.05.2019

Soil quality – Determination of pH. ISO 10390:1997P.

Soil quality – Determination of the potential cation exchange capacity and exchangeable cations using barium chloride solution buffered at pH = 8,1. ISO 13536:1995.

Soil quality – Determination of organic carbon by sulfochromic oxidation. ISO 14235:1998.

Soil quality – Determination of total nitrogen – Modified Kjeldahl method. ISO 11261:2002.

SZÁSZ-LEN A.M., HOLONEC L., PAMFIL D. 2016. *Mineral substances in stem wood tissue of European Beech (Fagus sylvatica L.).* ProEnvironment, 9: 41–55.

SZTEKE B., JĘDRZEJCZAK R., RĘCZAJSKA W. 2004. *Zawartość żelaza i manganu w wybranych roślinach jadalnych*. Roczn. PZH, Suppl., 55: 21–27.

USDA 2006. United States. Department of Agriculture-Handbooks, 1–690.

WOŹNIAK A., MAKARSKI B. 2012. *Content of minerals in grain of spring wheat cv. Koksa depending on cultivation conditions*. J. Elementol., 517–523, doi: 10.5601/jelem.2012.17.3.13.

ZAPAŁOWSKA A., PUCHALSKI C., HURY G., MAKAREWICZ A. 2017. *Influence of fertilization with the use of biomass ash and sewage sludge on the chemical composition of Jerusalem artichoke used for energy-related purposes*. J. Ecol. Eng., 18(5): 235-245. doi: 10.12911/22998993/76214.

Wapno PROFITKALK. Agro Trade Grupa, http://agrotradegrupa.com/pl/produkty/wapno-profitkalk, access: 17.01.2019.

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THE IMPACT OF NITROGEN FERTILIZATION STRATEGIES ON SELECTED QUALITATIVE PARAMETERS OF SPRING WHEAT GRAIN AND FLOUR

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Key words: grain quality; flour quality; nitrogen fertilization, protein, wheat.

Abstract

The quality of wheat grain determines the quality of flour and this becomes important in subsequent processing stages and impacts the end products. The objective of the studies was to determine whether a method of applying nitrogen both with and without compound fertilizers at different growth stages of spring wheat impacted the crop yield and qualitative parameters of grain and flour. A three-year field experiment was in north-eastern Poland at the Teaching and Research Station of the University of Warmia and Mazury in Olsztyn. Spring wheat was cultivated with the application of different treatments of nitrogen fertilization with a total dose of 120 kg ha^{-1}. The nitrogen fertilizers were applied to the soil or to soil and on leaves (foliar application) with and without microelements at the following growth stages: tillering, stem elongation and heading. It was concluded that the fertilization methods did not impact the grain yield. Soil urea application at doses of 40 kg ha^{-1} at the tillering and stem elongation stages mostly increased the protein content in grain and flour, vitreousness of grain, gluten content and alveographic – *W* parameter compared to the other fertilization variants. Soil application of the fertilizer with macronutrients and trace minerals generally produced worsened grain parameters, especially the protein grain content and the Zeleny index. The weather conditions most affected the grain yield and such grain and flour parameters as kernel weight, kernel diameter, hardness

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index and P/L index. The soil urea application at a dose of 40 kg ha^{-1} at the elongation stages contributes to better grain quality than foliar application of urea at doses of 20 kg N ha^{-1} at the stem elongation and heading stages.

Introduction

The quality of wheat grain is influenced by numerous factors, of which its genotype is the major one; in addition, it is also impacted by environmental factors during the growing season and cultivation techniques (KNAPOWSKI et al. 2010, STĘPIEŃ and WOJTKOWIAK 2016, HELLEMANS et al. 2018). It is believed that of the cultivation procedures and techniques, nitrogen fertilization plays a basic role in determining the quality of grains, with the key role being attributed to the dose and allocation of nitrogen during the cultivation cycle, the type of fertilizer and the time and method of application (RALCEWICZ et al. 2009, ABEDI et al. 2010, FUERTES-MENDIZÁBAL et al. 2010, BLANDINO et al. 2015b, RANSOM et al. 2016, XUE et al. 2016a). Wheat slowly uptakes and accumulates nitrogen during the early growth stages until tillering; afterwards, the uptake of nitrogen is rapid till the heading stage and then it slows down again after flowering till the milk ripening stage (MCGUIRE et al. 1998). The accumulation of nitrogen in the early and intermediate-early growth stages mainly serves to increase the grain yield, whereas during heading and afterwards its main purpose is for cereal protein synthesis. Under proper environmental conditions, the application of divided nitrogen doses results in higher nitrogen uptake by plants (ERCOLI et al. 2013). The use of divided nitrogen doses increases the total protein content in the grain in comparison with the same dose of nitrogen applied once. There are also some results indicating the lack of impact of divided nitrogen doses on the protein content in the grain (SCHULZ et al. 2015). The availability of nitrogen for plants is determined by the form of nitrogen found in a fertilizer. The source of nitrogen (urea, nitrate-N, and ammonium-N) may impact the protein content and quality of wheat mainly by changing the absorption of nitrogen by plants (XUE et al. 2016a). Numerous studies indicate a positive effect of nitrogen applied at the heading and anthesis stages, implicating it as a useful tool for increasing the protein content (BLANDINO et al. 2015a, DICK et al. 2016, RANSOM et al. 2016). In different wheat varieties, there is a tendency for varied reactions to nitrogen fertilization (RANSOM et al. 2016). The content of protein in the grain is often negatively correlated with the yield since higher grain filling is linked to increased starch storage (BLANDINO et al. 2015b). Protein is the main parameter in evaluating the quality of wheat grain since the content and composition of protein in

the grain (and consecutively in flour) impact the quality of bread (ZHANG et al. 2009). Dividing the total dose of nitrogen and applying it at different growth stages affects the quality of wheat flour because belated nitrogen application favours the accumulation of protein over starch and prolongs the grain filling time (XUE et al. 2016b). The other factors determining the quality of grain include: grain size, bulk density, hardness, flour extraction rate, ash content and flour colour – these are the basic parameters of grain milling quality (CAMPBELL et al. 2012, PAGANI et al. 2014). The quality of wheat grain determines the quality of flour and this becomes important in subsequent processing stages and impacts the end products (ZHANG et al. 2005, PAGANI et al. 2014).

The objective of the studies was to evaluate the impact of nitrogen application method, both with compound fertilizers added and without at different spring wheat growth stages on the content of protein and other qualitative parameters of the grain and flour.

Materials and Methods

The studies were carried out in north-eastern Poland at the Teaching and Research Station of the University of Warmia and Mazury in Olsztyn (5372N, 2042E). The field trial was conducted in 2009–2011 on brown soil of a granulometric composition of light loam. Its composition was as follows: 2.8–3.2% of fractions with their diameters below 0.002 mm; 30.4–31.9% of fractions with their diameters range – 0.002–0.050 mm, and 64.9–66.8% of fractions with their diameters range 0.050–2.00 mm, respectively.

Table 1

Chemical characteristics of soil before the start of the experiment

Measurement	Values
Soil acidity [1 mol L^{-1} KCl]	5.5–6.1
Humus [%]	2.02
Total N [g kg^{-1}]	1.23–1.25
P [mg kg^{-1}]*	63.7–97.2
K [mg kg^{-1}]*	141.1–199.2
Mg [mg kg^{-1}]*	46–56

*– available forms

The selected parameters of soil sampled before the trail are presented in Table 1. The experiment was based on a random block design in three repetitions. The area of cultivation plots was 6.25 m^2 and the harvesting plots were 4 m^2. Two varieties of spring wheat (*Triticum aestivum* ssp. *vulgare*) Parabola and Radunia were selected to compare the applied fertilization

methods. The wheat grains were sown at a density of 5 million grains ha^{-1}, with row spacing of 10.5 cm. The dose of 30.2 kg P ha^{-1} as 46% triple superphosphate and 83.1 kg K ha^{-1} as potassium salt was applied. The dosage of nitrogen fertilization was 120 kg ha^{-1} yet 40 kg ha^{-1} N as ammonium nitrate was applied before wheat was sown. The remaining nitrogen dose was delivered according to four different nitrogen fertilization strategies (T_1–T_4):

T_1, to soil at 40 kg ha^{-1} N as 46% urea at the tillering stage (GS 23) and to soil at 40 kg ha^{-1} N as 46% urea at the stem elongation stage (GS 31),

T_2, to soil at 20 kg ha^{-1} N as 46% urea and at 20 kg ha^{-1} N as the Azofoska fertilizer at the tillering stage (GS 23) and to soil at 40 kg ha^{-1} N as 46% urea at the stem elongation stage (GS 31),

T_3, to soil at 40 kg ha^{-1} N as 46% urea at the tillering stage (GS 23) and at 20 kg N as foliar application of 10% urea solution at the stem elongation stage (GS 31) and at 20 kg N ha^{-1} as foliar application as 10% urea solution at the heading stage (GS 52),

T_4, to soil at 40 kg ha^{-1} N as 46% urea at the tillering stage (GS 23) and at 20 kg N as foliar application of 10% urea solution with the Ekolist mikro Z product at a dose of 1 dm^{-3} ha^{-1} at the stem elongation stage (GS 31) and at 20 kg N as foliar application of 10% urea solution with the Ekolist mikro Z product at a dose of 1 dm^3 ha^{-1} at the heading stage (GS 52).

The amounts of macronutrients and trace minerals applied with the Azofoska fertilizer and the Ekolist mikro Z product are presented in Table 2. The fungicides (a.s. ciproconazole + propiconazole and azoxystrobin) were used to protect wheat against fungal diseases and a herbicide (a.s. florasulam + 2,4-dichlorophenoxyacetic acid) was applied to reduce weed infestation. At crop maturity, grain was harvested using a plot combine.

Table 2

Amounts of macronutrients and trace elements applied with Azofoska and Ekolist mikro Z fertilizers

Type of fertilizer	N	P	K	Mg	S	Cu	Zn	Mn	Fe	Mo	B
Azofoska [kg ha^{-1}]	20.0	2.69	23.3	3.98	13.52	0.26	0.07	0.39	0.25	0.06	0.07
Ekolist mikro Z [g ha^{-1}]	104.8	–	–	79.2	112.6	9.18	23.6	25.6	26.2	0.14	0.42

The grain samples were analysed for moisture content with the ICC 110/1 method. Hardness index (HI), kernel weight (KW), and kernel diameter (KD) were determined on 300 kernels using the Single Kernel Characterisation System (SKCS) type 4100, Perten Instruments North America Inc., Reno, USA (Method AACC 55-10, 2002). The evaluation of Test Weight (TW) was performed in accordance with the AACC 55-31 method. Grain vitreousness was assessed by analysing the cross sections of grain and expressed as a percentage of vitreous kernels on a 50-item sample.

Partially vitreous kernels were categorized as semi-vitreous grains and their number on a sample was multiplied by 0.5 factor. The protein content in the grain (g kg^{-1} dry matter, DM) was measured with the Kjeldahl (N 5.7) method on a KjelFlex K-360 apparatus (Buchi, Germany). The ash content in the grain and flour was determined following the ICC 104/1, 1990 method. Following preliminary humidification to 15% moisture content, grain was milled in a Quadrumat Jr laboratory Mill (Brabender, Germany) equipped with a 70 GG cylindrical sieve (PE 236 µm). The grain samples of 125 g were weighed on a WLC 2/A1 electronic scale (Radwag, Poland, d = 10 mg) and were then processed and milled according to the laboratory procedure. Three samples of each type of material were ground. Flour extraction rate was defined as the percentage of straight-grade flour. The wet gluten content in grain, Zeleny sedimentation index and flour moisture level, flour protein content (the result was expressed at 12% moisture basis – mb) and flour water absorption were determined on a NIR System Infratec 1241 Grain Analyser with flour module (FOSS, Hillerod, Denmark) that takes transmission measurements of near-infrared waves (570–1050 nm). This device was also used to determine two major alveographic parameters in flour: W – mechanical deformation of dough (energy) and P/L – the ratio of maximum resistance and elasticity of dough.

The results were statistically analysed with two-way analysis of variance (ANOVA) taking the treatment and variety as the independent variables. The ANOVA analysis was run individually for each year. The significance of differences between the means was determined with Tukey's test. The correlations between the individual parameters were evaluated with the Pearson's test. Statistical calculations were performed with STATISTICA for Windows v. 10 software (StatSoft Inc.). Statistical hypotheses were tested at $p \leq 0.05$.

Results and Discussion

In 2009–2011, the weather conditions during the growing season are presented in Table 3. Of the three wheat growing seasons, the highest temperature was recorded in 2010 (13.2°C) and it was higher than the average multiannual temperature. Considering the monthly distribution of temperatures, it was found that July and August in 2010 were much warmer than the corresponding months from the other growing seasons.

The average precipitation level from March till August 2009 was 320.1 mm and thus comparable to the average multiannual precipitation. In the 2010 and 2011 growing seasons, precipitation was high and higher than the amount of precipitation in 2009 (by 39.7% and 42.6%, respectively).

Table 3

Climate conditions during wheat vegetation

Factor/Years	Months						Average
	Mar.	Apr.	May	Jun.	Jul.	Aug.	
	Temperature [°C]						
2009	1.3	9.4	12.4	14.9	19.2	17.6	12.5
2010	2.1	8.1	12.0	16.4	21.1	19.3	13.2
2011	1.6	9.1	13.1	17.1	17.9	17.6	12.7
1961–2005	1.2	6.9	12.8	15.9	17.8	17.7	12.1
	Sums of monthly precipitation [mm]						Sum
2009	57.9	4.8	52.9	136.9	48.3	19.3	320.1
2010	36.7	18.2	131.9	84.8	80.4	95.3	447.3
2011	16.3	22.5	51.1	81.7	202.0	82.1	456.5
1961–2005	27.6	35.7	51.9	78.5	75.1	66.1	334.9

From May till the end of June, i.e. during tillering and stem elongation and when spring wheat has the highest demand for water, the lowest level of precipitation was recorded in 2011 (132.8 mm) while the highest was in 2010 (216.7 mm). The nitrogen fertilization treatments did not impact the grain yield (Table 4), which is consistent with the results presented by SCHULZ et al. (2015). However, due to different climatic conditions, the yield average varied in the individual growing seasons. In 2010, proper distribution of precipitation during tillering and stem elongation contributed to the highest grain yield. The varieties significantly differed in the grain yield in two growing seasons (2009 and 2011). A reaction of the varieties to weather conditions was different and this thus influenced the volume of crop yield. In 2009 the Radunia variety produced a significantly higher yield and the same was reported was the Parabola variety in 2011, whereas, in 2010, no significant differences were found. The differences in crop yield of the individual varieties may result from a varied capacity for nitrogen uptake by each of them (XUE et al. 2016a).

Grain test weight (TW) depended on the fertilization procedures and the variety itself, with higher test weight being reported for the Radunia variety. Foliar urea application at GS 31 and GS 52 (T_3) produced the highest value of grain test weight in each growing season (Table 4). RUSKE et al. (2003) reported a similar relation with increased test weight resulting from the application of additional nitrogen (as urea) at the anthesis stage. BLANDINO et al. (2015a) reported that TW was significantly increased when nitrogen was applied late in the growing season and in the form of ammonium nitrate. The author's research (data not shown) demonstrated a negative correlation between the grain yield and TW (r = -0.48).

It means that smaller wheat kernels were produced with a higher crop yield. Lower test weight may implicate smaller kernel filling, which translates into lower flour yield (RUSKE et al. 2003).

Table 4

Effect of N strategies and cultivar on grain yield, test weight, kernel weight, kernel diameter, hardness index and vitreousness in years 2009, 2010 and 2011

Years	Factor	Treatment	Grain yield [t ha^{-1}]	TW [kg hL^{-1}]	KW [mg]	KD [mm]	HI [-]	Vitreousness [%]
2009	N (*A*)	T_1	6.28^a	77.8^b	44.7^a	3.06^a	62.9^a	36^a
		T_2	6.16^a	77.8^b	43.7^a	3.02^a	63.3^a	26^b
		T_3	6.03^a	78.0^a	44.6^a	3.06^a	61.8^a	22^{bc}
		T_4	6.08^a	77.6^c	44.0^a	3.05^a	63.8^a	21^c
		P(F)	ns	***	ns	ns	ns	***
	cultivar (*B*)	Parabola	5.94^b	77.3^b	49.6^a	3.11^a	63.5^a	35^a
		Radunia	6.33^a	78.3^a	38.9^b	2.98^b	62.5^a	18^b
		P(F)	***	***	***	***	ns	***
	A x *B*	P(F)	ns	***	ns	ns	ns	***
2010	N (*A*)	T_1	6.82^a	76.4^a	41.4^a	2.95^a	64.4^{bc}	49^a
		T_2	6.79^a	76.4^a	40.8^a	2.93^a	64.1^c	42^b
		T_3	6.74^a	76.6^a	40.8^a	2.95^a	66.3^{ab}	46^{ab}
		T_4	6.80^a	76.1^b	40.5^a	2.95^a	67.6^a	44^b
		P(F)	ns	***	ns	ns	***	**
	cultivar (*B*)	Parabola	6.85^a	75.7^b	44.3^a	2.98^a	67.4^a	55^a
		Radunia	6.73^a	77.1^a	37.5^b	2.92^b	63.8^b	35^b
		P(F)	ns	***	***	***	***	***
	A x *B*	P(F)	ns	***	***	**	ns	***
2011	N (*A*)	T_1	5.68^a	78.1^b	46.8^{ab}	3.12^b	67.1^a	56^a
		T_2	5.92^a	78.1^b	45.8^b	3.10^b	66.8^a	56^a
		T_3	6.06^a	78.8^a	48.3^a	3.18^a	66.2^a	57^a
		T_4	5.75^a	77.9^c	45.8^b	3.09^b	66.7^a	49^a
		P(F)	ns	***	***	***	ns	*
	cultivar (*B*)	Parabola	6.27^a	77.3^b	48.9^a	3.16^a	66.9^a	53^a
		Radunia	5.43^b	79.1^a	44.5^b	3.09^b	66.4^a	55^a
		P(F)	***	***	***	***	ns	ns
	A x *B*	P(F)	ns	***	***	***	ns	***

a, *b*, *c* – averages designated by the different small letters in the columns of the table, separately for year, are significantly different at $p \leq 0.05$

*, **, *** Significant at the 0.05, 0.01 and 0.001 probability level, respectively

ns – not significant at the 0.05 probability level

The variety and climatic condition in the individual growing seasons influenced the kernel weight (KW) and kernel diameter (KD) the most. The interactions between the variety and nitrogen fertilization methods were significant in 2010 and 2011 for kernel weight and in 2011 for kernel diameter. Of the two varieties used in the trial, Parabola's wheat grain had higher weight and diameter. Weather conditions in the individual growing seasons influenced differently on the wheat varieties cultivated and therefore the Parabola variety had the highest kernel weight and diameter in 2009 and Radunia in 2011, but their yield was lowest in these seasons. The tested nitrogen fertilization treatments did not influence the kernel weight or diameter in the first two wheat growing seasons. In the third growing season (2011), there was an impact of foliar urea application at the GS 31 and GS 52 stages (T_3). In comparison with the other treatments, T_3 variant contributed to an increase in both the weight and diameter of the kernels.

Kernel hardness was most impacted by the weather conditions in the individual wheat growing seasons, with the grain from 2011 being the hardest. The wheat varieties and fertilization treatments affected the kernel hardness only in one growing season, i.e. in 2010. In this season significant differences in kernel hardness were demonstrated between the treatment that differed in the way nitrogen was applied: to soil (T_1 and T_2) and on leaves (T_3 and T_4). Foliar urea application combined with Ekolist mikro Z product and without it substantially increased the hardness of grain. The Parabola variety was harder than Radunia. Kernel hardness was significantly ($p < 0.001$) correlated with kernel vitreousness ($r = 0.78$); the data is not shown. Kernel vitreousness was most influenced by the year of cultivation. The variety and nitrogen fertilization variants exerted an impact on kernel vitreousness in the first two growing seasons (2009 and 2010). In these seasons, soil urea fertilization at the tillering (GS 23) and stem elongation (GS 31) stage (the T_1 option) produced the grains of the highest vitreousness, although this value was higher in the Parabola variety. The environmental condition, particularly the temperature and sunny weather, may have contributed to the differences in kernel vitreousness, as reported in other studies (Oury et al. 2015).

The grain protein content depended on nitrogen fertilization treatments (Table 5), but the outcome of such procedures was determined by weather conditions in the individual growing seasons (amount and distribution of precipitation) that affected the availability of nitrogen and its absorption by plants. Importantly, there was also an impact temperature during the grain filling phase. In the 2010 growing season, when higher temperatures were recorded in June and August than in the other seasons,

Table 5

Effect of N strategies and cultivar on grain protein content, gluten content, Zeleny index and grain ash content in years 2009, 2010 and 2011

Years	Factor	Treatment	Protein content [g kg^{-1} DM]	Gluten content [%]	Zeleny index [cm^3]	Ash content [% DM]
2009	N (*A*)	T_1	136.2^a	34.0^a	56.8^{ab}	1.92^a
		T_2	129.7^b	32.5^b	54.2^b	1.96^a
		T_3	131.4^{ab}	33.8^a	57.4^a	2.00^a
		T_4	128.2^b	33.6^a	57.2^a	1.95^a
		P(F)	**	***	*	ns
	cultivar (*B*)	Parabola	138.5^a	36.0^a	62.5^a	1.99^a
		Radunia	124.5^b	31.0^b	50.3^b	1.93^b
		P(F)	***	***	***	*
	A x *B*	P(F)	*	*	**	*
2010	N (*A*)	T_1	136.3^a	35.0^{ab}	55.9^b	2.00^a
		T_2	132.1^b	34.6^b	53.7^c	1.97^a
		T_3	136.7^a	35.1^a	55.8^b	2.01^a
		T_4	137.4^a	33.1^c	57.5^a	2.00^a
		P(F)	***	***	***	ns
	cultivar (*B*)	Parabola	138.2^a	36.8^a	56.5^a	2.05^a
		Radunia	133.1^b	32.1^b	55.0^b	1.95^b
		P(F)	***	***	***	***
	A x *B*	P(F)	***	***	***	*
2011	N (*A*)	T_1	138.5^a	36.1^a	59.7^a	2.03^a
		T_2	133.9^b	33.0^c	58.6^b	2.01^a
		T_3	131.9^b	32.8^c	58.8^b	1.98^a
		T_4	130.8^b	34.0^b	56.1^c	2.00^a
		P(F)	***	***	***	ns
	cultivar (*B*)	Parabola	134.9^a	34.9^a	56.9^b	2.01^a
		Radunia	132.7^b	33.1^b	59.7^a	2.00^a
		P(F)	*	***	***	ns
	A x *B*	P(F)	ns	***	***	ns

a, *b*, *c* – averages designated by the different small letters in the columns of the table, separately for year, are significantly different at $p \leq 0.05$

*, **, *** significant at the 0.05, 0.01 and 0.001 probability level, respectively

ns – not significant at the 0.05 probability level

the content of protein in kernels was highest. A positive impact of high temperature on protein synthesis during the grain filling phase in durum wheat was reported by DE STEFANIS et al. (2002). When hydration of wheat at the tillering and stem elongation stages (May 2009 and 2011) was com-

parable to the multiannual average value, soil urea application at GS 23 and GS 31 (T_1) increased the protein content in kernels in comparison to the other fertilization options. In these seasons, the biggest differences in grain protein content were demonstrated between the T_1 and T_4 fertilization variants, respectively 5.87 and 5.56%. However, in May 2010 when the precipitation level was over twice as high as the average multiannual precipitation level, soil urea application with Azofoska at GS 23 and urea at GS 31 resulted in a significant reduction of kernel protein content compared to the other fertilization variants (T_1, T_3, and T_4) by 3.18, 3.48 and 4.01%, respectively. Different nitrogen sources, such as urea, nitrate-N and ammonium-N, may affect the protein and quality of wheat mainly by modifying the uptake of nitrogen by plants (XUE et al. 2016a). The results of studies conducted by BLY and WOODARDA (2003) demonstrate that the total protein content is modified not only by the nitrogen dose but also by applying it at divided doses and at different time points. However, the finding of studies on the impact of divided nitrogen doses on the protein content are not unequivocal. The studies by SCHULZ et al. (2015) indicate a lack of the impact of divided nitrogen dose on the protein content in grain. In contrast, the results reported by XUE et al. (2016b) showed that dividing the nitrogen dose resulted in an increased content of cereal proteins, although the effect was more evident when nitrate-N was applied instead of urea. RANSOM et al. (2016) indicate that foliar urea ammonium nitrate application after flowering may increase the protein content in spring wheat as compared to applying a similar nitrogen dose at sowing. The collected data shows that divided nitrogen doses affects the protein content in kernels more than the grain yield, which was confirmed by the results reported in studies by BLANDINO et al. (2015b). Wheat yield and reaction of protein to nitrogen are divided into three phases: in the first phase, the yield of grains increases while the protein content drops as starch accumulation is more reactive than that of protein. In the second phase, the yield and protein content increase in response to increased nitrogen level and, in the third phase, the yield is maximized by nitrogen, but the protein content still increases together with higher N values (BROWN et al. 2005). In the presented studies, in 2010 the wheat crop yield was highest at the highest protein content, which may indicate the second reaction phase. In comparison with Radunia, the Parabola variety had higher protein levels in all growing seasons, with the highest protein content recorded in the season in which the grain yield was lowest, which may implicate a protein dilution effect in the other growing seasons. Considering the qualitative requirements for wheat grain for the milling industry (the minimum level of protein in food wheat should be 120 g kg^{-1} DM),

it was found that these requirements were met in all fertilization variants. Like for protein, the gluten content and Zeleny index were substantially modified by nitrogen fertilization, weather conditions in the individual growing seasons and the variety. The highest gluten level in wheat kernels in the 2009 and 2011 growing seasons was demonstrated with soil urea application at GS 23 and GS 31 (T_1) (though in 2009 the difference was insignificant) whereas in 2010 it was T_1 with urea applied to soil and T_3 with both soil and foliar application. The gluten content in Parabola grain was significantly higher than in the Radunia variety. The reaction of wheat, expressed with the Zeleny index, to different nitrogen fertilization strategies differed in each growing season. However, the Zeleny index was found to be substantially reduced when urea was applied together with Azofoska at GS 23 (T_2) in the first two wheat growing seasons. The value of this index was also much affected by the variety. For the first two growing seasons, it was demonstrated that the Zeleny index was significantly higher for Parabola, while in the third season, it was higher for Radunia. ABBAD et al. (2004) showed that the sedimentation index was substantially higher when nitrogen was applied during flowering. Different variants of nitrogen fertilization did not impact the content of ash in kernels. The variety influenced the ash level in the first two growing seasons, with a higher content recorded in the Parabola wheat grain.

Flour yield (FY) was determined by the applied fertilization variants and the wheat variety (Table 6). In two growing seasons (2009 and 2011), the highest FY value was produced with foliar urea application at GS 31 and GS 52 (T_3). In these seasons, the grain with the highest TW was also harvested with the same fertilization treatment, which translated into the largest flour extraction rate. Higher flour extraction was always reported for the Radunia wheat variety. As with the results for grain protein, in 2009 and 2011 soil urea fertilization at GS 23 and GS 31 (T_1) increased the flour protein level in comparison with the other fertilization variants. However, in 2010 the highest protein content was demonstrated in flour produced from the grain after foliar urea application (T_3). In each wheat growing season, the flour from Parabola wheat had a significantly higher protein content.

None of fertilization strategies affected the ash content in flour, flour water absorption or the P/L index. These parameters depended mainly on the wheat variety and growing season in which they were cultivated. Contrary to Radunia, the Parabola wheat flour was characterized with higher values of these parameters. The results of the present studies that did not demonstrate significant differences in the P/L values are consistent with the findings reported by BLANDINO et al. (2016) and FUERTES-MENDIZÁBAL et al. (2010).

Table 6
Effect of N strategies and cultivar on flour yield and some flour quality traits in years 2009, 2010 and 2011

Years	Factor	Treatment	Flour yield [%]	Protein content [g kg^{-1} at 12% mb]	Ash content [% DM]	Water absorption [%]	W [J 10^{-4}]	P/L [-]
2009	N (*A*)	T_1	67.6^{ab}	125.1^{a}	0.61^{a}	61.2^{a}	427^{a}	0.32^{a}
		T_2	67.2^{b}	119.4^{c}	0.61^{a}	60.2^{a}	404^{c}	0.22^{a}
		T_3	68.3^{a}	124.1^{ab}	0.61^{a}	61.2^{a}	418^{b}	0.35^{a}
		T_4	67.1^{b}	122.7^{b}	0.60^{a}	61.0^{a}	415^{b}	0.28^{a}
		P(F)	**	***	ns	ns	***	ns
	cultivar (*B*)	Parabola	66.1^{b}	128.8^{a}	0.65^{a}	63.8^{a}	441^{a}	0.38^{a}
		Radunia	69.0^{a}	116.9^{b}	0.57^{b}	58.0^{b}	391^{b}	0.22^{a}
		P(F)	***	***	***	***	***	ns
	A x *B*	P(F)	*	*	ns	ns	**	ns
2010	N (*A*)	T_1	68.7^{ab}	124.2^{b}	0.64^{a}	59.0^{a}	378^{a}	0.63^{a}
		T_2	69.2^{a}	122.3^{c}	0.64^{a}	58.1^{a}	370^{a}	0.68^{a}
		T_3	68.2^{bc}	125.7^{a}	0.63^{a}	59.2^{a}	370^{a}	0.67^{a}
		T_4	67.9^{c}	124.4^{b}	0.63^{a}	59.1^{a}	370^{a}	0.58^{a}
		P(F)	***	***	***	ns	ns	ns
	cultivar (*B*)	Parabola	67.6^{b}	126.4^{a}	0.67^{a}	61.0^{a}	377^{a}	1.12^{a}
		Radunia	69.5^{a}	121.9^{b}	0.60^{b}	56.7^{b}	368^{b}	0.17^{b}
		P(F)	***	***	***	***	***	***
	A x *B*	P(F)	**	***	ns	ns	ns	ns
2011	N (*A*)	T_1	68.5^{b}	125.2^{a}	0.64^{a}	62.7^{a}	359^{a}	1.62^{a}
		T_2	69.6^{ab}	120.4^{b}	0.64^{a}	61.5^{a}	344^{b}	1.47^{a}
		T_3	70.2^{a}	119.1^{b}	0.64^{a}	62.3^{a}	339^{bc}	1.65^{a}
		T_4	68.7^{b}	119.1^{b}	0.64^{a}	62.3^{a}	332^{c}	1.62^{a}
		P(F)	**	***	ns	ns	***	ns
	cultivar (*B*)	Parabola	68.4^{b}	122.2^{a}	0.67^{a}	64.1^{a}	343^{a}	2.11^{a}
		Radunia	70.1^{a}	119.7^{b}	0.61^{b}	60.3^{b}	344^{a}	1.07^{b}
		P(F)	***	***	***	***	ns	***
	A x *B*	P(F)	*	ns	ns	ns	**	ns

a, *b*, *c* – averages designated by the different small letters in the columns of the table, separately for year, are significantly different at $p \leq 0.05$
*, **, *** Significant at the 0.05, 0.01 and 0.001 probability level, respectively
ns – not significant at the 0.05 probability level

Soil urea fertilization at the tillering and stem elongation stages (T_1) increased the W index in comparison with the other fertilization variants. A similar effect was reported by BLANDINO et al. (2016) with applying ammonium nitrate to soil at the initial phase of heading.

Conclusions

The results of nitrogen fertilization strategies (time of application, type of fertilizer, and application techniques), expressed as the yield and the properties of grain and flour depended on weather conditions. Soil urea application at doses of 40 kg ha^{-1} at the tillering and stem elongation stages mostly increased the protein content in grain and flour, vitreous aspect of grain, gluten content and alveographic – *W* parameter compared to the other fertilization variants. Foliar application of urea at doses of 20 kg N ha^{-1} at the stem elongation and heading stages produced the highest value of grain test weight in each growing season. Soil application of the fertilizer with macronutrients and trace minerals generally produced worse grain parameters, especially the protein grain content and the Zeleny index. The weather conditions most affected the grain yield and such grain and flour parameters as kernel weight, kernel diameter, hardness index and P/L index. Our research shows, that soil urea application at a dose of 40 kg ha^{-1} at the elongation stages contributes to better grain quality than foliar application of urea at doses of 20 kg N ha^{-1} at the stem elongation and heading stages.

References

Abbad A.J., Lloveras., Michlena A. 2004. *Nitrogen fertilization and foliar urea effects on Durum wheat yield and quality and on residual soil nitrate in irrigated Mediterranean conditions.* Field Crops Res., 87: 257–269.

Abedi T., Alemzadeh A., Kazemeini S.A. 2010. *Wheat yield and grain protein response to nitrogen amount and timing.* Aust. J. Crop Sci., 5: 330–336.

Blandino M., Vaccino P., Reyneri A. 2015a. *Late-season nitrogen increases improver common and durum wheat quality.* Agron. J., 107: 680–690.

Blandino M., Marinaccio F., Vaccino P., Reyneri A. 2015b. *Nitrogen fertilization strategies suitable to achieve the quality requirements of wheat for biscuit production.* Agron. J., 107: 1584–1594.

Blandino M., Marinaccio F., Reyneri A. 2016. *Effect of late-season nitrogen fertilization on grain yield and on flour rheological quality and stability in common wheat, under different production situations.* Ital. J. Agron., 11: 107–113.

Bly A.G., Woodard H.J. 2003. *Foliar nitrogen application timing influence on grain yield and protein concentration of hard red winter and spring wheat.* Agron. J., 95: 335–338.

Brown B., Westcott M., Christensen N., Pan B., Stark J. 2005. *Nitrogen management for hard wheat protein enhancement.* A Pacific Northwest Extension Pub., Idaho Univ., Washington State Univ., PNW 578, pp.: 1–14.

Campbell G.M., Sharp C., Wall K., Mateos-Salvador F., Gubatz S., Huttly A., P. Shewry. 2012. *Modelling wheat breakage during roller milling using the Double Normalised Kumaraswamy Breakage Function. Effects of kernel shape and hardness.* J. Cereal Sci., 55: 415–425.

De Stefanis E., Sgruletta D., De Vita P., Pucciarmati S. 2002. *Genetic variability to the effects of heat stress during grain filling on durum wheat quality.* Cereal Res. Commun., 30: 117–124.

Dick C.D., Thompson N.M., Epplin F.M., Arnall D.B. 2016. *Managing late-season foliar nitrogen fertilization to increase grain protein for winter wheat.* Agron. J., 108: 2329–2338.

Ercoli L., Masoni A., Pampana S., Mariotti M., Arduini I. 2013. *As durum wheat productivity is affected by nitrogen fertilisation management in Central Italy.* Eur. J. Agron, 44: 38–45.

Fuertes-Mendizábal T., Aizpurua A., González-Moro M.B., Estavillo J.M. 2010. *Improving wheat breadmaking quality by splitting the N fertilizer rate.* Eur. J. Agron., 33: 52–61.

Hellemans T., Landschoot S., Dewitte K., Van Bockstaele F., Vermeir P., Eeckhout M., Haesaert G. 2018. *Impact of crop husbandry practices and environmental conditions on wheat composition and quality*. A Review. J. Agric. Food Chem., 66: 2491–2509.

Knapowski T., Spychaj-Fabisiak E., Ložek O. 2010. *Foliar nitrogen fertilization as a factor determining technological parameters of winter wheat*. Ecol. Chem. Eng. A, 17: 771–779.

McGuire A.M., Bryant D.C., Denison R.F. 1998. *Wheat yields, nitrogen uptake, and soil moisture following winter legume cover crop vs. fallow.* Agron. J., 90: 404–410.

Oury F.X., Lasme P., Michelet C., Rousset M., Abecassis J., Lullien-Pellerin V. 2015. *Relationships between wheat grain physical characteristics studied through near-isogenic lines with distinct puroindoline-b allele.* Theor. Appl. Genet., 128: 913–929.

Pagani M.A., Marti A., Bottega G. 2014. *Wheat milling and flour quality evaluation,* pp. 19–53: in: *Bakery Products Science and Technology.* Eds. W. Zhou, Y.H. Hui, I. De Leyn, M.A. Pagani, C.M. Rosell, J.D. Selman, N. Therdthai. Second Edition. John Wiley and Sons, Ltd, Chichester, UK.

Ralcewicz M., Knapowski T., Kozera W., Barczak B. 2009. *Technological value of spring wheat of Zebra cultivar as related to the way of nitrogen and magnesium application.* J. Cent. Eur. Agric., 10(3): 223–232.

Ransom J., Simsek S., Schatz B., Eriksmoen E., Mehring G., Mutukwa I. 2016. *Effect of a post-anthesis foliar application of nitrogen on grain protein and milling and baking quality of spring wheat.* Am. J. Plant Sci., 7: 2505–2514.

Ruske R.E., Gooding M.J., Jones S.A. 2003. *The effects of adding picoxystrobin, azoxystrobin and nitrogen to a triazole programme on disease control, flag leaf senescence, yield and grain quality of winter wheat.* Crop Prot., 22: 975–987.

Schulz R., Makary T., Hubert S., Hartung K., Gruber S., Donath S., Döhler J., Weiss K., Ehrhart E., Claupein W., Piepho H.P., Pekrun C., Müller T. 2015. *Is it necessary to split nitrogen fertilization for winter wheat? On-farm research on Luvisols in South-West Germany.* J. Agric. Sci., 153: 575–587.

Stępień A., Wojtkowiak K. 2016. *The effect of foliar application of Cu, Zn and Mn on the yield and quality indicators of the grain of winter wheat.* Chil. J. Agr. Res., 76: 220–227.

Xue C., Rücker S., Koehler P., Obenauf U., Mühling K.H. 2016a. *Late nitrogen application increased protein concentration but not baking quality of wheat.* J. Plant Nutr. Soil Sci., 179: 591–601.

Xue C., Schulte auf'm Erley G., Rossmann A., Schuster R., Koehler P., Muehling K.H. 2016b. *Split nitrogen application improves wheat baking quality by influencing protein composition rather than concentration.* Front Plant Sci., 7: 738.

Zhang Y., Tang J., Yan J., Xia X., He Z. 2009. *The gluten protein and interactions between components determine mixograph properties in an F6 recombinant inbred line population in bread wheat.* J. Cereal Sci., 50: 219–226.

Zhang Y., Quail K., Mugford D.C., He Z.H. 2005. *Milling quality and white salt noodle color of Chinese winter wheat cultivars.* Cereal Chem., 82: 633–638.

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CARCASS LEAN CONTENT IN YOUNG HEN AND TOM TURKEYS WITH SIMILAR BODY WEIGHT SLAUGHTERED AT DIFFERENT AGES

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Key words: age, carcass, lean meat, gender, turkeys.

Abstract

The experimental materials comprised 600 BIG-6 turkeys (360 ♂ and 240 ♀). Females were raised to a maximum of 16 weeks of age and males to 22 weeks of age in accordance with universally accepted standards. All birds were weighed at 14-day intervals. Males were slaughtered at 16, 18, 20 and 22 weeks of age, and females at 12, 14 and 16 weeks of age; 24 males and 24 females were slaughtered each time. Chilled carcasses were divided into parts and dissected. Pairwise comparisons (correlated observations) were performed. Pairs of females and males with similar body weight, slaughtered at different ages, were formed. The results of this study indicate that the percentage content of breast muscles, leg muscles and total lean meat in the carcasses of heavy-type male and female turkeys is influenced by body weight at slaughter and carcass weight rather than by age.

Introduction

In poultry, the length of the rearing period is determined by species, type and gender. The rearing period is the shortest in broiler chickens and the longest in turkeys. BOCHNO et al. (1993) demonstrated that the optimize productivity WAMA-1 female turkeys should be reared for 12 up to 16 week and males for 16 up to the 24 week of age. At the lower age

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limit, birds reach relatively high body weight (♀ > 4.0 kg, ♂ > 7.5 kg), a low feed conversion ratio and a low content of fat with skin in the carcass. However, they are also characterized by low carcass dressing percentage and low carcass lean content.

In the past 50 years, the length of the rearing period has been considerably shortened in all species of intensively farmed poultry, in particular broiler chickens and turkeys (HAFEZ and HAUCK 2005, CASE et al. 2010). Therefore, the question arises whether birds slaughtered at an early age can reach their full genetic potential for growth in terms of body weight and the most valuable muscles (breast and leg muscles), and which is more important: slaughter weight or slaughter age (WAWRO and BRZOZOWSKI 1998, WAWRO et al. 1999). Age at slaughter influences not only the body weight of birds, but also carcass quality and proportions between edible and non-edible components in the carcass (MURAWSKA et al. 2011, MURAWSKA 2012, 2013a, 2013b).

The aim of this study was to determine carcass lean content in heavy-type turkeys with similar body weight (within sex groups), slaughtered at different ages.

Material and Methods

The experimental materials comprised 600 BIG-6 turkeys (360 ♂ and 240 ♀). Females were raised to a maximum of 16 weeks of age and males to 22 weeks of age in accordance with the recommendations of AVIAGEN TURKEYS (2007). The experiment was approved by the Local Ethics Committee (authorisation No. 54/2010). Day-old poults were marked, weighed and allocated to 16 pens (10 pens of males and 6 pens of females). The chemical composition of diets and feeding periods are presented in Table 1.

Table 1

Chemical composition of diets

Item		Diet						
		R 280*	R 281	R 282	R 283	R 284	R 285	R 286
		feeding period [weeks]						
		1–2	3–5	6–8	9–11	12–14	15–17	> 18
Dry matter	[g kg^{-1}]	911	908	900	903	903	899	901
Crude ash	[g kg^{-1}]	92.7	89.8	72.0	65.2	58.0	51.2	49.5
Total protein	[g kg^{-1}]	272	255	230	226	195	182	165
Crude fat	[g kg^{-1}]	48.5	53.9	58.0	68.9	85.2	84.2	85.0
Crude fiber	[g kg^{-1}]	27.5	32.0	33.0	34.8	41.0	35.0	35.0
Metabolizable energy	MJ kg^{-1}	11.6	11.4	12.2	12.2	12.2	12.8	12.9

* Commercial indication of compound feeds

All birds were weighed at 14-day intervals. Females were slaughtered at 12, 14 and 16 weeks of age, and males at 16, 18, 20 and 22 weeks of age; 24 males and 24 females were slaughtered each time. To ensure sample representativeness, turkeys were arranged in ascending order based on their body weights, and stratified random sampling was carried out.

Turkeys were slaughtered after 12-h fasting. Carcasses were plucked and eviscerated, and the heads, shanks and wingtips were cut off. Chilled carcasses (approx. 4°C, 24 h) were divided into portions (KLECZEK et al. 2006) and dissected. Breast muscles (superficial and deep) were removed from the breast portion by cutting along the breastbone crest, clavicle and coracoids, and the line connecting the breast muscles to the ribs. In this article, total lean weight comprises the weight of muscle tissue from all carcass cuts excluding intermuscular fat that was separated during dissection, and skin with subcutaneous fat.

The statistical analysis included the determination of arithmetic means ($\bar{x}$), coefficients of variation (cv%) and significant differences in the analyzed parameters between turkeys of the same sex representing different age groups (*t*-test).

Table 2

Body weight, carcass weight and the content of the analyzed muscles in turkey carcasses

Item	Statistics	Gender and age [weeks]						
		♀			♂			
		12	14	16	16	18	20	22
Weight of body [g]	$\bar{x}$	7099^a	8984^b	10649^c	14200^a	16387^b	19692^c	21753^d
	cv	5.15	6.65	7.83	5.94	7.56	5.48	5.98
Carcass	$\bar{x}$	5895^a	7053^b	8416^c	11327^a	13200^b	15949^c	17577^d
	cv	5.81	7.05	8.39	5.93	7.82	6.17	6.76
Breast muscles	$\bar{x}$	1476^a	1996^b	2384^c	3219^a	3720^b	4553^c	5289^d
	cv	7.88	9.92	11.1	8.12	10.7	8.50	9.97
Leg muscles	$\bar{x}$	1243^a	1572^b	1822^c	2563^a	3031^b	3610^c	3978^d
	cv	6.08	6.77	10.2	6.14	8.96	6.21	8.60
Total lean meat	$\bar{x}$	3731^a	4338^b	5748^c	7856^a	9203^b	11096^c	12448^d
	cv	6.16	7.16	9.09	5.86	8.38	6.20	7.06
Content in carcass [%]: Breast muscles	$\bar{x}$	27.3	28.3	28.3	28.4^a	28.1^a	28.5^a	30.1^b
	cv	4.83	4.43	4.91	6.59	4.92	6.10	5.82
Leg muscles	$\bar{x}$	23.1^b	22.3^{ab}	21.6^a	22.6	22.9	22.6	22.6
	cv	4.13	5.17	4.38	3.19	3.55	3.80	5.48
Total lean meat	$\bar{x}$	69.1	68.6	68.3	69.4^a	69.7^a	69.6^a	70.8^b
	cv	1.98	3.00	1.99	1.73	1.65	1.23	1.14

Mean values in groups of males and females followed by different letters are significantly different at $\alpha \leq 0.05$

The presence of significant differences in average body weight between turkeys from different age groups (Table 2), which significantly affect carcass lean content, necessitated the use of a statistical method that would hypothetically eliminate the effect of increasing body weight on muscle deposition in growing turkeys. Therefore, pairwise comparisons (correlated observations) were performed. Pairs of females and males with similar body weight, slaughtered at different ages (2-week difference), were formed (Figure 1). The number of pairs in the analyzed groups was as follows: females slaughtered at 12 and 14 weeks of age – 8 pairs, females slaughtered at 14 and 16 weeks of age – 10 pairs; males slaughtered at 16 and 18 weeks of age – 12 pairs, males slaughtered at 18 and 20 weeks of age – 8 pairs, males slaughtered at 20 and 22 weeks of age – 10 pairs. The significance of differences in the mean values of the analyzed traits between turkeys representing two successive age groups was determined by the t-test (Statistica 10.0).

Results

Average body weight ranged from 7099 g at 12 weeks of age to 10 649 g at 16 weeks of age in females, and it exceeded 21753 g at 22 weeks of age in males (Table 2).

The weight of breast muscles, leg muscles and total lean meat in the carcass increased significantly with age, and the highest values of these traits were noted in the oldest turkeys, i.e. 16-week-old females and 22-week-old males (Table 2). The differences in the mean values of the analyzed parameters across different age groups of males and females were significant.

Different trends were observed in the percentage content of muscles in the carcasses of turkeys slaughtered at different ages (Table 2). Breast muscle content was only around 1% lower in the youngest females than in their 2 and 4 weeks older counterparts (27.3% vs. approx. 28.3%). In males, breast muscle content was significantly higher (30.1%) in the oldest birds (22 weeks of age) than in those aged 16 and 20 weeks. Different trends were noted in leg muscle content, which was highest in the youngest females (23.1%) and lowest in the oldest ones (21.6%). Age had no significant effect on the proportion of leg muscles in males across age groups (above 22.6% on average).

Total lean weight expressed as a percentage of total carcass weight (Table 2) was determined by the high content of breast and leg muscles. In females, total lean weight was highest (69.1%) in the youngest birds

(12 weeks of age) due to high leg muscle content. In males, total lean weight was highest (70.8%) in the oldest birds (22 weeks of age) due to high breast muscle content.

Figures 1–6 present the effects of age and gender on carcass lean content in heavy-type turkeys after the impact of body weight had been hypothetically eliminated. Breast muscle weight was determined by age and gender to a greater extent than by body weight only in 12- and 14-week-old females (Figure 1). Older females were characterized by significantly higher breast muscle weight than younger females, despite similar body weight at slaughter. In the remaining cases, breast muscle weight was not higher in older turkeys than in 2 weeks younger ones in both males and females.

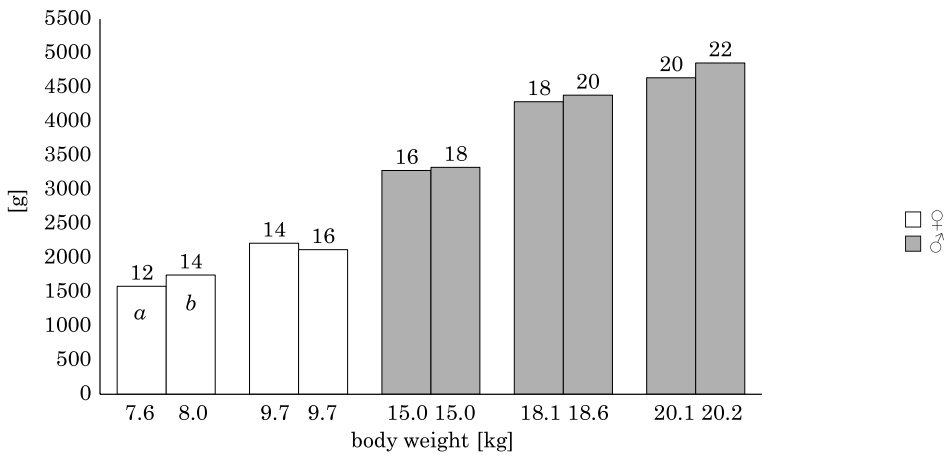

Fig. 1. Weight of breast muscles in turkeys (♂ and ♀) slaughtered at different ages. Birds' age [weeks] is shown inside the bars; *a*, *b* – significant differences at $\alpha \leq 0.05$

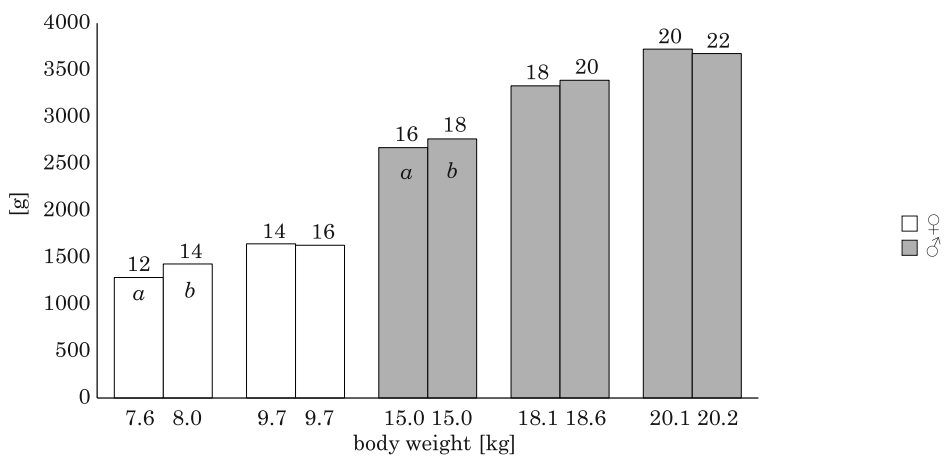

Fig. 2. Weight of leg muscles in turkeys (♂ and ♀) slaughtered at different ages. Birds' age [weeks] is shown inside the bars; *a*, *b* – significant differences at $\alpha \leq 0.05$

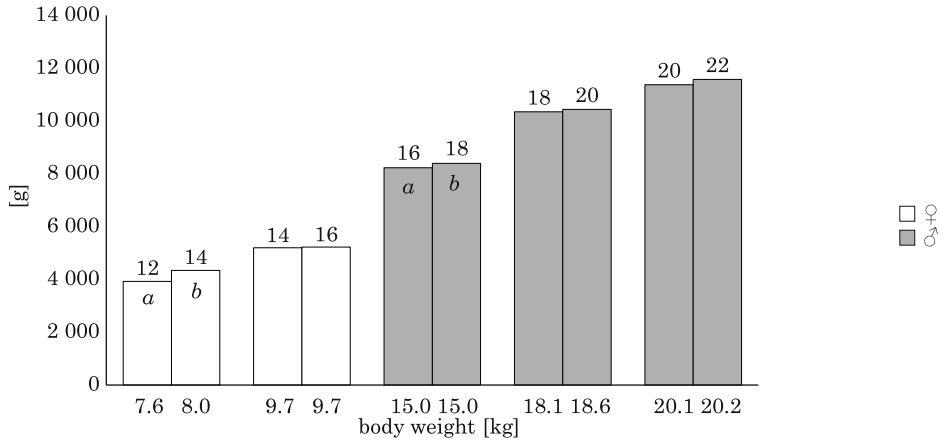

Fig. 3. Total lean weight in turkeys (♂ and ♀) slaughtered at different ages. Birds' age [weeks] is shown inside the bars; *a*, *b* – significant differences at $a \leq 0.05$

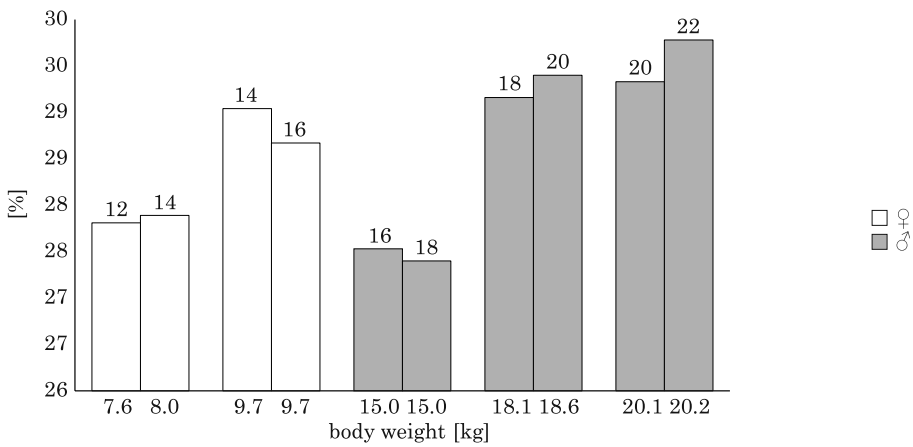

Fig. 4. Breast muscle weight expressed as a percentage [%] of total carcass weight in turkeys (♂ and ♀) slaughtered at different ages. Birds' age [weeks] is shown inside the bars

Age had a significant effect on leg muscle weight (Figure 2) and total lean weight (Figure 3) in female turkeys slaughtered at 12 and 14 weeks of age and in male turkeys slaughtered at 16 and 18 weeks of age. Two weeks older birds were characterized by significantly higher leg muscle weight and total lean weight than younger ones, despite similar body weight at slaughter.

The content of breast muscles (Figure 4), leg muscles (Figure 5) and total lean meat (Figure 6) expressed as a percentage of total carcass weight was similar in older and younger turkeys of both sexes. The obtained results indicate that the percentage content of the most valuable muscles (breast and leg muscles) and total lean meat in the carcasses of heavy-type turkeys is influenced by body weight at slaughter and carcass weight rather than by age.

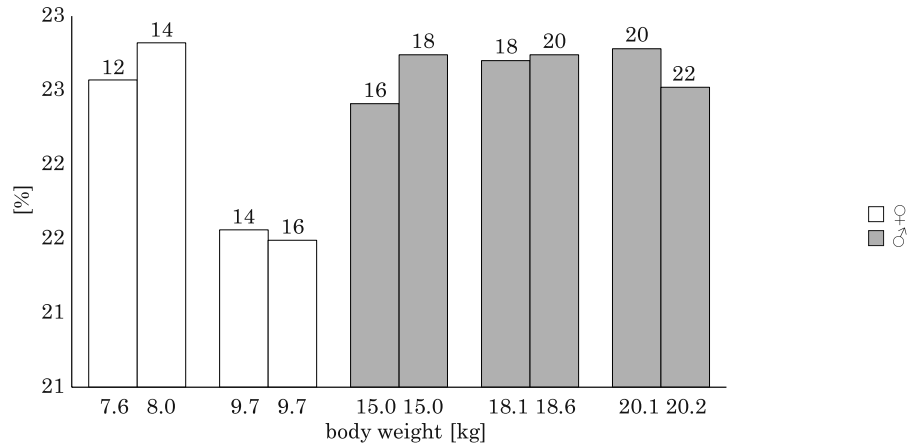

Fig. 5. Leg muscle weight expressed as a percentage [%] of total carcass weight in turkeys (♂ and ♀) slaughtered at different ages. Birds' age [weeks] is shown inside the bars

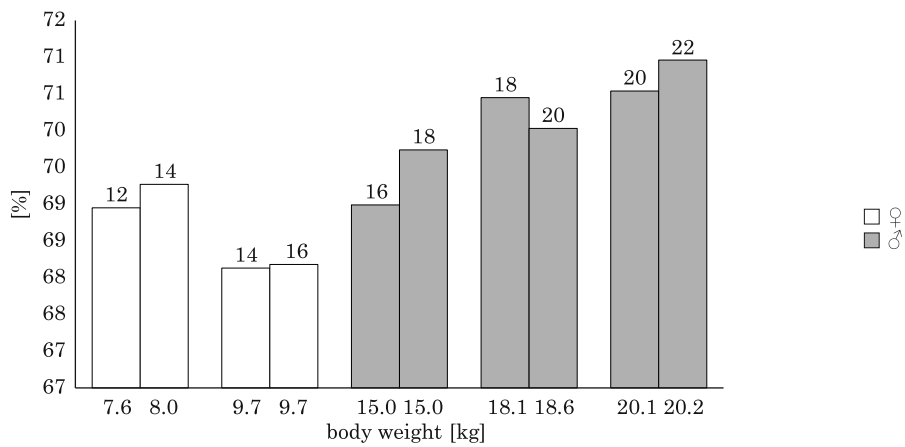

Fig. 6. Total lean weight expressed as a percentage [%] of total carcass weight in turkeys (♂ and ♀) slaughtered at different ages. Birds' age [weeks] is shown inside the bars

Discussion

Research results show that modern commercial turkeys bred for meat are characterized by a fast growth rate and high muscle deposition (HAVENSTEIN et al. 2007, TAHA and FARAON 2009, TAHA 2012). The results of our study, which investigated age-related changes in the body weight, breast muscle weight and leg muscle weight of turkeys, are consistent with the findings of other authors who also analyzed BIG-6 turkeys (MAKOWSKI et al. 2007, MIKULSKI et al. 2008, DAMAZIAK et al. 2012, MURAWSKA 2013a). The differences in the content of breast and leg muscles in the carcasses

of male and female turkeys slaughtered at different ages, noted in our study, partially corroborate the findings of other authors (WILKIEWICZ-WAWRO et al. 2004, MURAWSKA and BOCHNO 2008, MURAWSKA 2013a) who demonstrated that breast muscle weight increased and leg muscle weight decreased with age in different poultry species, in both males and females.

Previous research revealed that both body weight and muscle weight increased significantly in growing turkeys, and the proportions of different tissue components changed with age (BOCHNO et al. 1993, MURAWSKA 2013a). An important consideration is whether carcass lean content in turkeys is affected to a greater extent by slaughter weight or slaughter age. Earlier experiments involving Muscovy ducks (ROMBOLI and AVANZI 1997), Pekin ducks (WAWRO and BRZOZOWSKI 1998) and chickens (WAWRO et al. 1999) show that carcass lean content in young birds is determined primarily by age and gender, followed by body weight. The efficiency of poultry meat production is largely determined by the slaughter value of birds and meat yield. Correlations between the age and body weights of birds vs. the yields of most valuable muscles were described by other authors who stressed that birds raised for meat should be slaughtered at a specified, optimum age (WOOD 2009, CASE et al. 2010, MURAWSKA et al. 2015).

The results of this study indicate that the percentage content of breast muscles, leg muscles and total lean meat in the carcasses of heavy-type male and female turkeys is influenced by body weight at slaughter and carcass weight rather than by age. The research results also indicate that due to the slaughter value traits, the turkey body mass should be taken into account when optimizing the term of slaughter.

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References

AVIAGEN TURKEYS. 2007. *BUT Big 6 Commercial Performance Goals*.

BOCHNO R., LEWCZUK L., WAWRO K. 1993. *Attempt to determine the optimal slaughter age in WAMA-1 turkeys*. Zesz. Nauk. Prz. Hod., 8: 315–321.

BOCHNO R., MURAWSKA D., BRZOSTOWSKA U. 2006. *Age – related changes in the distribution of lean, fat with skin and bones in goose carcasses*. Poultry Sci., 85: 1987–1991.

CASE L.A., MILLER S.P., WOOD B.J. 2010. *Determination of the optimum slaughter weight to maximize gross profit in a turkey production system*. Can. J. Anim. Sci., 90: 349–356.

DAMAZIAK K., MICHALCZUK M., KUREK A. 2012. *Comparison of production performance of two genetic groups of turkeys reared in the semi-intensive system*. J. Cent. Eur. Agr., 13: 403–415.

HAFEZ H.M., HAUCK R. 2005. *Genetic selection in turkeys and broilers and their impact on health conditions*. Proceedings – World Poultry Science Association, 4th European Poultry Genetics Symposium, Dubrownik, Croatia, WPSA, Croatia Branch.

HAVENSTEIN G.B., FERKET P.R., GRIMES J.L., QURESHI M.A., NESTOR K.E. 2007. *Comparison of the performance of 1966-versus 2003-type turkeys when fed representative 1966 and 2003 turkey diets: growth rate, livability, and feed conversion*. Poultry Sci., 86: 232–240.

KLECZEK K., WAWRO K. WILKIEWICZ-WAWRO E., MAKOWSKI W. 2006. *Multiple regression equations to estimate the content of breast muscles, meat and fat in Muscovy ducks*. Poultry Sci., 85: 1318–1326.

MAKOWSKI W., WILKIEWICZ-WAWRO E., WAWRO K., KLECZEK K., SZEREMETA J. 2007. *Production results and slaughter quality of turkeys raised on different types of litter*. Proceedings – 14th Symposium, Česke Budějovice, Czech Republic, pp. 193–195.

MIKULSKI D., KOZŁOWSKI K., JANKOWSKI J., BLOK J., SOBOLEWSKI Z. 2008. *Efficacy of yeast extract Saccharomyces cerevisiae in turkey feeding*. Med. Weter., 64: 1331–1335.

MURAWSKA D. 2012. *The effect of age on the growth rate of tissues and organs and the percentage content of edible and non-edible carcass components in Pekin ducks*. Poultry Sci., 91: 2030–2038.

MURAWSKA D. 2013a. *Age – related changes in the percentage content of edible and non-edible components in turkeys*. Poultry Sci., 92: 255–264.

MURAWSKA D. 2013b. *The effect of age on the growth rate of tissues and the percentage content of edible and inedible components in Koluda White Geese*. Poultry Sci., 92: 1400–1407.

MURAWSKA D., BOCHNO R. 2008. *Age – related changes in the percentage content of tissue components in geese*. J. Cent. Eur. Agr., 9: 211–216.

MURAWSKA D., KLECZEK K., WAWRO K., MICHALIK D. 2011. *Age – related changes in the percentage content of edible and non-edible components in broiler chickens*. Asian-Aust. Anim. Sci., 24: 532–539.

MURAWSKA D., KOZŁOWSKI K., TOMASZEWSKA K., BRZOZOWSKI W., ZAWACKA M., MICHALIK D. 2015. *Age-related changes in the tissue composition of carcass parts and in the distribution of lean meat, fat with skin and bones in turkey carcasses*. Eur. Poultry Sci., 79, doi: 10.1399/eps.2015.103.

ROMBOLI J., AVANZI C.F. 1997. *Some data of differential growth of Muscovy duck tissues. Proceedings – International Conference Breeding and Geese Production*. Instytut Zootechniki, Kraków, Poland, pp. 228–236.

TAHA N.T. 2012. *Study the effect of local vs. imported heavy and light turkey strains on muscles and bones conformation of the drum-sticks*. Int. J. Poult. Sci., 11(60): 405–407.

TAHA N.T., FARRAN M.T. 2009. *Comparative study of thigh muscles and bones conformation and some carcass traits of local vs. imported turkey strain*. Int. J. Poultry Sci., 8: 368–372.

WAWRO K., BOCHNO R., BRZOZOWSKI W. 1999. *Carcass lean content in broiler chickens with similar body weight slaughtered at different ages*. Pr. Mater. Zoot., 54: 85–96.

WAWRO K., BRZOZOWSKI W. 1998. *The effect of age and sex on the muscle deposition in ducks*. Anim. Sci. Pap. Rep., 16: 113–122.

WILKIEWICZ-Wawro E., SZYPULEWSKA K., WAWRO K. 2004. *Age – related changes in tissue components distribution in Muscovy duck carcasses*. Arch. Geflügelk., 69: 128–134.

WOOD B. J. 2009. *Calculating economic values for turkeys using a deterministic production model*. Can. J. Anim. Sci., 89: 201–213.

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USING BEHAVIOURAL OBSERVATIONS TO ASSESS THE WELFARE OF RED-NECKED POND TURTLES (*MAUREMYS NIGRICANS*) KEPT IN A ZOO

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Key words: behaviour, animal welfare, captive turtles, Mauremys nigricans.

Abstract

The aim of this study was to use behavioural observations to assess the welfare of the red-necked pond turtles (*Mauremys nigricans*) kept in a zoo. In 2000, red-necked pond turtles were put on the critically endangered list. Today, the species appears to be extinct in the wild. The welfare of captive populations of the species will have an important impact on their survival. Due to unusual aspects of reptile biology and a lack of monitoring standards, the main criteria available for welfare assessment for these animals may be behavioural. Based on the results of this study, it can be inferred that the welfare of the observed turtles has been moderately well-preserved; however, the artificial conditions created by humans are not able to fully satisfy the behavioural needs of the studied animals.

Introduction

Welfare studies are usually restricted to "higher" vertebrates (mammals, birds) and are primarily referred to in the context of farm animals (pigs, cattle, poultry) and companion animals (dogs, cats, rodents) (KALETA 2013). Over the years, there has been a lot of concern regarding the welfare of cattle or pigs, but "lower" vertebrates are increasingly popular today (WABNITZ et al. 2003). Reptiles, amphibians and fishes have become very common among zoological gardens, private breeders and industrial farms, in which they are raised for hides and meat (KALETA 2013). Assess-

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ment of the welfare of fishes, amphibians and reptiles can be challenging compared with that of mammals and birds, due to relatively limited availability of guidance criteria, although some multi-taxa scientific sources are now available (WARWICK et al. 2018). The unique biology of reptiles and the existence of specific adaptive features make the task even harder. However, it is certain that both reptiles and other "lower" vertebrates are capable of experiencing stress, which manifests as unusual, altered behaviour (MOBERG 1985). Contrary to popular belief, reptiles exhibit a number of abnormal behaviours that are the result of excessive stress and are either atypical in nature and/or occur out of the context under stress conditions (WARWICK et al. 2013). Therefore, behavioural criteria should be the primary foci of research assessing the welfare of these animals. Ensuring adequate animal welfare and reducing the stress that animals experience should be the aim of every private person or institution that maintains living animals (BAYS et al. 2006).

Mauremys nigricans is a medium-sized turtle from the Geoemydidae family. These turtles are characterized by daily activity and strong sexual dimorphism. Females are much larger than males and can grow to 30 cm in length, while males do not exceed 20cm and have longer tails. The colour of the carapace of these turtles can vary from chestnut to black, and the tympanum region is decorated with several irregular, narrow, creamy or yellow strips stretching across the neck. The pharynx is covered in dark brown to black stripes interrupted by red streaks in males and cream streaks in females. The exposed parts of the forearms and hind legs may be characterized by creamy pigment, although in males these spots may be red (ANDERS and IVERSON 2012). The name of red-necked pond turtle was first used by IVERSON (1985) and is still valid today. Breeders also call the species the Kwantung River turtle.

The general range of this species is defined by the Pearl River in Guangdong and Guangxi Provinces in the People's Republic of China (IVERSON 1992). Current knowledge about the natural distribution of the red-necked pond turtle is derived from the reports of Mell (1922, 1929), who claimed that it was an upland species inhabiting mountain streams. The water temperature of the described streams was 16–17ºC in August. MELL (1922) also reported a decline in activity of the species from November to December and a rebound in activity in the spring. The above information were obtained through observations of captive specimens that were kept in external enclosures at latitudes consistent with the natural range of these turtles (ANDERS and IVERSON 2012). The natural diet of *M. nigricans* is not known, but the construction of the head and jaw suggests that it consists mainly of molluscs and crustaceans. Reproductive

behaviour has been observed only in captivity, but MELL (1929) reports that females of this species lay two eggs per year. The mating season begins in spring, when the males become intensely red in colour. According to ARTNER (2009) the female accepts the male by retracting her forearm and sliding the hind limbs so that the back of the shell and tail are raised. Some copulations can take aggressive character, evidenced by the male biting the neck of the female.

Mauremys nigricans is a species that is desirable by collectors around the world, and this combined with its very limited range has led to a significant decline in the species's population. Poachers trapping these turtles for the needs of Chinese folk medicine have contributed to the heavy decline in the number of *M. nigricans*. GAILLARD et al. (2017) reported that 500 grams of the red-necked pond turtle in 2015 reached a price of 80 000 dollars for males and 50 000–60 000 dollars for females. Another factor responsible for the population decline is the degradation of the turtle's natural habitat, in particular via deforestation, construction of hydroelectric power plants and stream liming. In 2007–2009, attempts to find *M. nigricans* in the provinces of Guangdong and Guangxi failed, suggesting that the species may have disappeared entirely from its natural habitat; no wild populations of these turtles are known today (ANDERS and IVERSON 2012).

Mauremys nigricans was listed on the Red List of Endangered Species in 2000 (IUCN, 2017). In 2011, this turtle was included on the IUCN's list of the 40 most endangered freshwater turtles (*Turtles in trouble*... 2011). Field trials and studies conducted on captive specimens may be useful for *M. nigricans* protection. It is also necessary to educate human societies, especially local ones, about *M. nigricans* conservation. The welfare of the individual turtles of this species that are kept in captivity must also be safeguarded. General welfare principles state that captive animals should be provided with five freedoms: freedom from hunger and thirst, freedom from discomfort, freedom from pain, injuries and diseases, freedom from fear and stress, and the ability to express normal behaviour (FAWC 1992). Unfortunately, in the case of reptiles it is believed that it can be very difficult to evaluate and ensure the conditions conducive to these five freedoms, particularly with regard to behaviour (KALETA 2013, WARWICK et al. 2013). The physiology and morphology, life history, and diversity of living environments of these animals makes it extremely difficult to identify consistent behavioural patterns for them (KALETA 2013). In addition, captive reptiles are usually restricted to small and often poorly-constructed enclosures, leading to physical and behavioural problems. Examples of behavioural disorders are given in Table 1.

Table 1

Abnormal behaviours of turtles kept in captivity (MORGAN and TROMBORG 2007, WARWICK et al. 2013, KALETA 2013, ARENA et al. 2014, SILVESTRE 2014)

Behaviour	Possible cause
ITB (interaction with transparent boundaries)	an inability to detect invisible barriers, stemming from adaptive limitations of these animals
Hyperactivity	excessive density of animals and poorly designed premises, stress, fear, lack of shelter, hunger
Hypoactivity	cold temperatures in the enclosures where animals are kept, infections, injuries, harassment by other occupants
Hyper-alertness	fear
Head-hiding	fear, excessive lighting
Co-occupant aggression	hunger, overcrowding of animals, lack of ability to escape in poorly designed enclosures
Human-directed aggression	fear
Freezing	fear
Grating of jaw	pain, fear, poorly designed enclosures, nutrient deficiencies
Prolonged retractions of head, limbs or tail	fear, pain, illness
Cloacal evacuations when handled	fear
Pseudovocalisation	fear, pain, illnesses
Atypical location of animals	diseases, injuries, discomfort, co-occupant aggression, hyperthermia, hypothermia
Accelerated body movements	stress, fear
Boundary exploration	excessive animal density, lack of shelter
Accumulation of individuals at the surface	excessive animal density, poorly designed enclosures
Anxiety behaviour	stress, fear, co-occupant aggression, pain
Cannibalism	excessive animal density, hunger, poorly designed enclosures

The aim of the present study was to assess the welfare of captive *M. nigricans* specimens based on observations of their behaviour. Observations consisted of documenting the turtles' time budget and defining individual behaviours. Observational data were used to establish hierarchy in the observed animal group. Observations also focused on the occurrence of abnormal behaviour, which may be caused by stress or disturbance, and additionally aimed to determine the nutritional preferences of the observed animals.

Materials and Methods

This study was conducted at the Wroclaw Zoo (Poland) in September and October of 2017. The focal group of turtles consisted of five individuals aged four years, whose gender has not been determined. The turtles averaged 10 cm in length. The animals were kept in an aquaterrarium with separate water and land portions; the "land" area consisted of numerous stones and roots placed in the water. The average water temperature in the aquaterrarium was 21°C and the average land temperature was 24°C. In the autumn and winter, the entire terrarium building must be heated, with the result that the temperature in the individual animal enclosures also increases. The turtle aquaterrarium was designed to provide numerous hiding places for the animals. The aquaterrarium had glass walls and overall dimensions of 120/50/50 cm. The rear wall was covered with mortar in imitation of rocks. A photograph of a turtle aquaterrarium is included in the supplementary materials.

The turtles were fed on Tuesdays, Thursdays and Fridays. Their diet consisted of dead suckling mice, fish, shrimp, earthworms and plants (chicory, plantain, dandelion). Food was dropped into the water. On Tuesdays the turtles were fed in the afternoon but on Thursdays and Fridays in the morning.

Observations were carried out during daylight hours for three hours per day over 20 days. Thus, the total time devoted to turtle observation was 60 hours. Observations consisted of documenting the turtles' time budget and identifying individual behaviours. Data included the quantity of time spent in water and on land, feeding, in quiescent rest (eyes closed, relaxed limbs), basking, and in aggressive behaviour. Chasing other specimens, biting, and fighting for food were classified as aggressive behaviours. Observations also focused on the occurrence of behaviours commonly regarded as abnormal, such as: interaction with transparent boundaries, hyperactivity, hypoactivity, hyper-alertness, freezing, grating of jaw, prolonged retraction of head and pseudovocalisation. Observations additionally aimed to determine the dietary preferences of the observed animals.

Behavioural observations assessing the time budget devoted to individual behaviours were analysed using *Statistica* ver. 13.1. A nonparametric Spearman's rank order correlation analysis was performed.

Evidence of abnormal behavioural data was examined to determine in what situations and with what intensity the behaviour manifested.

Animal nutritional preferences were determined on the basis of percentage of total food intake. The turtles were fed on a different type of food on each feeding day, and it was determined which type of food was taken most frequently.

Results and Discussion

The turtle's diet was varied, and the contribution of various elements to the diet is presented in Table 2.

Table 2

Percentage of individual components out of the total diet of red-necked pond turtles (*Mauremys nigricans*)

Component	Percentage [%]
Suckling mice	40
Fish	20
Shrimp	15
Earthworms	10
Plants (chicory, plantain, dandelion in equal proportions)	15

The most commonly consumed food was suckling mice, shrimp and earthworms, which were always completely eaten. When fish was offered, 75% of it was eaten. Greatest feeding interest occurred in the early stages of each feeding; after a few minutes, the turtles stopped hunting. The turtles were reluctant to eat the plants that were offered, consuming only 10–15% of the total plant feed available.

Table 3 presents the correlation coefficient and time budget devoted to each individual behaviours.

Table 3

Time budget and correlation coefficients for individual behaviours

Specification	Mean ± SD	Time spent in water	Time spent on land	Time spent in quiescent rest	Time spent on basking	Time spent on feeding	Time spent in aggressive behaviour
Time spent in water	138 min 15 s ± 7.86	1	-1*	0.43	-0.97*	0.07	0.13
Time spent on land	41 min 45 s ± 7.86	-1*	1	-0.43	0.97*	-0.07	-0.13
Time spent in quiescent rest	58 min 50 s ± 3.85	0.43	-0.43	1	-0.45	0.41	0.02
Time spent on basking	35 min 30 s ± 6.68	-0.97*	0.97*	-0.45	1	-0.17	-0.15
Time spent on feeding	10 min 40 s ± 1.61	0.07	-0.07	0.41	-0.17	1	0.7*
Time spent in aggressive behaviour	1 min 31 s ± 0.45	0.13	-0.13	0.02	-0.15	0.7*	1

*significant differences at $P < 0.05$

The observed turtles spent 76.75% of their time in the water and 23.25% of their time on land. Time spent feeding was positively correlated with the time spent in water. This was to be expected, due to the fact that many species of turtles are unable to swallow food while on land. Time of day did not affect food intake; turtles consumed food willingly in the morning and afternoon. The test animals always fed together, since individual animals were not separated during feeding time.

Rest was quantified as the combination of quiescent rest and basking. The animals rested both as a group and individually. Time spent in quiescent rest was positively correlated with the time spent in water and negatively with time spent on land; this may be due to the fact that turtles resting this way also tried to hide from the light, which was impossible on land. Turtles often spent quiescent rest in hiding places that also occur in the wild. Time spent basking was positively correlated with time spent on land and negatively with time spent in water, since the temperature on land was higher than the water temperature. Quiescent rest was usually observed in the mornings, and after some time the turtles went ashore to bask and then returned underwater again. Additionally, the animals basked after feeding. Time devoted to aggressive behaviours was positively correlated with time spent in water and time spent feeding. Turtles exhibited aggression against each other only at feeding times. This aggression was demonstrated to other individuals as a consequence of turtles competing for food. No aggressive behaviour was observed in other situations. Turtles investigated the environment regardless of the time of day, and in the vast majority of cases they did so individually. Investigation of the environment usually consisted of calmly swimming to the gaps between the rocks and examining them. Sometimes turtles tried to swim against the glass or attempted to climb walls. These were symptoms of interaction with transparent boundaries. No hierarchy structure was observed within the group. None of the turtles initiated group activities such as foraging or basking together.

SCHOFIELD et al. (2006) created a simplified ethogram for loggerhead sea turtles (*Caretta caretta*). They divided the investigated behaviours of the turtles into individual and group behaviours. According to that study, among individual behaviours one can distinguish: locomotive behaviour (surface rest, resting at the bottom, swimming vertically, horizontally and near the surface, patrolling), digestive behaviour (searching and eating) and comfortable behaviour (self-care) (SCHOFIELD et al. 2006). Group behaviours, on the other hand, include agonistic behaviours (male conflict, conflict between females) and reproductive behaviours (aesthesia, copulation, copulation with the assistance of another male) (SCHOFIELD et al. 2006).

The observations of the Schofield et al. (2006) study differed slightly from those carried out in the course of this study. The turtles observed in the Wroclaw Zoo exhibited specific individual and group behaviours. Exploration of the environment took place in an individual manner, as was found by SCHOFIELD et al. (2006). Consumption of food among the animals observed in the Wroclaw Zoo also took place in a group, probably as a result of certain feeding times. The turtles could not search for and take food at any time, so they took the opportunity to take food when it was distributed. Self-care behaviours were not observed in this study. Conflicts between males and females in this study were impossible to identify because the sex of the turtles was not determined. There were no conflicts between turtles observed in this study other than those observed during feeding. However, these behaviours were the result of natural co-occupation rivalry. Although they are a natural phenomenon, these behaviours can result in undesirable injuries and infections if left unmanaged. No reproductive behaviour was observed in this study, because of the young age of turtles.

Observations carried out in the Wroclaw Zoo revealed several abnormalities in turtle behaviour. One of these was evidence of interaction with transparent boundaries, resulting from the fact that the walls of the turtle enclosure were made of glass. The turtles did not see transparent barriers and therefore pressed against them, trying to get out of the aquaterrarium. Theoretically, the turtles could also see their reflection in the glass. In this case, pressing on the glass would not be an effect of the interaction with transparent boundaries, but rather an attempt to interact with the turtle's own reflection. However, it is difficult to determine whether this was occurring. It is also abnormal that there was no hierarchy in the group examined in this study. Under normal conditions, animals living in a group usually develop a hierarchical structure. BOICE et al. (1974) stated that hierarchies are established after natural cycles such as hibernation. However, no such natural cycles occurred for this observed group, and this could be responsible for the lack of hierarchy. The resting patterns of the animals in this group are also noteworthy. According to SCHOFIELD et al. (2006), resting should be an individual behaviour. Therefore, resting in a group may be a form of previously unspecified disorder resulting from a limited living space. On the other hand, it may also be that for this species group resting is normal behaviour resulting from an increased tolerance of these turtles towards their co-occupants. Other observed behaviours included turtles hiding their head, limbs and tails in the shell, excreting urine or faeces and hesitating during food catching, but these behaviours are typical in the face of danger, so they were not considered to be abnormal.

Normal behaviour of reptiles associated with a sufficient level of welfare includes environmental exploration, species-specific behaviour (gearing, thermoregulatory behaviour) and behavioural diversity (IZZO et al. 2011, but see BASHAW et al. 2016). According to WARWICK et al. (2013), normal behaviours include quiet environmental exploration, subtle changes in body posture and orientation, calm food intake and quiet breathing.

The turtles examined in this study demonstrated behaviours consistent with these standards. The only exception was the lack of calm feeding, which was probably tied to group maintenance and specific feeding times.

The aim of this study was to determine the welfare of the focal turtles on the basis of observations of their behaviour. It was shown that the observed turtles were characterized by a number of normal behaviours. However, there were also a few behaviours that indicated a reduced level of animal welfare. These behaviours may have resulted from the moderate stress associated with the large number of people visiting the zoological garden in Wroclaw, in combination with the adaptive limitations of the examined animals, as well as a limited and controlled living space. Based on the results obtained here, it can be inferred that the welfare of *M. nigricans* has been moderately well-preserved, but that the artificial conditions created by humans are not able to fully satisfy the behavioural needs of the studied animals.

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References

ANDERS B., IVERSON J.B. 2012. *Mauremys nigricans (Gray 1834) – Red-necked Pond Turtle, Chinese Red-necked Turtle, Kwangtung River Turtle, Black-necked Pond Turtle*. In: *Conservation biology of freshwater turtles and tortoises. A compilation project of the IUCN/SSC Tortoise and Freshwater Turtle Specialist Group*. Eds. A.G.J. Rhodin, P.C.H. Pritchard, P.P. van Dijk, R.A. Saumure, K.A. Buhlmann, J.B. Iverson, R.A. Mittermeier. Chelonian Research Monographs No. 5: 1–9.

ARENA P.C., WARWICK C., STEEDMAN C. 2014. *Welfare and environmental implications of farmed sea turtles*. Journal of Agricultural and Environmental Ethics, 27(2): 309–330.

ARTNER H. 2009. *Successful breeding of the Chinese red-necked pond turtle* Mauremys [Chinemys] nigricans (Gray, 1834). Emys, 16(2): 4–22.

BASHAW M.J, GIBSON M.D., SCHOWE D.M., KUCHER A.S. 2016. *Does enrichment improve reptile welfare? Leopard geckos (*Eublepharis macularis*) respond to five types of environmental enrichment*. Applied Animal Behaviour Science, 184: 150–160.

BAYS T., LIGHTFOOT T., MAYER J. 2006. *Exotic pet behavior, birds, reptiles and small mammals*. Saunders-Elsevier, St. Louis.

BOICE R., QUANTY C.B., WILLIAMS R. C. 1974. *Competition and possible dominance in turtles, toads and frogs*. Journal of Comparative and Physiological Psychology, 86(6): 1116–1131.

Farm Animal Welfare Council (FAWC), 1992. *FAWC updates the five freedoms*. Veterinary Record, pp. 131–357.

FUSTER J. F., PAGES T., PALACIOS L. 1997. *Effect of temperature on oxygen stores during aerobic diving in the freshwater turtle,* Mauremys caspica leprosa. Physiol. Zool., 70: 7–18.

GAILLARD D., LIU L., HAITAO S., SHUJIN L. 2017. *Turtle soup: local usage and demand for wild caught turtles in Qiongzhong County, Hainan Island*. Herpetological Conservation and Biology, 12(1): 33–41.

IUCN. 2017. *IUCN Red List of Threatened Species*. Version 2017.2, www.iucnredlist.org.

IVERSON J.B. 1985. *Checklist of the Turtles of the World with English Common Names*. Society for the Study of Amphibians and Reptiles, Herpetological Circulars, 14: 1–14.

IVERSON J.B. 1992. *A revised checklist with distribution maps of the turtles of the world*. Richmond, IN: Privately published, p. 363.

KALETA T. 2013. *Zachowanie się niższych kręgowców trzymanych przez człowieka jako wskaźnik ich dobrostanu*. Życie Weterynaryjne, 88(10): 860–866.

MELL R. 1922. *Beiträge zur Fauna sinica.* I. *Die Vertebraten Südchinas; Feldlisten und Feldnoten der Säuger, Vögel, Reptilien, Batrachier*. Archiv für Naturgeschichte, 88: 1–134.

MELL R. 1929. *Beiträge zur Fauna sinica.* IV. *Grundzüge einer Ökologie der chinesischen Reptilien und einer herpetologischen Tiergeographe Chinas*. Berlin, p. 282.

MOBERG G. 1985. *Animal stress*. American Physiological Society, Bethesda.

MORGAN K.N., TROMBORG C.T. 2007. *Sources of stress in captivity*. Applied Animal Behaviour Science. Conservation, Enrichment and Animal Behaviour, 102: 262–302.

SCHOFIELD G., KATSELIDIS K.A., DIMOPOULOS P., PANTIS J.D., HAYS G.C. 2006. *Behaviour analysis of the loggerhead sea turtle* Caretta caretta *from direct in-water observation*. Endangered Species Research, 2: 71–79.

SILVESTRE A.M. 2014. *How to assess stress in reptiles*. Journal of Exotic Pet Medicine, 23(3): 240–243.

Turtles in trouble. The world's 25+ most endangered tortoises and freshwater turtles – 2011. 2011. Turtle Conservation Coalition. Eds. A.G.J. Rhodin, A.D. Walde, B.D. Horne, P.P. van Dijk, T. Blanck, R. Hudson, MA: IUCN/SSC Tortoise and Freshwater Turtle Specialist Group, Turtle Conservation Fund, Turtle Survival Alliance, Turtle Conservancy, Chelonian Research Foundation, Conservation International, Wildlife Conservation Society, and San Diego Zoo Global, Lunenburg, p. 54.

WABNITZ C., TAYLOR M., GREEN E., RAZAK T. 2003. *From ocean to aquarium. The global trade in marine ornamental species*. UNEP.

WARWICK C., ARENA P., LINDLEY S., JESSOP M., STEEDMAN C. 2013. *Assessing reptile welfare using behavioural criteria*. In Practice, 35(3): 123–131.

WARWICK C., JESSOP M., ARENA P., PILNY A., STEEDMAN C. 2018. *Guidelines for inspection of companion and commercial animal establishments*. Front. Vet. Sci., 5: 151.

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INTENSIFICATION OF BIOGAS PRODUCTION IN THE PROCESS OF CO-FERMENTATION OF SILAGES FROM PERENNIAL GRASSES BLENDED WITH MAIZE OR WASTE FROM THE AGRO-FOOD INDUSTRY

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Key words: apple pomace, beet pulp, miscanthus, *Spartina,* big bluestem, switchgrass.

Abstract

The aim of this study was to determine the possibility of increasing the efficiency of biogas production from perennial grasses by their co-fermentation with maize or waste from the agro-food industry. Biomass of miscanthus, *Spartina,* switchgrass, and big bluestem was harvested on October (second harvest, autumn regrowth) and ensiled. Silages were made also from sugar beet pulp and particular grasses mixed with maize or apple pomace in the weight ratio of 50:50. The silages produced were of good quality. The methane fermentation of silages from grasses blended with maize or particular waste enabled achieving from a few to several dozen percent higher biogas production compared to the mono-fermentation of grass silages. It was concluded that co-digestion of perennial grass silages with apple pomace or beet pulp is an useful method for post-production waste utilization. Moreover, using perennial grasses for biogas production as blends with maize affords an opportunity for the partial replacement of maize as the main substrate with no loss of biogas and methane yield.

Introduction

Agricultural biogas is produced as a result of methane fermentation of raw materials of plant or animal origin, derived mainly from the agro-food industry. Most of the crops with a high biogas potential constitute a group

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of the so-called strategic raw materials and their wider use for energy production may affect the balance of the food production chain (GOŁASZEWSKI 2011, RAMA et al. 2013). Therefore, perennial plants that are not foodstuffs nor feedstuffs, are becoming increasingly important in renewable energy production. The reason behind that is that maize silage, which is nowadays used as a basic substrate for biogas production, is going to be replaced by cheaper and easily accessible organic waste from the agro-food industry. An increasing role of agro-food waste in renewable energy production is due to the fact that using this kind of substrate for biogas production is also a means of waste management and that it fits into the circular economy trend that is currently in force in the EU.

The efficiency of biogas production in a given installation may be increased through various solutions, like e.g.: using a specially designed system for fermentation bulk mixing (YADVIKA et al. 2004), controlling the concentration of ammonia throughout the process (NIELSEN and ANGELIDAKI 2008), adding micronutrients to increase the activity of microflora in the fermentation tanks (DEMIRCI and DEMIRER 2004) or to manipulate basic process parameters such as temperature, retention time, and degree of substrate disintegration (YADVIKA et al. 2004). New methane fermentation systems are being developed, the aim of which is to maximize biogas production through the use of modern solutions in the construction of fermentation tanks (NDEGWA et al. 2008, KAPARAJU et al. 2009).

Another way to increase the efficiency of biogas production is to ferment properly composed blends of different substrates, i.e. using the co-fermentation process (GELEGENIS et al. 2007). Co-fermentation offers multiple advantages, like e.g. enrichment of the fermentation bulk with additional nutrients, improvement of carbon to nitrogen ratio (C/N), and attenuation of the negative impact of inhibitors (DUBROWSKIS et al. 2012). As a result, an increase in daily biogas production can be obtained from the unit volume of the reactor and an increase in methane content in biogas (SOSNOWSKI et al. 2003). Optimization of batch parameters (e.g. dry matter content) and an increase of the amount of bioavailable carbon, makes co-fermentation superior over mono-fermentation, as it enables the processing of a wider range of substrates which cannot be used as monosubstrates, e.g. due to their low biogas potential, tendency to undergo biochemical conversion in a different direction than methane fermentation, or the presence of fermentation inhibitors (MYCZKO et al. 2011).

The increase of methane content in biogas and daily biogas production was observed by KACPRZAK et al. (2009) who in their study co-fermented two and three different substrates. As a result of co-fermentation of silage from maize and wheat grain, they produced 33 dm^3 of biogas/day. The

addition of glycerin to these substrates enabled a daily increase in biogas production to 55 dm^3. The use of grass silages in the form of co-substrates in a mixture with slurry is also a very good way to utilize animal feces, which due to their low methanogenic potential cannot be used as mono-substrates in biogas installations (FUGOL and SZLACHTA 2010). MURPHY et al. (2013) agrees with XIE et al. (2011), that the addition of slurry to grass silage stabilizes the pH value in the digester, counteracts the inhibiting effect of ammonia, increases the content of micronutrients, and optimizes the C/N ratio of fermentable bulk.

This study aimed to determine the possibility of increasing the efficiency of biogas production from perennial grasses through their co-fermentation with maize or waste from the agro-food industry.

Materials and Methods

Materials

The following perennial grasses were used in the study: miscanthus (*Miscanthus sinensis x giganteus* JM Greef & M. Deuter), *Spartina* (*Spartina pectinata* Bosc ex Link), switchgrass (*Panicum virgatum* L., variety Dacotah), and big bluestem (*Andropogon gerardii* L., variety Bison). Biomass was harvested in October (the second harvest, autumn regrowth) at the collection of energy crops located in Skierniewice (central Poland), belonging to Department of Agriculture and Biology of the Warsaw University of Life Sciences (SGGW). Maize (*Zea mays* L., variety Ulan V270) originated from the Agricultural Experimental Station SGGW in Żelazna and was harvested on October. After harvesting, the biomass was cut into 1–2 cm pieces (using a forage harvester so as to reflect the conditions that exist in an industrial environment) and ensiled in barrels (10 kg per barrel, in triplicate). Biomass was compacted very thoroughly, no head space was left.

Fresh pomace was obtained from an agro-food processing plant located in the Mazovian Voivodship, while beet pulp was obtained from a sugar factory in Glinojeck. The beet pulp and the pomace were ensilaged in barrels (in triplicate). Silages were made also from particular grasses blended with maize or apple pomace in the weight ratio of 50:50.

All barrels were filled completely, tightly closed, and stored at room temperature for 3 months. During the ensilaging process (lactic acid fermentation), the resulting gas was removed by the valves located on barrel closures.

Analytical methods

After 3 months of storage, the barrels were opened and chemical parameters of the silages were analyzed. Dry mass (DM) and organic dry mass (ODM) contents of ensiled biomass were determined according to Polish Standards PN-EN 12880 and PN-EN 12879, respectively. Chemical components of grass and maize silages were determined by NIRS method with a NIRFlex N-500 spectrometer according to the Polish Standard PN-EN ISO 12099. Parameters of silages from waste (beet pulp and apple pomace) were analyzed by classical methods: mono sugars were determined by the Luff-Schoorl method, crude fiber content was determined according to the Polish Standard PN-ISO 5498:1996, and protein content was determined using the Kjeldahl method. In all silages, pH value was determined with the potentiometric method and the content of organic acids was determined with the enzymatic method using UV tests (r-Biopharm, Germany).

Anaerobic fermentation

Biogas production from the ensiled plant material was analyzed in batch anaerobic digestion tests (BMP tests), in 1.3 L fermenters (glass bottles with special construction). 5 g of the substrate and 100 mL of the inoculum (content of secondary digester from an agricultural biogas plant after five days of incubation at 39°C to completely decompose the remaining organic matter) were added to each fermenter (in triplicate). The control assays were also prepared by adding 100 mL of the inoculum into the fermenters but without the substrate. The fermenters were encapped with measuring heads of OxiTop® Control (WTW, Germany) pressure monitoring system, flushed with N_2 to remove the air from the headspace, and incubated in a thermostatic cabinet on mixing platforms (WTW, Germany). During methane fermentation, an increase of biogas pressure was measured and saved every day by manometric sensors in measuring OxiTop® heads. Anaerobic digestion was conducted at 39°C for at least 30 days until *plateau* has achieved. At the end of the fermentation process, data was wirelessly transmitted (infrared) from the measuring heads to the OxiTop® OC 110 (WTW, Germany) controller and then transferred to a PC and processed in Excel program. Volume of the gas pressure was converted into the amount of biogas (in moles) using the ideal gas equation:

$$pV = nRT \quad (1)$$

where:
p – pressure [Pa]
V – reactor capacity [m^3]
T – temperature [K]
R – universal gas constant 8,31 [J · (mol K)-1]
n – number of moles.

The amount of biogas was then converted into the volume of biogas referring to normal conditions (1013.25 hPa, 273.15 K) and expressed in cubic meters [N m^3]. The amount of biogas produced from the inoculum itself (control assays) was subtracted from the amount of biogas obtained from the tested substrates.

Biogas composition was analyzed using a gas analyzer (COMBIMASS®GA-m, Germany).

Statistical analysis

The analysis of variance (ANOVA) was performed, after checking if the data meet the assumption of ANOVA (normality of distribution and equality of variance). In the case of significant differences between particular mean values, a post hoc analysis was performed (Tukey test). For all results, the level of significance was set at 0.05. The analysis was performed using Statistica 8.0 (Statsoft, Poland).

Results

The chemical composition of silages made from particular grasses was presented in Table 1.

Table 1

Chemical composition of silages made from perennial grasses or maize

Material	DM [%]	ODM [% DM]	Protein [% DM]	Crude fibre [% DM]	Mono sugars [% DM]	pH	Organic acids [g kg^{-1} DM]	
							lactic	acetic
Maize	28.4±0.50	94.5±0.50	9.9±0.60	23.8±0.40	6.7±0.30	3.9±0.00	11.6±0.21	1.9±0.10
Miscanthus	23.5±0.30	93.0±1.18	13.3±0.33	27.6±0.36	3.5±0.34	5.3±0.06	44.0±8.70	4.0±0.14
Spartina	35.7±0.62	93.8±1.68	10.1±0.32	29.4±0.50	2.7±0.21	5.2±0.03	2.5±0.42	2.2±1.62
Switchgrass	35.5±0.47	94.4±0.85	10.1±0.53	28.8±0.21	6.1±0.41	4.9±0.05	18.4±2.63	10.8±2.44
Big bluestem	28.8±0.50	94.0±0.66	11.1±0.39	28.6±0.60	5.9±0.07	5.2±0.07	15.8±3.87	6.1±2.02

± standard deviations
DM – dry mass
ODM – organic dry mass

Maize silage was of good quality, it had a low pH value (under 4.2) and no butyric acid – being an indicator of the spoilage processes (data not presented). Thus, the excellent usefulness of maize for ensiling was confirmed (KHAN et al. 2015).

Silages from perennial grasses were also of good quality, as evidenced by a lack of molds and butyric acid (data not presented) – Table 1.

Silages from beet pulp and apple pomace had low pH values, with no signs of molds growth (Table 2). The characteristics of beet pulp silages (with reference to the content of lactic acid exceeding acetic acid content and the lack of butyric acid) was similar to that of beet pulp silages presented by DULCET et al. (2011).

Table 2

Characteristics of ensiled waste from the agro-food industry

Material	DM [%]	ODM [% DM]	Protein [% DM]	Crude fiber [% DM]	Mono sugars [% DM]	pH	Organic acids [g kg^{-1} DM]	
							lactic	acetic
Apple pomace	33.2±0.50	98.0±0.10	6.3±0.02	72.0±0.90	1.9±0.20	3.4±0.00	16.8±0.50	5.8±0.25
Beet pulp	17.2±0.50	96.5±0.60	4.2±0.10	18.7±0.25	0.4±0.03	4.0±0.00	8.1±0.45	0.3±0.12

± standard deviations
DM – dry mass
ODM – organic dry mass

A new solution, proposed in this paper, is to ensile the blends of perennial grasses with waste from the agro-food industry or with maize. Silages made from blends of perennial grasses and maize had lower pH values than the silages made only from the grasses. In contrast, silages made from blends of particular perennial grass with apple pomace had lower pH values than the silages made only from particular grass, and were characterized by a delicate smell of acetic acid, the content of which in silages was higher than that of lactic acid (Table 3). The higher content of acetic acid compared to the lactic acid resulted probably from decomposition of pentosanes (e.g. xylan) by heterofermentative lactic acid bacteria. Pentosanes built of xylose or arabinose are constituents of hemicellulose, the content of which in lignocellulose biomass amounts to 20-35% (LIU et al. 2008).

In this study, a high biogas yield was obtained from grass silages, i.e.: from 445.7 m^3 Mg^{-1}_{ODM} (*Spartina*) to 622.8 m^3 Mg^{-1}_{ODM} (miscanthus) – Figure 1. The content of methane in biogas ranged from 54.6 to 55.1%. In turn, maize silage enabled gas production at 737.8 m^3 Mg^{-1}_{ODM} (data not

presented in Figure 1) with 55% methane content. PIĄTEK et. al. (2016) obtained comparable results of biogas yield from *Spartina* (404.5 m^3 Mg^{-1}_{ODM}) and big bluestem (546.6 m^3 Mg^{-1}_{ODM}), while from miscanthus they achieved less biogas yield (487.9 m^3 Mg^{-1}_{ODM}). Biogas yield from grass silages in this study was comparable with the biogas yield from maize silages (456.6–599.7 m^3 Mg^{-1}_{ODM}) reported by VERVAEREN et. al. (2010) or KLIMIUK et. al. (2010).

Table 3

Chemical composition of silages made from a mixture of perennial grasses and maize or apple pomace

Grass	pH	DM [%]	ODM [% DM]	Lactic acid [g kg^{-1} DM]	Acetic acid [g kg^{-1} DM]
Grass + maize (50:50)					
Miscanthus	4.9±0.06	29.0±0.50	93.6±0.15	13.4±1.85	0.8±0.26
Spartina	4.8±0.00	32.4±0.42	93.5±0.06	13.4±2.78	0.5±0.25
Switchgrass	4.8±0.06	33.5±0.31	94.2±0.26	12.5±4.37	0.3±0.15
Big bluestem	4.6±0.06	30.9±0.35	93.4±0.44	17.9±4.12	0.3±0.06
Grass + apple pomace (50:50)					
Miscanthus	3.9±0.00	28.6±0.50	93.4±0.36	11.1±1.27	14.1±2.35
Spartina	3.9±0.06	35.6±0.15	93.0±0.36	11.0±2.35	39.0±7.38
Switchgrass	3.9±0.06	36.2±0.80	94.1±1.06	7.1±2.21	37.6±1.93
Big bluestem	3.8±0.06	33.3±0.80	93.9±1.06	12.5±2.21	15.3±1.93

± standard deviations
DM – dry mass
ODM – organic dry mass

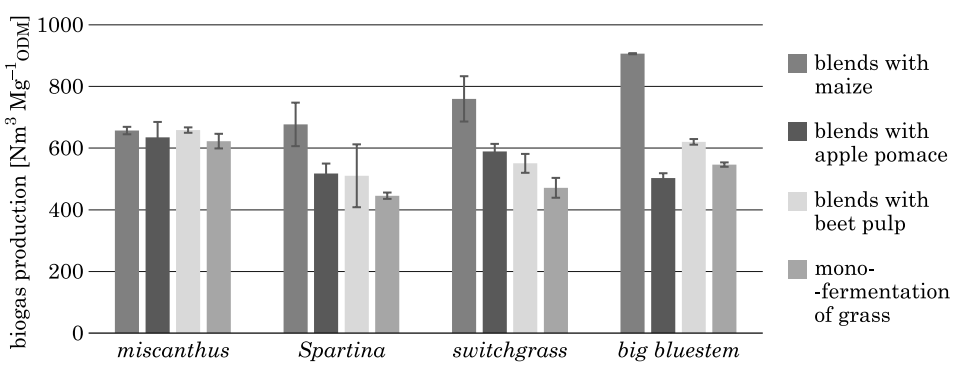

Fig. 1. Biogas production (mean values and ± standard deviations) from silages made of perennial grasses through mono- and co-fermentation with maize or agro-food waste

As a result of co-fermentation of silages made from blends of perennial grasses with maize or apple pomace or grass silages blended with ensiled beet pulp, in most cases a significant increase in biogas yield was observed in comparison to the mono-fermentation of grasses ($P \leq 0.05$). The co-fermentation process of silage from grasses with maize resulted in from 5.5 (blends with miscanthus) to 65.8% (blends with big bluestem) significantly higher biogas production compared to the mono-fermentation of particular perennial grass (Figure 1). Co-fermentation did not affect the percentage of methane in biogas, which in the case of fermentation of blends ranged from 53.9 to 58.4%.

In the case of co-fermentation of grasses ensiled with apple pomace, the increase in biogas yield in comparison to grasses mono-fermentation was from 1.9 (blends with miscanthus) to 25% (blends with switchgrass). In the case of ensiled blends of apple pomace with big bluestem, a reduction in biogas yield was observed compared to the mono-fermentation of the grass, but the difference was not significant (Figure 1). Methane content in biogas produced from the blends of particular grass and apple pomace ranged from 55.6 to 56.5%.

In the case of co-digestion of ensiled grasses with ensiled beet pulp, an increase in biogas yield was also observed in relation to the mono-fermentation of particular grass silages. The increase in biogas yield in the co-fermentation process was the lowest in the case of the blend of beet pulp and miscanthus (by 5.7%) and the highest in the case of the blend of beet pulp and switchgrass (16.8%) – Figure 1. Methane content in biogas from the blends of particular grasses and beet pulp was in the range of 55.5–57.6%.

The co-fermentation of silages from the grasses with maize or waste caused no significant changes in methane content in biogas as compared to methane content in biogas obtained as a result of the mono-fermentation of particular grass species.

Discussion

After harvesting the biomass should be preserved, mainly via the ensiling process. The quality of silage is of great importance to the methane fermentation process. The use of spoiled and moldy silages affects the reduction of biogas production (KALAČ 2011). Perennial warm-season grasses, such as miscanthus, may be difficult to ensile due to their little-soluble carbohydrates and a high buffering capacity (DOS SANTOS et al. 2013). For this reason, demonstrating that perennial grasses are susceptible to ensiling is in this case a valuable observation.

Ensiling is an excellent means to preserve quickly spoiling waste from the agro-food industry. Beet pulp is a difficult to store substrate. Due to the high content of sugars, it is an excellent medium for microorganisms development during storage, e.g. in heaps, which results in the loss of sugars and the overall technological value of beet pulp. Alcoholic fermentation may occur during beet pulp storage, as it was reported by FUGOL and PILARSKI (2011). The use of spoiled beet pulp for biogas production resulted in the loss of control over the methane fermentation process and, as a consequence, in its inhibition. Apple pomace is also a quickly spoiling substrate due to the high content of water (up to 73%) and easily fermentable sugars, which causes the rapid development of microorganisms and alcoholic fermentation (KUMIDER 1996). This paper confirms the usefulness of the ensiling process for preservation of this kind of waste.

A new solution, proposed in this paper, is to ensile the blends of perennial grasses with waste from the agro-food industry or with maize. Ensiling the blends of different substrates brings many benefits, for example when one of the raw material has too low dry matter content. The ensiling of plants with dry matter content below 28% may result in a large amount of leachate appearing during storage of silage and thus in energy losses (in the case of storing silages e.g. on piles while the emerging cell juice is discharged to the sewage). The addition of a substrate with a higher dry matter content is aimed at absorbing effluent cell juice (MYCZKO 2011). In this study, the silage made from miscanthus biomass had a low dry matter content (23.5%), which could cause the appearance of leachate during prolonged storage. In the silage made from the mixture of miscanthus and maize or apple pomace, the dry matter content increased (to 29 and 28.6% respectively).

In this study, co-fermentation of grass silages with maize or agro-food waste increased biogas production compared to the mono-fermentation of grass silages. This may be due to a better carbon to nitrogen ratio during co-digestion. The excessive carbon to nitrogen ratio occurs during an anaerobic digestion process of crop materials, especially lignocellulosic biomass (ZHONG et al. 2012). Agro-food waste, such as beet pulp or apple pomace, as an external nitrogen and a source of nutrients, is responsible for new quality of feedstock obtained and thus for higher biogas production compared to the mono-fermentation of perennial grasses.

The co-fermentation of lignocellulosic biomass with easily biodegradable material increased biogas yield also in the study described by YANG et al. (2009). As a result of the mono-fermentation of *Spartina alterniflora* 358.5 $m^3\ Mg^{-1}_{ODM}$ of biogas were produced, while during the co-fermentation of the mixture of spartina with potato waste in a mass ratio of 4:1

and 6:1, biogas production reached 433.6 and 460.1 $m^3\ Mg^{-1}_{ODM}$, respectively. Analyses of structural changes of *Spartina alterniflora* after anaerobic digestion indicated that the co-digestion improved hemicellulosic degradation.

In another work apple pomace or maize silage was co-fermented with ensiled lignocellulosic biomass of knotweed bohemica (*Reynoutria* × *bohemica* Chrtek & Chrtkova). As a result of mono-fermentation of knotweed 327 $m^3\ Mg^{-1}_{DM}$ of biogas was obtained, while co-fermentation with maize or apple pomace resulted in biogas production increase to 650.6 and 494.3 $m^3\ Mg^{-1}_{DM}$ respectively (KUPRYŚ-CARUK et. al. 2014).

Conclusion

The co-fermentation of ensiled lignocellulosic biomass of perennial grasses with maize or waste from the agro-food industry is a way to increase biogas yield compared to the mono-fermentation of grasses. The use of the proposed blends of ensiled materials for biogas production brings many benefits, including: waste management and the possibility of partial replacement of maize silage with perennial grass silage with no loss of biogas and methane yield. Study results indicate that ensiled perennial grasses can be alternative sources of biogas and may be successfully used as substitutes or supplements to the main substrates used in agricultural biogas plants based on maize silage.

References

DĘBOWSKI M., DUDEK M., ZIELIŃSKI M., GRALA A. 2013. *Technological effectiveness of methane fermentation of prairie cordgrass (Spartina pectinata).* Proceedings of ECOpole, 7(1): 49–58.

DEMIRCI G., DEMIRER G. 2004. *Effect of initial COD concentration, nutrient addition, temperature and microbial acclimation on anaerobic treatability of broiler and cattle manure.* Bioresource Technology, 93: 109–117.

DUBROVSKIS V., KOTELENCECS V., CELMS A., ZABOROVSKIS E. 2012. *Co-fermentation of biomass with high content of lignocelluloses for biogas production.* In: *Conference materials. Renewable energy and energy efficiency*. Latvia University of Agriculture, Jelgawa, pp. 121–126.

DULCET E., DORSZEWSKI P., KASZKOWIAK J., BOROWSKI S., RAMA R., BUJACZEK R., CHOJNACKI J. 2011. *Analiza jakości kiszonek z wysłodków buraczanych sporządzonych przy użyciu prasy zwijającej do materiałów rozdrobnionych*. Acta Sci. Pol., Technica Agraria, 10(3–4): 19–26.

FRĄC M., ZIEMIŃSKI K. 2012. *Methane fermentation process for utilization of organic waste*. International Agrophysics, 26(3): 317–330.

FRIGON J.C., MEHTA P., GUIOT S. 2012. *Impact of mechanical, chemical and enzymatic pre-treatments on the methane yield from the anaerobic digestion of switchgrass.* Biomass and Bioenergy, 36: 1–11.

FUGOL M., PILARSKI K. 2011. *Burak cukrowy jako substrat do biogazowni*. Inżynieria Rolnicza, 5(130): 63–71.

GELEGENIC J., GEORGAKAKIS D., ANGELIDAKI I., MAVRIS Y. 2007. *Optimization of biogas production by co-digesting whey with diluted poultry manure*. Renewable Energy, 32(13): 2147–2160.

GOŁASZEWSKI J. 2011. *Wykorzystanie substratów pochodzenia rolniczego w biogazowniach w Polsce*. Postępy Nauk Rolniczych, 2: 69–94.

KACPRZAK K., KRZYSTEK L., LEDAKOWICZ S. 2009. *Anaerobic co-digestion of agricultural products and industrial wastes*. Environmental Protection Engineering, 35(3): 215–224.

KALAČ P. 2011. *The required characteristics of ensiled crops used as a feedstock for biogas production: a review*. J. Agrobiol., 28: 85–96.

KAPARAJU P., ELLEGAARD L., ANGELIDAKI I. 2009. *Optimisation of biogas production from manure through serial digestion – Lab-scale and pilot-scale studies*. Bioresource Technology, 100: 701–709.

KHAN N., YU P., ALI M., CONE J., HENDRIKS W. 2015. *Nutritive value of maize silage in relation to dairy cow performance and milk quality*. Journal of the Science of Food and Agriculture, 95(2): 238–252.

KLIMIUK E., POKÓJ T., BUDZYŃSKI W., DUBIS B. 2010. *Theoretical and observed biogas production from plant biomass of different fibre contents*. Bioresource Technology, 101: 9527–9535.

KUPRYŚ-CARUK M., PODLASKI S., WIŚNIEWSKI G. 2014. *Suitability of knotweed bohemica (Reynoutria x bohemica Chrtek & Chrtkova) for biogas production*. Zeszyty Problemowe Postępów Nauk Rolniczych, 579: 27–36.

LIU Z., SAHA B., SLININGER P. 2008. *Lignocellulosic biomass conversion to ethanol by Saccharomyces spp*. In: *Bioenergy*. Eds. J. Wall, C. Harwood, A. Demain. ASM Press, Washington, D.C.

MURPHY J., WALL D., O'KIELY P. 2013. *Second generation biofuel: biomethane from co-digestion of grass and slurry*. In: *Proceedings of the 17th Symposium of the European Grassland federation*, Akureyri, Iceland, pp. 505–513.

MYCZKO A., MYCZKO R., KOŁODZIEJCZYK T., GOLIMOWSKA R., LENARCZYK J., JANAS Z., KLIBER A., KARŁOWSKI J., DOLSKA M. 2011. *Budowa i eksploatacja biogazowni rolniczych. Poradnik dla inwestorów zainteresowanych budową biogazowni rolniczych*. Wydawnictwo ITP, Warszawa – Poznań.

NDEGWA P., HAMILTON D., LALMAN J., CUMBA H. 2008. *Effects of cycle-frequency and temperature on the performance of anaerobic sequencing batch reactors (ASBRs) treating swine waste*. Bioresource Technology, 99: 1972–1980.

NIELSEN H., ANGELIDAKI I. 2008. *Strategies for optimizing recovery of the biogas process following ammonia inhibition*. Bioresource Technology, 99(17): 7995–8001.

PIĄTEK M., LISOWKI A., KASPRZYCKA A., LISOWSKA B. 2016. *The dynamics of an anaerobic digestion of crop substrates with an unfavourable carbon to nitrogen ratio*. Bioresource Technology, 216: 607–612.

RAMA R., BOROWSKI S., DULCET E. 2013. *Biogazownie rolnicze konkurencją dla rynku żywności*. Inż. Ap. Chem., 52(2): 60–61.

DOS SANTOS J.,LIRA M., GUIM A., DOS SANTOS M., JUNIOR J., DE LAO DE MELLO A. 2013. *Elephant grass clones for silage production*, Sci. Agric., 70: 6–11.

SOSNOWSKI P., WIECZOREK A., LEDAKOWICZ S. 2003. *Anaerobic co-digestion of sewage sludge and organic fraction of municipal solid waste*. Advances in Environmental Research, 7: 609–616.

VERVAEREN H., HOSTYN K., GHEKIERE K., WILLEMS G. 2010. *Biological ensilage additives as pretreatment for maize to increase the biogas production*. Renew. Energy, 35: 2089–2093.

YADVIKA, SANTOSH, SREEKRISHNAT T.R., KOHLI S., RANA V. 2004. *Enhancement of biogas production from solid substrates using different techniques – a review*. Bioresource Technology, 95, 1–10.

YANG S., LI J., ZHENG Z., MENG Z. 2009. *Lignocellulosic structural changes of Spartina alterniflora after anaerobic mono- and co-digestion*. International Biodeterioration & Biodegradation, 63(5): 569–575.

ZHONG W., ZHANG Z., LUO Y., QIAO W., XIAO M., ZHANG M. 2012. *Biogas productivity by co-digesting Taihu blue algae with corn straw as an external carbon source*. Bioresour. Technol., 114: 281–286.

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HEAVY METALS POLLUTION OF SMALL URBAN LAKES SEDIMENTS WITHIN THE ONEGO LAKE CATCHMENT AREA*

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Key words: sediments, heavy metals, geo-accumulation index, pollution.

Abstract

A geochemical investigation of two sediment cores retrieved from Plotich'e and Kitaiskoe lakes (Republic of Karelia, Russia) has been carried out. The content of eight heavy metals (Pb, Cd, Zn, Cr, Ni, Cu, Mn, and V) in the modern bottom sediments was determined. The sources of pollution of the sediments of the lake have been revealed. In order to estimate the negative impact of human activities on the urban lakes, the geo-accumulation index has been calculated. It is noted that Plotich'e and Kitaiskoe lakes are contaminated by heavy metals in different ways.

Introduction

Waterbodies, which are located either within urban areas or nearby towns, are permanently subjected to anthropogenic load. The extent of the load can be determined via the investigation of the chemical composition of both water and sediments. Lakes, as a rule, are considered as a landscape depression, since they are capable of the accumulation of natural material (DAUVALTER et al. 2011), delivered from the catchment area through rivers as well as temporary flows. As a result, lacustrine sediments (especially closed-basin lakes sediments) are considered as perfect archi-

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ves (SUN et al. 2009). These can be used for reconstructing past sedimentation processes, assessment of the modern ecological conditions, and prognostication of possible ways of changing in the future.

The purposes of this survey are to define the heavy metals content in the lake sediment cores, which were retrieved from the central parts of Plotich'e and Kitaiskoe lakes (Medvezhyegorsk town, Republic of Karelia) and to the main sources of heavy metals input to these waterbodies.

Research area, Materials and Methods

Medvezhyegorsk is a small town with population of 15 thousand people, which is situated in the central part of Karelia Republic. The town is washed by Poveneckii bay (Onego lake) from the south. The main possible sources of urban environment pollution are railway and automobile mean of transport, a crushed stone quarry, a milk plant, a bakery plant and a number of woodworking enterprises.

Plotich'e lake (Figure1) is a small water body, which is situated in the West part of Medvezhyegorsk. This water object is actively used in household and recreation purposes (swimming, fishing). In March of 2016, a 28-cm sediment core was retrieved from the central part of the lake (the site's depth is roughly 20 m) through a Limnos sampler. The total number of samples obtained from this lake was 13. The sediment core, except the bottom 4 cm, was split into 2 cm layers and sealed in plastic containers.

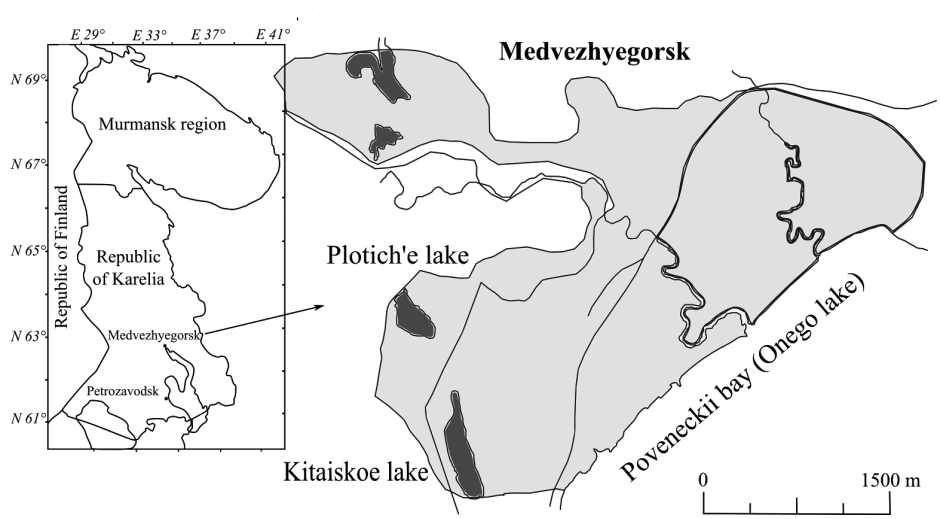

Fig 1. Map of the research area

Kitaiskoe lake (Figure 1) is also a small water body, which is located in the South-West part of the town. This lake is mostly used by domestic fishermen. In June of 2016, a 20-cm sediment core was retrieved from the central part of the lake (depth is approximately 26 m) through a Limnos sampler. The total number of samples obtained from this lake was 10. The sediment core was split into 2 cm layers and sealed in plastic containers.

Each 2 cm layer was put into a refrigerator bag and transported to the Institute of Geology RAS, Petrozavodsk. Dried sediment samples were then decomposed by acid autopsy with an acid mixture in an open system. The method for preparing the samples for chemical analysis was described in (SLUKOVSKII and POLYAKOVA 2016). Metal concentrations in the bottom sediment samples were estimated using the mass-spectral method on a XSeries-2 ICP-MS instrument at the Analytical Centre of the Institute of Geology, KarRC, RAS, Petrozavodsk. Each sample was measured in three repetitions. The final concentration is an average of these repetitions. The geo-accumulation index I_{geo} of heavy metals in bottom sediments was calculated using the formula:

$$I_{\text{geo}} = \log_2\left(\frac{C}{1.5B}\right),$$

where C is the metal concentration in the layer of interest, B is background metal concentration measured in the deepest layer of the core (GUPTA et al. 2014). The concentrations of heavy metals in the lowest layers were accepted as the background for each of the researched lakes.

Results and Discussion

Table 1 and Table 2 represent information about the concentration of eight heavy metals in sediments of Plotiche and Kitaiskoe lake. As it can be seen from the data, the ***lead*** concentration reaches its peak (155 mg kg^{-1}) in the 6–8 cm layer of sediment core retrieved from Plotiche lake, while the maximal rate of the element (42.6 mg kg^{-1}) in Kitaiskoe sediment core was detected in the 4–6 cm layer. In general, both lakes have similar lead content through sediments columns. The largest values were ascertained in the upper 14 cm layers. Then there is a noticeable decrease in the lead concentration in the underlying layers. The average concentration of the element in sediments accounts for 62.7 mg kg^{-1} for the former lake and 26.4 mg kg^{-1} for the latter.

Table 1

Concentration of heavy metals in Plotiche lake sediments [mg kg^{-1}]

Specification	Pb	Cd	Zn	Cr	Ni	Cu	Mn	V
0–2	79.4	1.24	577	41.7	40.8	**790**	363	101
2–4	89.6	1.53	563	42.8	41.5	105	306	96.6
4–6	113	1.81	**624**	53.6	45.4	70.5	330	106
6–8	**155**	**2.03**	571	60.2	45.4	90.9	353	95.4
8–10	104	1.62	339	60.7	43.6	61.9	**371**	78.9
10–12	77.8	1.28	191	45.3	34.6	47.0	338	76.4
12–14	55.7	1.38	123	**73.1**	**60.6**	54.7	301	103
14–16	32.6	0.80	113	23.5	24.3	21.3	228	121
16–18	30.7	0.58	123	25.8	25.7	23.6	240	128
18–20	24.0	0.46	67.7	19.3	21.1	16.9	205	109
20–22	26.2	0.52	70.5	20.8	22.6	18.9	215	109
22–24	24.6	0.46	63.2	20.1	21.9	17.1	213	109
24–28	2.60	0.34	97.6	21.3	18.1	19.8	164	**160**
X Average	62.7	1.08	271	39.1	34.3	103	279	107

Table 2

Concentration of heavy metals in Kitaiskoe lake sediments [mg kg^{-1}]

Specification	Pb	Cd	Zn	Cr	Ni	Cu	Mn	V
0–2	35.4	1.24	**391**	19.9	30.3	**47.6**	**1713**	26.2
2–4	39.8	1.27	384	19.8	27.6	44.5	1578	26.7
4–6	**42.6**	1.28	380	20.9	**31.0**	47.1	1600	**27.2**
6–8	42.4	**1.31**	361	20.1	30.1	45.4	1453	24.8
8–10	38.6	0.85	283	19.4	29.2	42.1	1127	23.2
10–12	27.0	0.73	228	18.4	27.1	33.9	1178	19.2
12–14	23.3	0.71	215	**21.5**	26.2	38.9	1041	18.5
14–16	7.06	0.24	82.0	18.6	24.8	37.3	690	16.4
16–18	2.74	0.17	45.2	16.9	24.1	31.5	550	15.6
18–20	5.36	0.25	85.3	17.9	25.0	53.7	428	21.9
X Average	26.4	0.81	245	19.3	27.5	42.2	1136	22.0

According to the tables, the **cadmium** concentration has its maximal rate in the 6–8 cm layer in both lakes. It amounts to 2.03 mg kg^{-1} in Plotiche and 1.31 mg kg^{-1} in Kitaiskoe lakes sediments. As in the case with lead distribution, the highest concentrations of cadmium were detected in the upper 14 cm layers of both sediment cores, while the rates of the heavy metal in the underlying layers do not exceed the average concentrations for both columns.

The upper 10 cm of a Plotich'e lake sediment core is considerably contaminated with **zinc**. The 4–6 cm layer has the maximal rate of the element – 624 mg kg^{-1}. The minimal concentration – 63.2 mg kg^{-1} was detected in the 22–24 cm layer. The highest rates of zinc in Kitaiskoe lake sediments core has been concentrated in the first 14 cm layers with a maximum of 391 mg kg^{-1} in the 0–2 cm layer. The lower concentration of the element was ascertained in the 16–18 cm layer.

The maximal concentration of **chromium** – 73.1 mg kg^{-1} in sediments of Plotich'e lake was detected in the 12–14 cm layer. The minimal rate of the element – 19.3 mg kg^{-1} is concluded in the 18–20 cm layer. In general, the chromium average rate of the upper 14 cm of the column is roughly twice higher than the bottom 14 cm one. In contrast, the content of chromium in Kitaiskoe lake sediments varies inconsiderably throughout the column. The maximal rate of the element – 21.5 mg kg^{-1} was detected in the 12–14 cm layer, while the minimal chromium concentration – 16.9 mg kg^{-1} was found in 16–18 cm layer.

As in the case with chromium distribution in the Plotich'e lake sediment core, the maximal rate of **nickel** – 60.6 mg kg^{-1} was found in the 12–14 cm layer of the column retrieved from the lake. The minimal concentration of the heavy metal –18.1 mg kg^{-1} was detected in the bottom 4-cm layer. The maximal value of nickel in Kitaiskoe lake sediment core – 31.0 mg kg^{-1} was detected in the 4–6 cm layer, while the minimal content of the element – 24.1 mg kg^{-1} was found in the 16–18 cm layer.

The concentration of **copper** ranging from a maximum of 790 mg kg^{-1} in the first 2-cm layer to a minimum of 16.9 mg kg^{-1} in the 18–20 cm layer in Plotiche lake sediments and from the largest of 47.6 mg kg^{-1} in the first 2-cm layer to a low of 31.5 mg kg^{-1} in the 16–18 cm layer in Kitaiskoe lake sediments.

The highest concentrations of **manganese** were established in the uper sediment 14-cm layers of both lakes. However, there is a substantial difference in the values of the heavy metal in these two lakes. The content of manganese for Plotiche lake ranging from a maximum of 371 mg kg^{-1} in the 8–10 cm layer to minimum of 164 mg kg^{-1} in the 24–28 cm layer, while sediment core retrieved from Kitaiskoe lake has the maximal rate of the element equals to 1713 mg kg^{-1} (found in the 0–2 cm layer) and minimal rate equals to 428 mg kg^{-1} (found in the 18–20 cm layer).

The concentration of **vanadium** in sediments of both lakes varies inconsiderably throughout the columns. In Plotiche lake sediments the highest concentration of the element – 160 mg kg^{-1} was found in the bottom 4-cm layer. In sediments of Kitaiskoe lake, on the contrary, the maximal rate of vanadium was detected in 4–6 cm layer.

In order to estimate the extent of anthropogenic influence on the heavy metals concentrations in the sediments of these waterbodies, the geoaccumulation index was used. The index is calculated through the formula: $I_{geo} = \log_2 (C/1.5B)$, where C is the measured concentration of the element in the sediments fraction, B is the geochemical background value, 1.5 is the correction factor due to lithological variations (MÜLLER 1979). According to FORSTNER et al. (1993), the geoaccumulation index classification consists of 7 grades (Table 3).

Table 3

Index of geoaccumulation and contamination levels

Sediment Igeo contamination value	Geoaccumulation class Intensity	Index, Igeo (sediment quality)
>5	6	very strong
>4–5	5	strong to very strong
>3–4	4	strong
>2–3	3	moderate to strong
>1–2	2	moderate
>0–1	1	uncontaminated to moderate
>0	0	practically uncontaminated

Source: GUPTA et al. (2014)

As it can be seen from the Figure 2, the first 2-cm layer of Plotich'e lake sediment core has a very strong Cu contamination (class 6), strong to very strong Pb contamination (class 5), and moderate to strong Zn contamination (class 3). The 2–6 cm layer is polluted by Pb (class 5), Cu (class 3), and Zn (class 3). The 6–8 cm layer is also contaminated by Pb (class 5), Cu and Zn (class 3) plus there appeared Cr contamination (class 3). The 8–10 cm layer is polluted by Pb (class 5) and Cr (class 3). In the 10–12 cm layer just Pb contamination (class 4) was found. The 12–14 layer is contaminated by Pb (class 4), Cr (class 3), and Ni (class 3). The 14–24 cm layer is only contaminated by Pb, values for this heavy metal are in class 3. The bottom 4 cm layer of the sediment core does not consist of any heavy metals contamination footprints.

The upper 8 cm layer of Kitaiskoe lake sediment core is characterized by high values for Mn (class 4), Pb (first 2 cm layer – class 3, 4–8 cm layer – class 4), and Zn (class 3) – Figure 3. The 8–14 cm layer is strongly polluted by Mn (class 4) and has moderate to strong Pb pollution (class 3). In the 14–18 cm layer moderate to strong Mn contamination was detected. The bottom 2 cm layer has no heavy metal pollution.

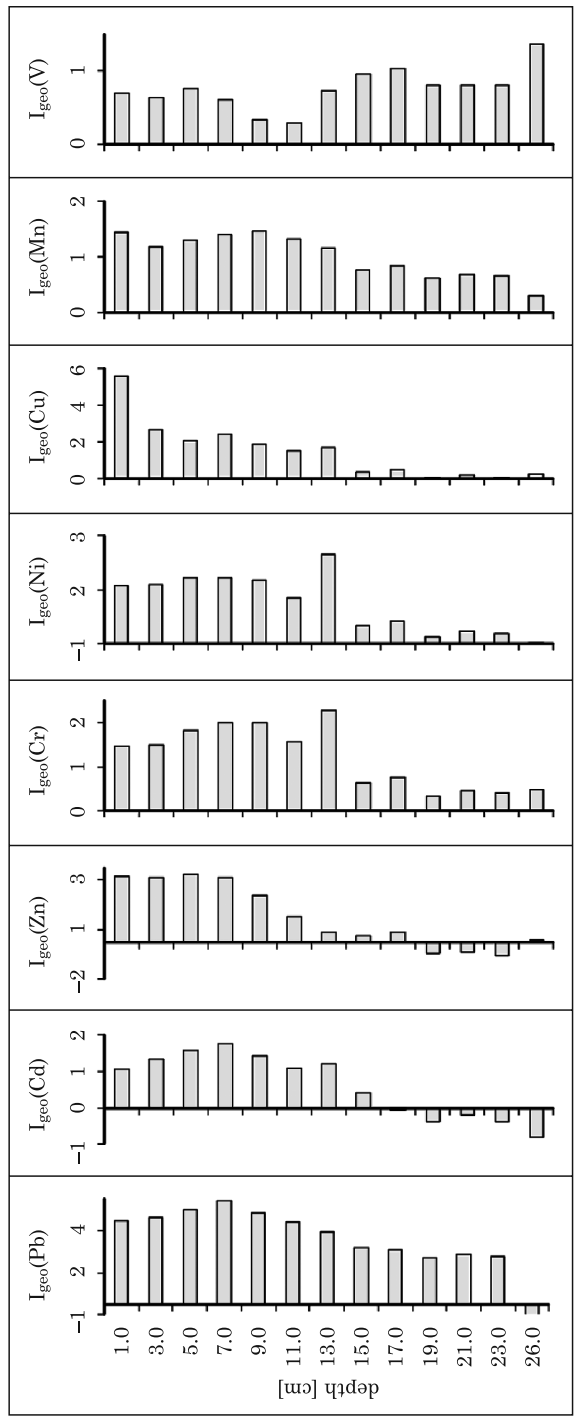

Fig. 2. Values of the geo-accumulation index (I_{geo}) of heavy metals in Plotich'e lake sediments

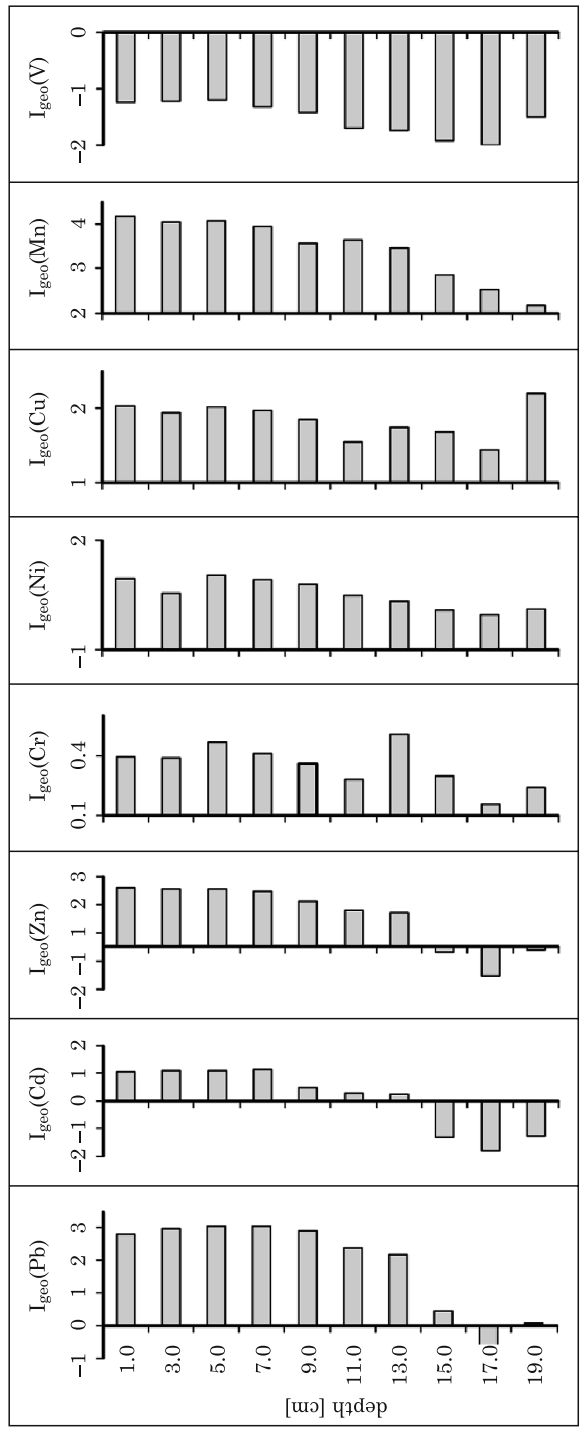

Fig. 3. Values of the geo-accumulation index (I_{geo}) of heavy metals in Kitaiskoe lake sediments

According to the calculations, copper has the maximal value of geoaccumulation index (5.6) among the eight heavy metals and it is observed only in the upper 2 cm layer of the sediment core retrieved from Plotich'e lake. Kitaiskoe lake, on the contrary, doesn't have high levels of the element throughout the whole sediment column. It could be connected with the fact that copper is used in agriculture (fertilizer and pesticides) (KABATA-PENDIAS and MUKHERJEE 2007). Because Plotich'e lake is located in a zone of housing with a plethora of private kitchen-gardens, which are located up to the territory of the lake, it is possible that this heavy metal is delivered into the waterbody through the surface flows. Seemingly, copper precipitates and gets "fixed" in the surficial sediment layer of the lake, which could be considered as a vertical geochemical barrier between the water and sediments. Kitaiskoe lake, by contrast, is rather secluded from any dwelling and agricultural objects. So, the Cu content in the sediments of the lake is considerably lower, than in the Plotich'e lake sediments.

Lead also has a rather high value of the geoaccumulation index, especially in the upper 10 cm layer of sediment core sampled from Plotich'e lake, reaching its peak at 4.9 in the 6–8 cm layer. The maximal value of the geoaccumulation index for Pb in Kitaiskoe lake sediments (3.1) was found in the 4–8 cm layer. Exceeded lead content in the sediments is probably connected with the effect of global pollution by Pb compounds (MCCONNEL and EDWARDS 2008, NORTON et al. 1990), such as tetraethyl lead, which was actively used in knock-sedative dope in gasoline in the middle of the XX century (NRIAGU 1990, KOMAREK et al. 2008). This metal together with Cd and Sb from the lake sediments of the Republic of Karelia, the Murmansk Region and Finland, including Arctic zone of these territories, behave in a similar manner (UKONMAANAHO et al. 1998, VERTA and TOLONEN 1998, DAUVALTER and KASHULIN 2010, DAUVALTER et al. 2011, SLUKOVSKII et al. 2018). The influence of the long-distance air transport of heavy metals is observed in these cases (VINOGRADOVA et al. 2017).

Moderate to strong zinc pollution is revealed in the upper 8 cm sediments layer of both lakes. Zinc contamination could be related to non-ferric metal industry (KABATA-PENDIAS and MUKHERJEE 2007). Possibly, the high level of this metal in the sediments caused by the activity of the Nadvoickii aluminum plant, which is located 100 km northward from Medvezhyegorsk town. The plant was placed in operation in 1954, for this reason, Zn contamination is observed only in the first 8 cm of both sediment cores.

Manganese contamination from strong to moderate is observed through the whole sediment core of Kitaiskoe lake, except of the bottom 2 cm. Sediments from Plotich'e lake, by contrast, do not have any traces of high

Mn concentration. Inasmuch as the manganese content could be connected with igneous rocks and glacial depositions (MARTYNOVA 2012), it is possible that it has entered into the lake owning to intensive weathering, occurring within the local catchment area of Kitaiskoe lake.

Moderate to strong chromium pollution was found in 6–10 cm and 12–14 cm layers of Plotich'e lake sediments. Cr is used in woodworking industry as preservatives (KABATA-PENDIAS and MUKHERJEE 2007, BESSONOVA and IVANCHENKO 2011), so the chromium contamination in Plotich'e lake sediments may be caused by the activity of woodworking enterprises, which are located in Medvezhyegorsk town.

critical values

U_{Cr}	
$p \leq 0.01$	$p \leq 0.05$
27	37

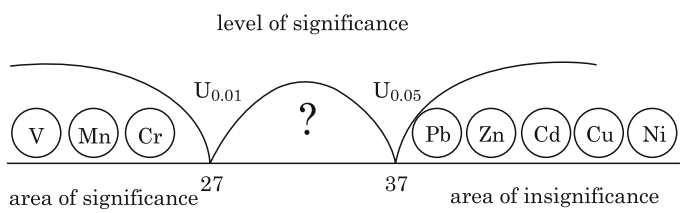

Fig 4. The Mann-Whitney U-test for heavy metals content in sediments of Plotich'e and Kitaiskoe lakes

In order to determine if there is a difference in the value of parameters between two columns of sediments, Mann-Whitney U-test was run. The test is meant to be used for comparing two independent samples, when the assumption of normality of equality of variance is not met (PEREIRA and LESLIE 2010). Figure 4 demonstrates that there is a discrepancy in V, Mn and Cr content in sediments of Plotich'e and Kitaiskoe lake, whereas the three heavy metals are in the zone of significance.

Conclusions

Thus, sediments of Plotich'e lake are mostly polluted by copper (the highest value of *I*geo – 5/6 found in the upper 2 cm layer), lead (the highest value of Igeo – 4.9 found in the 6–8 cm layer), zinc (the highest value of Igeo – 2.8 found in the 4–6 cm layer), and chromium (the highest value of Igeo – 2.3 found in the 12–14 cm layer). Also, it is considerable to point

out that there is no manganese contamination in the sediment core retrieved from Plotich'e lake, while sediments of Kitaiskoe lake are contaminated by this heavy metal (the highest value of Igeo for Mn – 3.7 found in the first 2 cm layer). Other valuable contaminants revealed in sediments of the lake are lead (the highest value of Igeo – 3.1 found in the 4–8 cm layer) and zinc (the highest value of Igeo – 2.1 found in the upper 4 cm layer).

In general, it could be concluded that the highest values of pollutants are concentrated in the upper 8 cm layer of both sediment columns. The basic pollution sources of the lakes sediments are the railway and automobile means of transport, fertilizers, woodworking industry, and an aluminum plant.

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References

Bessonova V., Ivanchenko O. 2011. *Chromium in the environment.* Bioindication and Ecology Questions, 1: 13–29.

Dauvalter V., KASHULIN N. 2010. *Chalcophile elements (Hg, Cd, Pb, As) in lake Umbozero, Murmansk Province.* Water Resources, 37(4): 497–512.

Dauvalter V., Kashulin N., Sandimirov S., Terentjev P., Denisov D., Amundsen P. 2011. *Chemical composition of lake sediments along a pollution gradient in a Subarctic watercourse.* Journal of Environmental Science and Health, Part A, 46: 1020–1033.

Forstner U., Ahlf W., Calmano W., *1993. Sediment quality objectives and criteria development in Germany.* Water Sci. Technol., 28: 307–316.

Gupta S., Vinod J., Matic N., Kapralova V. Solanki J., 2014. *Assessment of geo-accumulation index of heavy metal and source of contamination by multivariate factor analysis.* Int. J. Hazardous Mater., 2: 18–22.

Gusev O. N., Smirnova N.M., Liskovich A.L., Klintevich V., Bolshakova L., Sokolova L. 2000. *Assessment of the status and prospects of development of the mineral resource base of peat and sapropel on the North-West of the Russian Federation.* Respublika Kareliya, SPb.

Kabata-Pendias A., Mukherjee A. 2007. *Trace elements from soil to Human.* Springer-Verlag.

Komárek M., Ettler V., Chrastný V., Mihaljevi M. 2008. *Lead isotopes in environmental sciences:* A review Environ. Int.V. 34(4): 562–577.

Martynova M.V. 2012. *Manganese forms, their contents, and transformation in freshwater sediments (analytical review).* Ekol. Khim., 21(1): 38–52.

McConnell J., Edwards R. 2008. *Coal burning leaves toxic heavy metal legacy in the Arctic.* Proceedings of the National Academy of Sciences, 34: 12140–12144.

Nriagu J. 1990. *The rise and fall of leaded gasoline.* Science of the Total Environment, 92: 13–28.

Norton S.A., Dillon P.J., Evans R.D. 1990. *The history of atmospheric deposition of Cd, Hg and Pb in north America: Evidence from lake and peat bog sediments.* Sources, Deposition and Capony Interactions, III: 73–101.

Pereira S.M.C, Leslie G. 2010. *Testing differences between two samples of continuous data.* Australian Critical Care, 23: 160–166.

Slukovskii Z.I., Polyakova T.N. 2017. *Analysis of accumulation of heavy metals from river bottom sediments of the urban environment in the bodies of Oligochaetes*. Inland Water Biology, 10(3): 315–322.

SLUKOVSKII Z.I., SHELEKHOVA T.S., SIROEZHKO E.V. 2018. *A response of diatoms from the small lake on heavy metals effect in an urban environment, Republic of Karelia.* Vestnik of Saint Petersburg University. Earth Sciences, 63(1): 103–123.

SUN Q., WANG S., ZHOU J., CHEN Z., SHEN J., XIE X, WU F., CHEN P. 2009. *Sediment geochemistry of Lake Daihai, north-central China: implications for catchment weathering and climate change during the Holocene.* J. Paleolimnol., 43: 75–87.

UKONMAANAHO L., STARR M., HIRVI J.P., KOKKO A., LAHERMO P., MANNIO J., PAUKOLA T., RUOHO-AIROLA T., TANSKANEN H. 1998. *Heavy metal concentrations in various aqueous and biotic media in Finnish Integrated Monitoring catchments.* Boreal Environment Research, 3: 235–249.

VERTA M., TOLONEN K. 1998. *History of heavy metal pollution in Finland as recorded by lake sediments.* The Science of Total Environment, 87/88: 1–18.

VINOGRADOVA A., KOTOVA E., TOPCHAYA V. 2017. *Atmospheric transport of heavy metals to regions of the North of the European territory of Russia.* Geography and Natural Resources, 38(1): 78–85, https://doi.org/10.1134/S1875372817010103.

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NITRILE-METABOLIZING BACTERIAL STRAINS ASSOCIATED WITH MUNICIPAL WASTE TIPS IN THE LAGOS METROPOLIS, NIGERIA

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Key words: cyanide, pollutant, solid waste, *Corynebacterium* sp., *Bacillus* sp., detoxification, bioremediation.

Abstract

Cyanide is one of the dominant pollutants in the environment. This study aimed at exploring the potential of microbes in the detoxification of cyanogenic substances. *Bacillus* sp. WOD8 KX774193 and *Corynebacterium* sp. WOIS2 KX774194 strains were isolated from solid waste leachates. The doubling times of strain WOD8 and strain WOIS2 when grown on glutaronitrile and benzonitrile (without supplementing glucose) were 12.2 and 7.86 d (specific growth rate, μ: 0.057 and 0.088 d^{-1}) and 15.75 and 13.33 d (specific growth rate, μ: 0.044 and 0.052 d^{-1}) respectively. Also, strains WOD8 and WOIS2 grew on glutaronitrile and benzonitrile (with supplementing glucose) with doubling times of 9.76 and 7.62 d (μ: 0.071 and 0.091 d^{-1}) and 10.5 and 8.15 d (μ: 0.066 and 0.085 d^{-1}) respectively. The results from the present study suggest that the nitrile-metabolizing capabilities of these bacterial isolates can potentially be explored for the degradation and bioremediation of nitrile contamination in the environment.

Introduction

Nitriles are cyano group (-C ≡ N) containing organic compounds with the general formula RC ≡ N. These compounds are cyanide-substituted carboxylic acids and are produced naturally as well as synthetically. These

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are found naturally in plants, bone oils, insects, and microorganisms (SMITH 1965, DIGERONIMO and ANTOINE 1976, MUKRAM et al. 2015) and it has been proved that microorganisms can also synthesize them (KNOWLES 1976, BANERJEE et al. 2002). The synthetic nitriles have extensively been used in the manufacture of solvents, extractants, pharmaceuticals and drug intermediates (BANERJEE et al. 2002). Besides, they have also been used in the manufacture of herbicides such as dichlobenil (2,6-dichlorobenzonitrile), bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) (ASHTON and CRAFTS 1973, MUKRAM et al. 2015), the synthesis of polymers and plastics, and are widely used as organic solvents (HENAHAN and IDOL 1971, MUKRAM et al. 2015). Several studies on the application of nitrilases in chemical synthesis have been carried out, since the discovery of first nitrilase in the early 1960s (THIMANN and MAHADEVAN 1964, GONG et al. 2012). The common causes of nitrile entry into the environment include effluents from industrial processes either engaged in nitrile production or processing (WYATT and KNOWLES 1995, RODRIGUEZ 2014), accidental spillage of nitrile from the storage tank (DESHKAR et al. 2003), the use of nitrile compounds as chemical herbicides (VOSAHLOVA et al. 1997) and processing of shale deposit for oil extraction may lead to significant contamination of air, soil, and groundwater (HAWTHORNE et al. 1985, AISLABIE and ATLAS, 1988, RODRÍGUEZ 2014). Therefore, the removal of nitrile from industrial effluents and contaminated places should be of prime importance. Nevertheless, nitrilases play a key role in the bioremediation of hazardous nitriles from the contaminated air, soil, and water systems. Meanwhile, nitrilase-mediated bioremediation is an efficient method for scavenging highly toxic nitriles from environmental wastes and contaminants contaminants (MARTINKOVA et al. 2009, Vesela et al. 2010). The present investigation describes the isolation and characterization of two tropical bacterial species capable of metabolizing glutaronitrile and benzonitrile and a variety of other cyanogenic molecules.

Materials and Methods

Sample collection

Solid waste samples were collected from the dump sites at two locations, Olusosun, Ojota (Coordinates: N 6°29'21.8"; E 003°23'29.3") and Oke-Afa, Isolo (N6°27'11.0002"; E3°23'44.9999") in sterile sample bottles, properly labeled and stored at 4°C, and processed within 24 h. Figure 1 shows the satellite view of dump sites and sampling points.

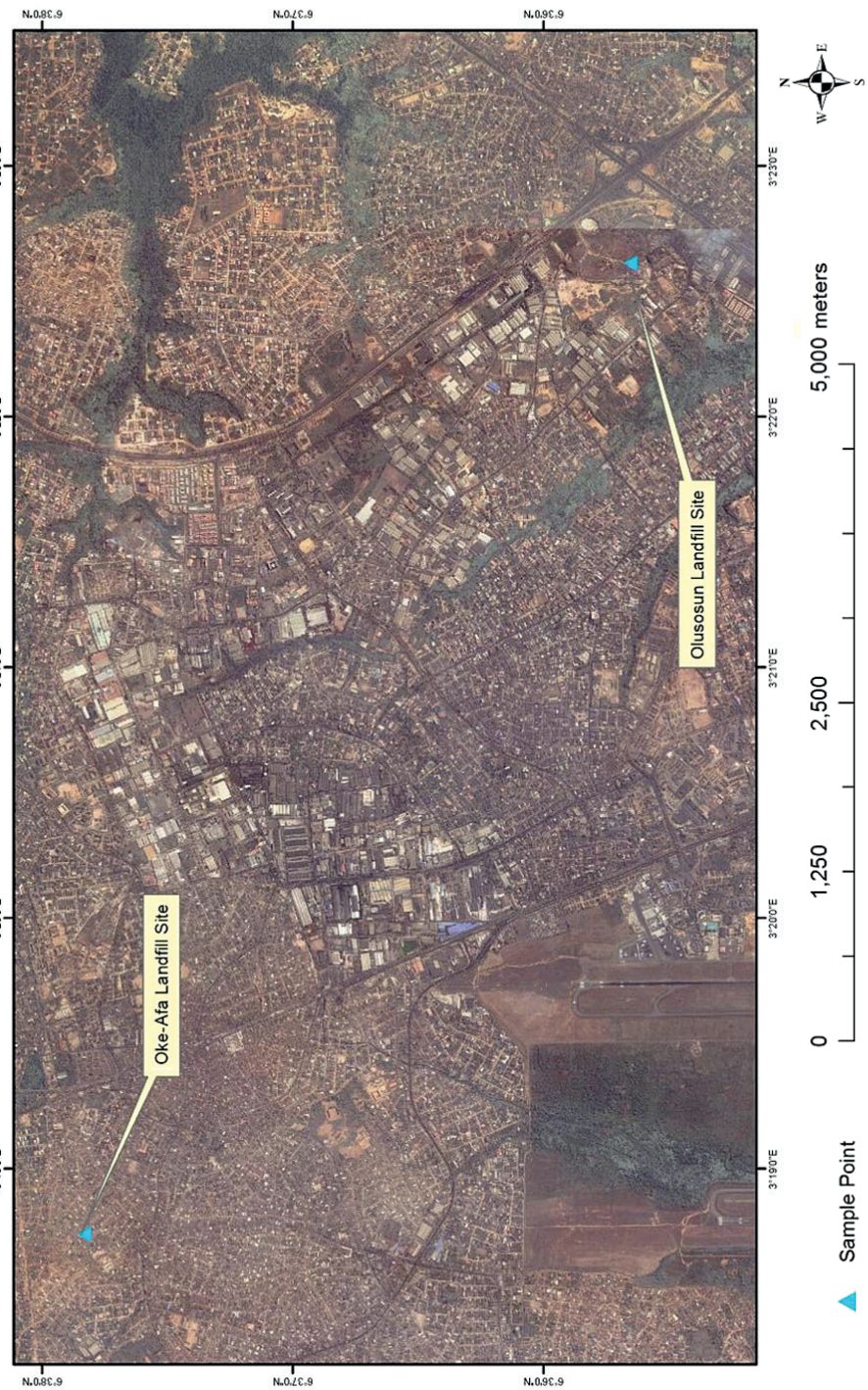

Fig. 1. Satellite image of Olusosun and Oke-Afa Landfill sites in Lagos State, Nigeria

Culture conditions and microbial bacterial enrichment

The bacteria capable of metabolizing glutaronitrile as the sole carbon and nitrogen source were isolated from solid waste leachates by selective enrichment culture technique (SANTOSHKUMAR et al. 2011). About 1.0 g of each of the solid waste samples was suspended in 50 ml of mineral salts medium (MSM) (K_2HPO_4 2.5; KH_2PO_4, 2.0; $MgSO_4.7H_2O$, 0.5; $MnSO_4.4H_2O$, 0.1; $CaCl_2.2H_2O$, 0.06; $FeSO_4.7H_2O$, 0.1; $Na_2MoO_7.2H_2O$, 0.006) supplemented with glutaronitrile (0.2% $^{v}/_{v}$) in a 250 ml Erlenmeyer flask. The flasks were incubated in an orbital shaker (180 rpm) at 32 °C for 7 days. For further enrichment, 5% inoculum was transferred to fresh MSM containing the same concentration of glutaronitrile. After several repeated subcultures, the culture was streaked on MS agar plates containing glutaronitrile. The colonies that grew on agar plates containing glutaronitrile but not on control plates (without glutaronitrile) were selected for further identification. The purity of the culture was checked periodically by plating on agar plates.

Cultural, morphological characteristics and microscopy

The cultural attributes of the isolates were observed visually on nutrient agar plates using a magnifying lens. Cellular morphology was observed using a light microscope. The fresh culture samples were used for the microscopy. The samples were sub-cultured on a fresh nutrient agar plate, smeared and then Gram-stained as described by OGUNYEMI et al. (2010). They were examined for Gram's staining status and other characteristics by using a compound microscope (Hitachi S-3500N model, ThermoNaran, Hitachi technologies, Schaumburg, Illinois, USA).

Biochemical characteristics

Various biochemical assays such as catalase reaction, oxidase, urease, indole, citrate utilization, nitrate reduction, methyl-red-Vogues Proskauer reaction and sugar fermentation were carried out as described by LANYI (1987). The pure cultures of the bacterial isolates were identified according to the identification criteria of Bergey's manual of determinative bacteriology (HOLT et al. 1994).

VITEK identification system

The available identification system was applied to the two (2) isolates obtained and the assays were performed according to the manufacturer's recommendations. VITEK 2 fluorescent card assay were carried out on the

two isolates and the results with very good identification of species of *Corynebacterium* sp. strain WOIS2 and *Bacillus* sp. strain WOD8 were subjected to 16S rRNA gene sequencing.

16S rDNA gene sequencing

The genomic DNA of the strains was extracted and purified following standard protocol for bacterial genomic DNA preparations using Jena Bioscience DNA preparation kits (Germany). 16S rDNA was amplified by Polymerase Chain Reaction (PCR) (94°C for 5 min, 30 cycles consisting of 94°C for 30 s, 55°C for 30 s, 72°C for 90 s followed by a terminal incubation at 72°C for 10 min) using universal 16S rDNA forward 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and reverse 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') primers. The quality of the PCR amplified DNA segments where check by electrophoresis on a 1.5% agarose gel stained with ethidium bromide, using 100 bp DNA marker (Promega, USA) as DNA standard, Millipore water (blank) was used as negative control. The gel was run for 80 min at 100 V, and the amplified products were observed and imaged by Kodak fluorescent imaging equipment, model IS 4000R (Kodak image station, care stream molecular imaging health Inc. Rochester, NY, USA.). Furthermore, the PCR amplified product was purified and the nucleotide sequence was determined with an automated sequencing apparatus (ABI PRISM 377, PE Biosystems Inc.). The 16S rDNA sequences of the strains were searched for homology with the sequences in public databases using the BLAST search program (http://www.ncbi.nlm.nih.gov/) to find closely related bacterial 16S rDNA gene sequences. The generated sequences were deposited in the National Centre for Biotechnology Information (NCBI) nucleotide sequence database as accession numbers KX774193 and KX774194. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 (TAMURA et al. 2007). Neighbor-Joining method was used to construct the phylogenetic tree.

Growth and substrate utilization studies

About 1.0 ml of 48 h grown pure isolate was inoculated in a 250 ml conical flask containing mineral salts medium (MSM 100 ml, pH 7.2, nitrile 0.2% v/v) and incubated at 30°C and 150 rpm. The aliphatic, as well as aromatic nitrile compounds, were tested for their abilities to support the growth of the nitrile-metabolizing bacteria. In order to determine the effect of supplementing glucose with glutaronitrile and benzonitrile in MSM for enhancing the growth of strains of WOD8 and WOIS2, a comparative growth analysis of strains was performed using mixture of each of

the nitrile (glutaronitrile and benzonitrile) and glucose (0.5% w/v). The culture broths were sampled at an interval of 48 h and tested for nitrilase activities over a period of 12 days. For each substrate, two sets of controls (the uninoculated: MSM with the substrate and the inoculated MSM with glutaronitrile and glucose and MSM with benzonitrile and glucose; without any substrate, were put in place to monitor the growth rate of each isolate and to rule out contamination. The bacterial growth was determined by recording the turbidity of the growth medium against the controls in UV-visible spectrophotometer (Thermo Fisher Scientific, USA) at 600 nm. The hydrolysis of glutaronitrile and benzonitrile was measured using spectrophotometer estimation of ammonia release at 630 nm (GUPTA et al. 2010, ALMATAWAH et al. 1999). The release of ammonia is an indicator of cyanide cleavage which simultaneously increases the pH of the medium.

Nitrilase assay

Culture filtrates collected at 48 h intervals from each of the culture flasks were centrifuged (10,000 g, 4°C, 10 min). The supernatants were used as the source of enzymes. The reaction mixture (3.0 ml) comprised of culture supernatant (1.0 ml), glutaronitrile (1.0 ml) and 1.0 ml phosphate buffer (0.2 M, pH 7.2). Nitrilase activity was measured as described earlier (GUPTA et al. 2010, ALMATAWAH et al. 1999) by monitoring the production of ammonia using a UV-visible spectrophotometer at 630 nm for 10 min. One unit of enzyme activity was defined as 1.0 mM of glutaronitrile oxidized per minute. The uninoculated medium was used as the control in all the experiments. All the experiments were carried out in triplicate.

Results

Morphological and biochemical characteristics

By selective enrichment culture technique, eight bacterial strains metabolizing glutaronitrile as the sole carbon source were isolated from solid waste leachates, out of which two were found to grow efficiently on aliphatic as well as aromatic nitrile compounds. Various morphological, physiological and biochemical characteristics of the strain WOD8 and WOIS2 are summarized in Table 1. The light microscopy showed that the strains were Gram's positive (Figure 2). The colonies of strain WOD8 appeared cream-colored, spreading with noticeable smell or pigmentation, while the colonies of strain WOIS2 were white-colored, glossy and non--spreading without any noticeable smell or pigmentation. The strain WOD8 was rod-shaped, while strain WOIS2 was rod-shaped either straight or slightly curved. They were catalase positive and oxidase negative.

Table 1

Morphological, physiological and biochemical characteristics of test strains

Characteristics	WOD8	WOIS2
Gram reaction	+	+
Shape	*R*	*R*
Colour	*C*	*W*
Motility	–	+
Growth	+	+
Catalase	–	+
Oxidase	–	–
Urease	–	+
H_2S	–	+
Indole	–	+
Citrate	+	+
MR	–	–
VP	–	–
Glucose	+	+
Lactose	+	+
Arabinose	+	+
Mannitol	+	–
Maltose	+	+
Putative identification	*Bacillus* sp.	*Corynebacterium* sp.

+ – positive reaction; – negative reaction; *C* – creamy, *R* – rod shaped

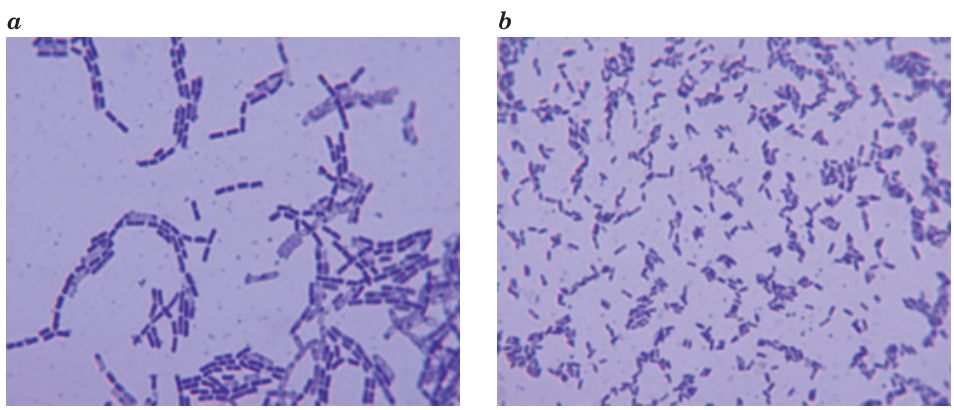

Fig. 2. Light microscopy of *Bacillus* sp. strain WOD8 (*a*) and *Corynebacterium* sp. strain WOIS2 (*b*) at x 1000 magnification. The scale bar indicates 10 μm

The isolates were capable of fermenting glucose, lactose, arabinose, mannitol, and maltose, except the strain WOIS2, which could not ferment mannitol and maltose. They utilized citrate and WOIS2 were found to be positive for H_2S production, urease and indole tests, whereas WOD8 was found to be negative for the same tests. They tested negative to methyl red and the Voges Proskauer test. The isolates were able to produce gas from glucose. The bacterial species were further characterized using VITEK 2 identification system.

Genotypic identities of isolates

The 16S rRNA gene sequences determined the positive identification of isolates. The corresponding genes of strains WOD8 and WOIS2 were sequenced (approximately 1500 bp each) with GenBank accession numbers KX774193 and KX774194, respectively. The homology search showed that the 16S rRNA genes of strain WOD8 had 99% similarity to *Bacillus* sp. Ba29b KU851836, whereas the 16S rRNA genes of strain WOIS2 shared 99% homology with *Corynebacterium* sp. 1031B 12EMannit KU644524 (Table 2).

Table 2
Genotypic identities of nitrile-metabolizing bacterial isolates from amplified sequences of 16S rRNA fragment of genomic DNA

Bacteria strain	Tentative identity	GenBank accession number	Closest strain (s)	% identity	GenBank accession number
WOD8	*Bacillus* sp.	KX774193	*Bacillus* sp. Ba29b	99	KU851836
WOIS2	*Corynebacterium* sp.	KX774194	*Corynebacterium* sp. 1031B12 12EMannit	99	KU644524

The dendrogram constructed for comparing the sequences of some other bacterial strains associated with nitrile degradation in the GenBank indicated that strain WOD8 belongs to the genus *Bacillus,* while WOIS2 belongs to the genus *Corynebacterium* (Figure 3). The dendrogram showed two major distinct clusters; the *Bacillus* group that was closely related and likely to have evolved from same ancestors, and the *Corynebacterium* group that was also closely related but distant from the *Bacillus* group. Strain WOIS2 belongs to cluster one having *Corynebacterium* sp. 1031B 12EMannit KU644524 as member while strain WOD8 belongs to cluster two which had *Bacillus* sp. Ba29b KU851836, as member (Figure 3).

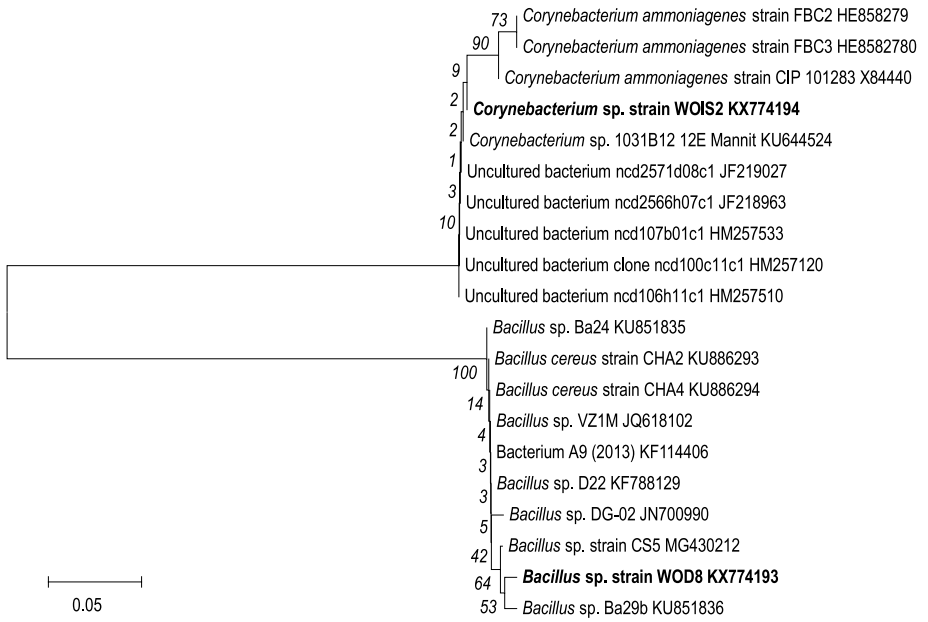

Fig. 3. Phylogenetic tree (dendrogram) of nitrile-degrading *Bacillus* and *Corynebacterium* species based on 16S rRNA sequences using the neighbor-joining method (SAITOU and NEI 1987). Bootstrap test = 1000 replicates. The evolutionary distances were computed using the Kimura[41] parameter method. Analysis involving 20 nucleotide sequences was computed using Mega 6 software

Growth studies of bacterial strains

The growth patterns are shown in Figure 4 and Table 3 revealed the nitrile-metabolizing potential of bacteria. They exhibited exponential growth patterns in the first two days. The highest growth exhibited by *Bacillus* sp. strain WOD8 on glutaronitrile (without supplementing glucose) was 0.552 (O.D 600 nm) with a specific growth rate of 0.057 d^{-1} and doubling time of 12.16 d. Whereas *Corynebacterium* sp. strain WOIS2 recorded highest growth of 0.859 (O.D 600 nm) with a specific growth rate of 0.088 d^{-1} and doubling time of 7.86 d on glutaronitrile (without supplementing glucose).

Table 3

The growth potentials of nitrile-metabolizing bacteria on glutaronitrile and benzonitrile in mineral salts medium

Isolate	μ (d^{-1})				T_d (d)			
	G	*G* + *g*	*B*	*B* + *g*	*G*	*G* + *g*	*B*	*B* + *g*
WOD8	0.057	0.071	0.044	0.066	12.16	9.76	15.75	10.5
WOIS2	0.088	0.091	0.052	0.085	7.86	7.62	13.33	8.15

μ – specific growth rate, T_d – doubling time, *G* – glutaronitrile (without supplementing glucose), *G* + *g* – glutaronitrile plus glucose (with supplementing glucose); *B* – benzonitrile (without supplementing glucose); *B* + *g* – benzonitrile plus glucose (with supplementing glucose)

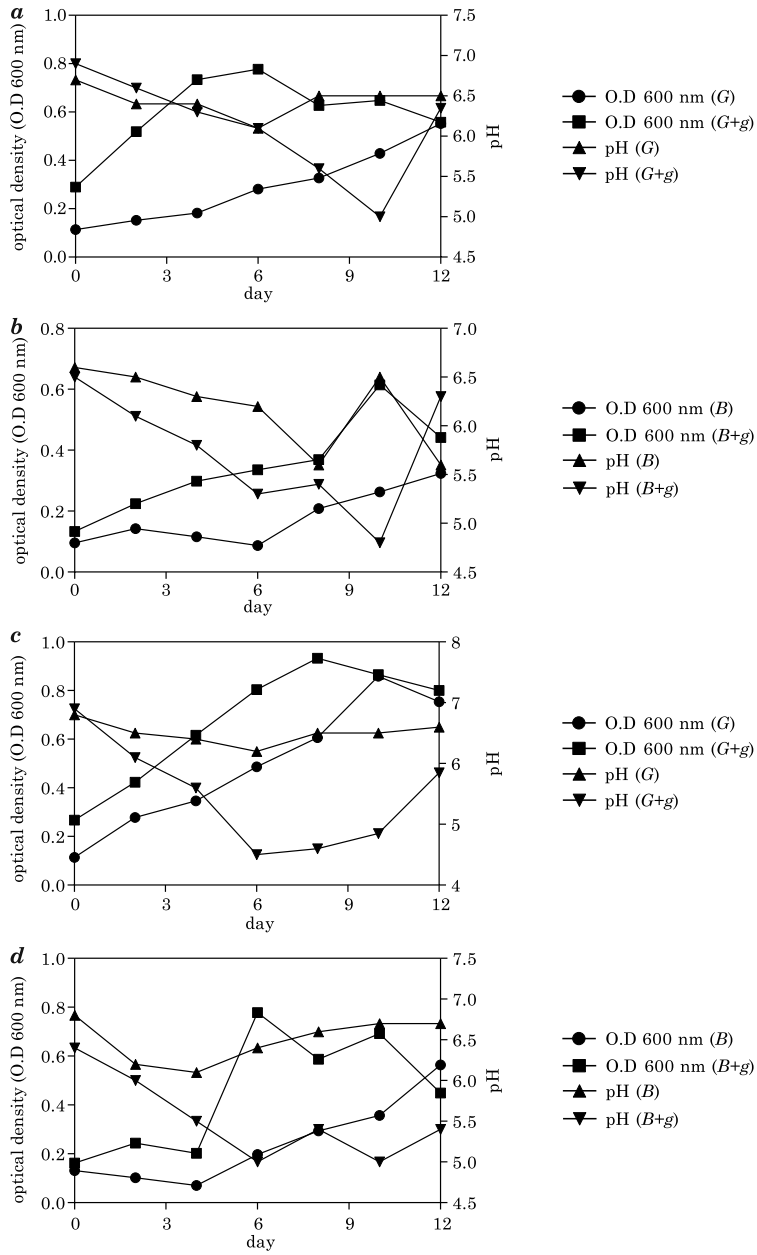

Fig. 4. Growth profiles and pH changes in the culture fluids of *Bacillus* sp. strain WOD8 and *Corynebacterium* sp. strain WOIS2 on glutaronitrile and benzonitrile: *a* – *Bacillus* sp. strain WOD8 on glutaronitrile (with or without supplementing glucose in mineral salts medium); *b* – *Bacillus* sp. strain WOD8 on benzonitrile (with or without supplementing glucose in mineral salts medium); *c* – *Corynebacterium* sp. strain WOIS2 on glutaronitrile (with or without supplementing glucose in mineral salts medium); *d* – *Corynebacterium* sp. strain WOIS2 on benzonitrile (with or without supplementing glucose in mineral salts medium). *G* – glutaronitrile (without supplementing glucose); *G* + *g* – glutaronitrile plus glucose (with supplementing glucose); *B* – benzonitrile without supplementing glucose; *B* + *g* – benzonitrile plus glucose (with supplementing glucose)

While the maximum growth recorded by *Bacillus* sp. strain WOD8 on supplemented glutaronitrile (with supplementing glucose) was 0.777 (O.D 600 nm) with a specific growth rate of 0.071 d^{-1} and doubling time of 9.76 d. whereas *Corynebacterium* sp. strain WOIS2 showed maximum growth of 0.933 (O.D 600 nm) with a specific growth rate of 0.091 d^{-1} and doubling time of 7.62 d on supplemented glutaronitrile (with supplementing glucose). Similarly, *Bacillus* sp. strain WOD8 had highest growth of 0.324 (O.D 600 nm) with a specific growth rate of 0.044 d^{-1} and doubling time of 15.75 d when grown on benzonitrile (without supplementing glucose). Whereas *Corynebacterium* sp. strain WOIS2 recorded highest growth of 0.564 (O.D 600 nm) with a specific growth rate of 0.052 d^{-1} and doubling time of 13.33 d on benzonitrile (without supplementing glucose). While *Bacillus* sp. strain WOD8 had maximum growth of 0.614 (O.D 600 nm) with a specific growth rate of 0.066 d^{-1} and doubling time of 10.5 d on supplemented benzonitrile (with supplementing glucose). Whereas *Corynebacterium* sp. strain WOIS2 had maximum growth of 0.778 (O.D 600 nm) with a specific growth rate of 0.085 d^{-1} and doubling time of 8.15 d on supplemented benzonitrile (with supplementing glucose).

The change in pH

Continuous monitoring of pH revealed a drop in pH (7.2–5.0) and (7.2–6.1) of the medium containing glutaronitrile (with or without supplementing glucose), respectively, inoculated with *Bacillus* sp. strain WOD8 (Figure 4). While on benzonitrile (with or without supplementing glucose), the pH of the culture medium with the same strain, sharply dropped from 7.2 to 4.8 and from 7.2 to 5.6, respectively (Figure 4). Similarly, when *Corynebacterium* sp. strain WOIS2 was grown on glutaronitrile (with or without supplementing glucose), the pH of the culture medium considerably dropped from 7.2 to 4.5 and from 7.2 to 6.2, respectively (Figure 4). While the growth of the same strain on benzonitrile (with or without supplementing glucose), the pH of the culture medium significantly dropped from 7.2 to 5.0 and from 7.2 to 6.1, respectively (Figure 4). From these analyses, it can be concluded that the supplementation of glucose in the culture medium promoted the substrate utilization by the test organisms.

Nitrilase activity

Bacillus sp. strains WOD8 and *Corynebacterium* sp.WOIS2 had maximum nitrilase activities of $3.44 \cdot 10^{-2}$ mg ml^{-1} min^{-1} (day 6) and $2.63 \cdot 10^{-2}$ mg $ml^{-1}min^{-1}$ (day 8) respectively when grown on glutaronitrile (without supplementing glucose) (Figure 5). While optimum nitrilase activities

of $3.94 \cdot 10^{-2}$ mg $ml^{-1}min^{-1}$ (day 6) and $3.89 \cdot 10^{-2}$ mg ml^{-1} min^{-1} (day 8) were obtained by the same strains on supplemented glutaronitrile (with supplementing glucose) (Fig. 5). On the other hand, the maximum nitrilase activities of $1.89 \cdot 10^{-2}$ mg $ml^{-1}min^{-1}$ (day 8) and $2.12 \cdot 10^{-2}$ mg ml^{-1} min^{-1} (day 8) were observed by strains WOD8 and WOIS2 when grown on benzonitrile (without supplementing glucose). While maximum nitrilase activities of $2.52 \cdot 10^{-2}$ mg $ml^{-1}min^{-1}$ (day 6) and $2.56 \cdot 10^{-2}$ mg $ml^{-1}min^{-1}$ (day 10) on were recorded by the same strains on supplemented benzonitrile (with supplementing glucose) – Figure 5. In the previous studies,

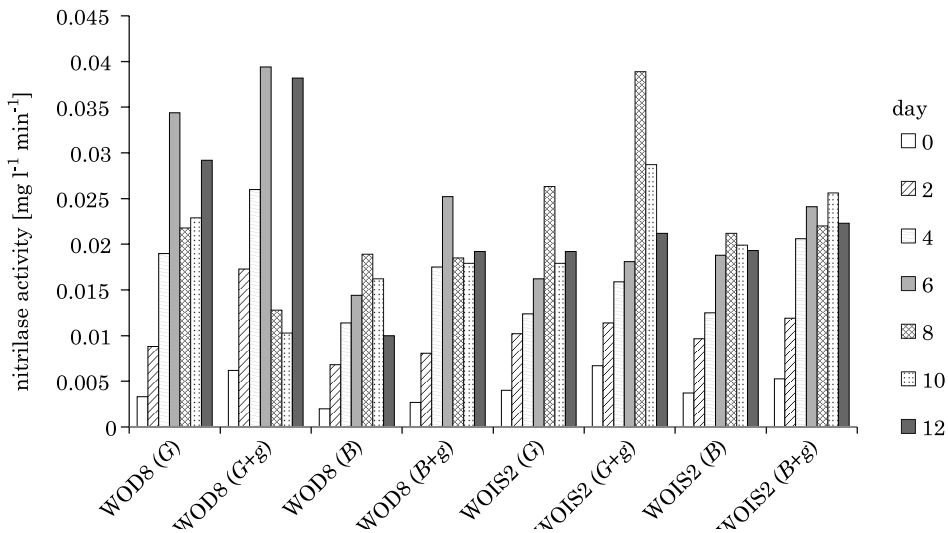

Fig. 5. Niltrilase activities of *Bacillus* sp. (WOD8) and *Corynebacterium* sp. (WOIS2) in growth cultures:
G – Glutaronitrile (without supplementing glucose); *G* + *g* – Glutaronitrile plus glucose (with supplementing glucose); *B* – Benzonitrile (without supplementing glucose); *B* + *g* – Benzonitrile plus glucose (with supplementing glucose)

the selected strains showed varying detoxifying potentials when cyanogenic natural growth substrates were used (OGUNYEMI et al. 2019). In general, the supplementation of glucose with each of the nitrile substrates promoted the nitrilase activity.

Discussion

This study represents the first reported characterization of two nitrile-metabolizing bacterial species from solid waste leachates in Nigeria. The two nitrile-metabolizing bacterial species were isolated from solid waste leachates by selective enrichment technique, which were identified

as *Bacillus* sp. strain WOD8 KX774193 and *Corynebacterium* sp. strain WOIS2 KX774194. The combination of morphological, cultural, and the biochemical characteristics, as well as the 16S rRNA gene sequences identified strains WOD8 and WOIS2 to belong to the genus *Bacillus* and *Corynebacterium,* respectively. The characteristic features of the strains were similar to those reported previously by BRENNAN et al. (2001), YASSIN et al. (2003), LOGAN et al. (2009) and LUDWIG et al. (2007). The result of the phylogenetic tree shows that both the organisms are closely related to each other. *Bacillus* sp Ba29b KU851836 is closest to the strain WOD8, while *Corynebacterium* sp. 1031B12 12EMannit KU644524 is the nearest neighbor of strain WOIS2. The sequence analysis of 16S rRNA gene and unrooted phylogenetic tree showed that *Bacillus* and *Corynebacterium* spp. are likely to have evolved from the same ancestor; however, belong to two different clusters. Although, YAMADA et al. (1980) isolated identified *Pseudomonas* sp. strain K-9; as capable of utilizing only glutaronitrile but failed to utilize other nitrile compounds as growth substrates. Also, DIGERONIMO and ANTOINE (1976) isolated a strain of *Nocardia rhodochrous* that metabolized a selected number of nitrile compounds and their derivatives to carboxylic acids and ammonia. NAWAZ et al. (1989) isolated bacterium capable of utilizing high concentrations of acetonitrile as the sole source of carbon and nitrogen from which had increased growth rates in the media containing range of nitrile compounds including range of nitrile compounds including butyronitrile, glutaronitrile, isobutyronitrile, methacrylonitrile, propionitrile, succinonitrile, valeronitrile, and their corresponding amides, such as acetamide, butyramide, isobutyramide, methacrylamide, propionamide, and succinamide. KAO et al. (2006) and MUKRAM et al. (2015) reported utilization of acetonitrile, propionitrile, benzonitrile, phenyl acetonitrile and butyronitrile by *Klebsiella oxytoca*. *Rhodococcus* sp. MTB5, capable of utilizing benzonitrile as the sole source of carbon and nitrogen, was isolated from a nitrile-contaminated agricultural soil sample by selective enrichment culture technique. In this study, *Bacillus* sp. strain WOD8 and *Corynebacterium* sp. strain WOIS2 isolated were able to utilize both aliphatic (glutaronitrile) and aromatic nitriles (benzonitrile) as a carbon source. BAXTER et al. (2006) explored the potential of a known acetonitrile-metabolizing organism *Rhodococcus* sp. AJ270 for the degradation of acetonitrile and investigated its effects on soil bacterial community, and postulated that the use of such microorganism could play an important role in the detoxification of the toxic compound and thereby, decreasing the risk of environmental contamination. KOBAYASHI and SHIMIZU (1998) proposed that the use of specialized consortia of microorganisms could be a viable alternative to activated sludge for the degradation

and management of toxic chemical wastes. Likewise, KOHYAMA et al. (2006) developed a process for the treatment of acetonitrile-containing wastes by employing two nitrile-degrading microorganisms, *viz., Rhodococcus pyridinivorans* S85-2 and *Brevundimonas diminuta* AM10-C as a source of NHase and amidase, respectively. These strains rapidly established in the nitrile soil community and successfully carried out the bioremediation of nitrile contamination and the addition of acetonitrile significantly affected the composition of the bacterial community in the soil. The drift in pH of the culture medium within 12 days of incubation further confirmed changes in the composition of nitrile substrates possibly degraded by nitrilases (ZHOU et al. 2005). The microbial degradation of nitriles often leads to the production of carboxylic acids and ammonia (HOWDEN and PRESTON 2009). Hence, the formation of acids may probably lead to the reduction in pH levels, which advocates the nitrile utilizing abilities of the strains. Also, in this study, the concentration of ammonia in the residual medium was estimated at different time intervals for the highest accumulation. The prolonged incubation of the culture failed to increase the concentration of ammonia and pH of the medium. The time course study showed a decrease in the concentrations of glutaronitrile and benzonitrile accompanied by the accumulation of ammonia. It was observed that the growth of the selected strains was optimum in glutaronitrile than benzonitrile. Interestingly, the strains displayed substrate dependent nitrilase activities. Furthermore, the bacterial species were grown in cassava effluent and solid waste leachates as natural cyanogenic substrates (sole carbon sources) and they efficiently degraded the cyanogens (OGUNYEMI et al. 2019). The current study claims that the culture conditions and the nature of substrate are the most important factors in the production of nitrilase.

Conclusion

In the present study, the bacterial strains capable of utilizing glutaronitrile and benzonitrile were isolated, characterized, and identified as *Bacillus* sp. strain KX774193 and *Corynebacterium* sp. strain KX774194. These strains were able to grow successfully in aliphatic as well as aromatic nitriles as determined by the growth kinetics. Besides, the strains have the ability to utilize the natural substrate of both cassava effluent as well as solid waste leachates. Therefore, these two strains may be used as the promising tool for the remediation of sites contaminated with both aliphatic and aromatic nitriles. Further research is going on that would focus on deciphering the metabolic pathways and determining the degradative enzymes involved and their metabolic products.

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References

AISLABIE J., ATLAS R.M. 1998. *Biodegradation of nitriles in shale oil*. Appl. Environ. Microbiol., 54: 2197–2202.

ALMATAWAH C.A.R., CRAMP R., COWACH. D.A. 1999. *Characterization of an inducible nitrilase from a thermophilic Bacillus*. Extremophiles, 3: 283–291.

ASHTON F.M., CRAFTS A.S. 1973. *Mode of action of herbicides*. John Wiley & Sons, Inc., New York, pp. 236–255.

BANERJEE A., SHARMA R., BANERJEE U.C. 2002. *The nitrile-degrading enzymes: current status and future prospects*. Appl. Microbiol. Biotechnol., 60: 33–44.

BAXTER J., GARTON N., CUMMINGS S.P. 2006. *The impact of acrylonitrile and bioagumentation on the biodegradation activity and bacterial community structure of a topsoil*. Folia Microbiol., 51: 591–597.

BRENNAN N.M., BROWN R., GOODFELLOW M., WARD A.C., BERESFORD T.P., SIMPSON P.J., FOX P.F., COGAN T.M. 2001. *Corynebacterium mooreparkense sp. nov. and Corynebacterium casei sp. nov., isolated from the surface of a smear-ripened cheese*. Int. J. Syst. Evol. Microbiol., 51: 843–852.

DESHKAR A., DHAMORIKAR N., GODBOLE S., KRISHNAMURTHI K., SARAVANADEVI S., VIJAY R. 2003. *Bioremediation of soil contaminated with organic compounds with special reference to acrylonitrile*. Ann. Chim., 93: 7297–37.

DIGERONIMO M.J., ANTOINE A.D. 1976. *Metabolism of acetonitrile and propionitrile by Nocardia rhodochrous LL1100–21*. Appl. Environ. Microbiol., 31: 900–906.

GONG J.S., LU Z.M., LI H., SHI J.S., ZHO Z.M., XU Z.H. 2012. *Nitrilases in nitrile biocatalysis: recent progress and forthcoming research*. Microbial Cell Factories, 11: 142–160.

GUPTA V., GAIND S., VERMA P.K., SRIVASTAVA A.K. 2010. *Purification and characterization of intracellular nitrilases from Rhodococcus sp.-potential role of periplasmic nitrilases*. African J. Microbiol. Res., 4(11): 1148–1153.

HAWTHORNE S.B., SIEVERS R.E., BARKLEY R.M. 1985. *Organic emissions from shale oil wastewaters and their implications for air quality*. Environ. Sci. Technol., 19: 992–997.

HENAHAN J.F., IDOL J.D. 1971. *Setting the world of nitrile chemistry afire*. Chem. Engin. News, 49: 16–18.

HOLT J.G., KRIEG, N.R., SNEATH P.H.A., STALEY J.T., WILLIAMS S.T. 1994. *Bergey's manual of determinative bacteriology*, 9th edn. Baltimore, Williams & Wilkins.

HOWDEN A.J., PRESTON G.M. 2009. *Nitrile enzymes and their role in plant microbe interactions*. Micro. Biotech., 2(4): 441–451.

KAO C.M., CHEN K.F., LIU J.K., CHOU S.M., CHEN S.C. 2006. *Enzymatic degradation of nitriles by Klebsiella oxytoca*. Appl. Microbiol. Biotechnol., 71(2): 228–233.

KNOWLES C.J. 1976. *Microorganisms and cyanide*. Bacteriol. Rev., 40: 652–680.

KOBAYASHI M., SHIMIZU S. 1998. *Metalloenzyme nitrile hydratase: structure, regulation and application to biotechnology*. Nat. Biotechnol., 16: 733–736.

KOHYAMA E., YOSHIMURA A., AOSHIMA D., YOSHIDA T., KAWAMOTO H., NAGASAWA T. 2006. *Convenient treatment of acetonitrile containing wastes using the tendem combination of nitrile hydratase and amidase-producing microorganism*. Appl. Microbiol. Biotechnol., 72: 600–606.

LANYI B. 1987. *Classical and rapid identification methods for medically important bacteria.* Methods Microbiol., 19: 1–67.

LOGAN N.A., BERGE O., BISHOP A.H., BUSSEH.J., DE VOS P., FRITZE D., HEYNDRICKX M.R.A., KÄMPFER P., RABINOVITCH L., SALKINOJA-SALONEN M.S., SELDIN L.,VENTOSA A. 2009. *Proposed minimal standards for describing new taxa of aerobic, endospore-forming bacteria.* Int. J. Syst. Evol. Microbiol., 59: 2114–2121.

LUDWIG W., SCHLEIFER K.H., WHITMAN W.B. 2007. *Revised road map to the phylum Firmicutes.* Bergey's Manual Trust website, http://www.bergeys.org/outlines/Bergeys,Vol 3, Outline.pdf (2007).

MARTÍNKOVÁ L., UHNÁKOVÁ B., PÁTEK M., NEŠVERA J., V. KŘEN V. 2009. *Biodegradation potential of the genus Rhodococcus.* Environ. Int., 35: 162–177.

MUKRAM I., NAYAK A.S., KIRANKUMAR B., MONISHA T.R., REDDY P.V., KAREGOUDAR T.B. 2015. *Isolation and identification of a nitrile hydrolyzing bacterium and simultaneous utilization of aromatic and aliphatic nitriles.* Int. Biodete. Biodegr., 100: 165–171.

NAWAZ M.S., CHAPATWALA K.D., WOLFRAM J.H. 1989. *Degradation of Acetonitrile by Pseudomonas putida*. Appl Environ Microbiol., 55(9): 2267–2274

OGUNYEMI A., AMUND O., OKPUZOR J., ADEIGA A., IDIKA N., AHMED O. 2010. *Physicochemical properties of municipal refuse in Lagos metropolis and cellulolytic activities of resident microorganisms associated with organic matter degradation.* Int. J. Biol. Chem. Sci. 4 (1): 209–217.

OGUNYEMI A.K., SAMUEL T.A., AMUND O.O., ILORI M.O. 2019. *Cassava wastewater and Solid waste leachate as substrates for the Growth of Nitrile and Linamarin-Utilizing Bacteria.* J. Trop. Life Sci., 9(1): 79–87.

RODRÍGUEZ J.R. 2014. *Understanding nitrile-degrading enzymes: classification, biocatalytic nature and current applications.* Revista Latinoamericana de Biotecnología Ambiental y Algal, 5(1), 8–25.

SANTOSHKUMAR M., VEERANAGOUDA Y., KYOUNG L., KAREGOUDAR T.B. 2011. *Utilization of aliphatic nitrile by Paracoccus sp. SKG isolated from chemical waste samples.* Int. Biodeterior. Biodegrad., 65(1):153–159.

SMITH P.A.S. 1965. *Open chain nitrogen compounds*, vol. 1. W.A. Benjamin, Inc., New York.

TAMURA K., DUDLEY J., NEI M., KUMAR S. 2007. MEGA4. *Molecular evolutionary genetics analysis (MEGA) software version 4.0.* Mol Biol. Evol., 24: 1596–1599.

THIMANN K.V., MAHADEVAN S. 1964. *Nitrilase.* I. *Occurrence, preparation, and general properties of the enzyme.* Arch. Biochem. Biophys., 105: 133–141.

VESELÁ A., FRANC M., PELANTOVÁ H., KUBÁČ D., VEJVODA V., ŠULC K., BHALLA. T., MACKOVÁ M., LOVECKÁ P., JANŮ P., DEMNEROVÁ K., MARTÍNKOVÁ L. 2010. *Hydrolysis of benzonitrile herbicides by soil actinobacteria and metabolite toxicity.* Biodegr., 21: 761–770.

VOSAHLOVA J., PAVLU L., VOSAHLO J.V. 1997. *Degradation of bromoxynil, ioxynil and dichlobenil and their mixtures by Agrobacterium radiobacter.* Plastic Sci., 49: 303–306.

WYATT J., KNOWLES C. 1995. *Microbial degradation of acrylonitrile waste effluents: the degradation of effluents and condensates from the manufacture of acrylonitrile.* Int. Biodeterior. Biodeg., 35: 227–248.

YAMADA H., ASANO Y., TANI Y. 1980. *Microbial utilization of glutaronitrile.* J. Ferment. Technol., 6: 495–500.

YASSIN A.F., KROPPENSTEDT R.M., LUDWIG W. (2003). *Corynebacterium glaucum sp. nov.* Int. J. Systematic Evolutionary Microbiol., 53: 705–709.

ZHOU Z.M., HASHIMOTO Y., KOBAYASHI M. 2005. *Nitrile degradation by Rhodococcus. Useful microbial metabolism for industrial productions.* Actinomycetologica, 19: 18–26.

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COMPARISON OF OLEOGELS PROPERTIES OBTAINED WITH DIFFERENT STRUCTURE-FORMING SUBSTANCES

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Key words: ethyl cellulose, monoacylglycerols, structured fats, mechanical properties, stability.

Abstract

Evaluation of selected quality parameters of oleogels made with one type of lipid solvent – rapeseed oil was done. The effect of two gelling agents: ethyl cellulose (EC) and monoacylglycerol (MAG) on the hardness, lubricity and stability of organogels was determined. Oleogels containing 7% and 8% w/w of single EC or MAG were prepared, as well as mixed variants with 3.5% MAG & 4.0% EC and EC 3.5% & 4.0% MAG in rapeseed oil. It has been shown that both: ethyl cellulose and monoacylglycerol had structural-forming properties. The greatest hardness and the least spreadability was found in the oleogel with an 8% polysaccharide addition. EC showed good ability of gelling rapeseed oil but organogels with EC were less stable in time compared to the single-monoacylglycerol oleogels. Mixed variants had the lowest stability compared to oleogels with the addition of a single gelling substance.

Introduction

Vegetable oils containing essential unsaturated fatty acids are a desirable ingredient in the daily diet. Due to the presence of acids from the group of n-3, n-6, n-9, antioxidants and the lack of cholesterol are recommended for consumption by both food technologists and dieticians (Lin et al. 2013). Confectionery or bakery products require the presence of solid

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lipids in order to obtain the appropriate sensory and physical quality (ŻBIKOWSKA 2010, ŻBIKOWSKA et al. 2015). In technological terms, liquid oils are not, therefore, recommended substitutes for shortening or other solid bakery fats, which contain nutritional undesirable fatty acids isomers in trans configuration (JANG et al. 2015, HWANK et al. 2016, DOAN et al. 2017). The search for an alternative to these lipid food ingredients has become one of the reasons for the formation of oleogelation (MARANGONI and GARTI 2015, PATEL 2015). It is a process of forming semi-solid consistency of liquid oil with the use of a gelling substance (MARANGONI 2012). Oleogelation is a physical transformation, based on the dissolution of gelators in a lipid medium whose chemical properties do not change during process (PATEL and DEWENTTINCK 2016). Gelling substances that are currently used in the production of oleogels are, among others, polysaccharides (e.g., ethyl cellulose, hydroxypropyl methyl cellulose) monoacylglycerol, natural waxes, or low molecular weight gelators (LMWGs) (WRIGHT and MARANGONI 2007, HUGHES et al. 2009, HWANK et al. 2016, SAGIRI et al. 2017, MENG et al. 2018).

The most commonly described compound in the literature, having the ability to gel vegetable oils is ethyl cellulose (EC). It consists of 1,4-D-glucose molecules linked to the ethyl groups at 2.3 and 6 carbon atoms (ROGERS et al. 2014). It is the only cellulose derivative that has the ability to be directly dispersed in liquid oil (PATEL and DEWETTINCK 2016). The non-smooth, rough structure of the network formed by ethyl cellulose ensures the maintenance of oil particles (ROGERS et al. 2014). This biopolymer is one of the most effective gelling agent used in the food industry (DAVIDOVICH-PINHAS et al. 2016). It is generally permitted for use in food in accordance with the principle of *quantum satis* as an additional substance (Regulation of the Minister of Health… 2010). According to STORTZ et al. (2013), ethyl cellulose is one of the gelling agents with the ability to create a network that maintains fat molecules, e.g. in chocolate or nut butter. In the form of oleogel, EC could be used as a lipid component of frankfurters (ZETZL et al. 2012).

Another substance that has the ability to form a gel in a lipid solvent is monoacylglycerol (MAG). It is a single chain of fatty acid (most commonly palmitic or stearic acid), connected by an ester bond with a glycerol molecule (EICHMANN and KNITTELFELDER 2015, CHEN and TERENTJEV 2015). According to GOLDSTEIN et al. (2012), monoacylglycerols exist in two crystalline forms: α and β. The first of these is used to produce baked products, e.g. cakes, because it facilitates the insertion of air into the fat phase. The crystalline form β is in turn more suitable for harder products, such as biscuits, due to its greater hardness.

Monoacylglycerol-contained organogels may be produced using one of the two methods: as an oil solutions or water-lipid mixtures. However, those that have water in their structure are four times less durable (GOLDSTEIN et al. 2012). According to CHEN and TERENTJEV (2015), monoacylglycerol organogels, in particular, could be used in the production of ice cream, as ingredients that create their creamy consistency.

This paper compares two additional substances, belonging to different groups of chemical compounds: ethyl cellulose (polysaccharide) and monoacylglycerol (lipid), as single and mixed gelators of rapeseed oil. Their impact on selected qualities of oleogels (hardness, spreadability, as well as centrifugal and filtration stability) were evaluated.

Materials and Methods

Raw materials and preparation of organogels

Ethyl cellulose (EC) and monoacylglycerol oleogels (MAG) were made in two variants. The first one contained 7%, the second 8% of a single gelling substance. The organogels, being a mixture of the mentioned gelators, consisted of 3.5% EC (EC45 Std Premium – a gift from Dow Chemical Company, Germany) and 4.0% MAG (Lasenor, Spain), or 4.0% EC and 3.5% MAG (two variants). In all oil gels, rapeseed oil (ZT "Kruszwica" S.A., Poland) was used as a lipid solvent.

Preparation of organogels consisted in dissolving a gelling substance of a specified concentration in rapeseed oil at a temperature allowing the dissolving of gelators, i.e. 90°C for monoacylglycerol lipid gels and 140–160°C for ethyl cellulose and mixed oleogels. Prior to qualitative determinations, the oleogels were allowed to solidify for 24 hours at a temperature of 20 ± 1°C.

Analysis of fats

The rapeseed oil was subjected to derivatization into fatty acid methyl esters (*Animal and vegetable...* ISO 5509:2001) to the content analysis of FA by the GC method in gas-liquid partition, according to recommendations in the ISO standards (*Animal and vegetable...* ISO 15304:2003, *Animal and vegetable...* ISO 5508:1996). The analysis was carried out using the gas chromatograph Agilent Technologies 6890 II with the software ChemStation with an FID detector, equipped with an SGE BPX 70 capillary column. The temperature during the analysis was maintained between

140 to 220°C. Identification of peaks of fatty acids was carried out by comparing them with the retention time of samples of fatty acid methyl esters (Supelco 37, Sigma Aldrich, St. Louis, MO, USA) – Table 1.

Table 1

The fatty acids composition of rapeseed oil (g FA/100 g FA)

Fatty acids									
C16:0	C18:0	C18:1	C18:2	C18:3	C20:0	C22:0	C20:1	C22:1	another
3.6±0.02	2.4±0.01	57.9±1.02	21.1±0.05	9.8±0.01	2.1±0.01	0.6±0.02	1.0±0.02	1.1±0.03	0.4±0.02

Hardness of oleogel

The hardness of oleogels were shown using penetration test. Samples were tested using a TX.AT plus device (Micro Stable Systems, UK). It consisted of dipping the cylinder shape tip (P/0.5R), with a diameter of 1 cm in the sample to a depth of 5 mm. Samples were poured into glass weighing dishes (diameter 3.5 cm) to a height (H) of approx. 2.5 cm. The speed at which the rod moved was 1 mm s^{-1}. Results were obtained in the form of curves on the graph, illustrating the force (y-axis) in time (x-axis). The highest penetration value obtained, expressed in Newton, was the hardness of the gel. The measurements were realized executed at room temperature. A total of twelve determinations were carried out for each type of oleogel.

Spreadability of olegel

The spreadability determination was based on the penetration test, which consists in submerging the tip of the measuring apparatus in the shape of a cone (spreadability ring) with a diameter of 45 mm, in a special container perfectly suited to it. The speed at which the upper working element was lowered (until 1 mm gap obtained between the two measuring parts) was 3 mm s^{-1}. The test was performed using a TX.AT plus device (Micro Stable Systems, UK) at room temperature. Spreadability was determined as a total value of twelve for each type of oleogel in the N mm unit, as the area under the curves on the graph illustrating the force (y-axis) in time (x-axis).

Stability of oleogels – centrifugal separation method

The stability of the oleogels was determined on the modified method of Da Pieve et al. (2010). The oil-gel samples were transferred to empty tubes (mt) at 3.00 g weight. The whole samples were centrifuged at 3500 rpm. in the MPW-340 centrifuge (MPW Med. Instruments, Poland). The duration

of the process was 20 minutes, after which the mass of the gel and the centrifuged oil (mgo) was determined. The liquid collected at the top of the test tube was drained. The residue was weighed with the tube [mg]. Each variant was subjected to nine determinations.

The stability of the oleogel determined by the centrifugal method (ST) was calculated from the modified YILMAZ and ÖĞÜTCÜ (2014) formula:

$$ST = \frac{(mg - mt)}{(mgo - mt)} \cdot 100\%$$

Oil binding capacity

The oil-binding measurements were managed using modified BLAKE et al. (2014) method. It consisted in measuring mass (gram) of rapeseed oil leakage, filtered through a 150 mm diameter filter paper (Munktell-Ahlstrom, Germany) at 20 ± 1°C. Thirty-gram samples of all the oleogels were placed on glass funnels with filter paper located in pre-weighed conical flasks. After 15, 30, 45, 60, 90, 120, 180, 240, 300, 360 minutes, and after 24, 48 and 72 hours, rapeseed oil leakage was weighed. The oil binding capacity of gelling agents was determined as the percentage loss of the initial weight of organogel. It was measured in sixplicate.

Statistical analysis

Statistical preparation was carried out using the software Statgraphics Plus 4.1. Analysis of the obtained results of physical parameters of oleogel was performed using one-way analysis of variance and regression. The assessment of the significance of differences between the means was performed using Tukey Test ($p \leq 0.05$). The regression method was used for model determination. Based on the experimental data for each dependent variable (y – mass of liquid fraction – the size of mass loss) equations were formulated ($y = \alpha_0 + \alpha_1 + \ldots \alpha_k x$) in which $\alpha_{0,1}, \ldots_k$ are appropriate coefficients determining intercepts, and x is the time of measurement.

Results and Discussion

The effect of gelling agents on the textural parameters of oleogels

Hardness is a mechanical texture feature, expressing the force that causes a specific deformation. The results showed that the highest hardness was characterized by oleogels with the highest percentage of single

gelling ingredient, i.e. 8% polymers (EC) and 8% monoacylglycerol (MAG). The maximum force causing their deformation in the penetration test was 1.8 N and 1.7 N (Figure 1). This proves that the higher the concentration of a single gelling substance in rapeseed oil, the harder the oleogel structure. Organogels, being a mixture of two gelling substances, were characterized by lower hardness (more than twice) in the case of lower EC content (for the variant 3.5% MAG & 4.0% EC – 0.7 N and for 3.5% EC & 4.0 % MAG – 0.3 N). This results suggesting that polysaccharide gelator causes an increase in the hardness of the organogels.

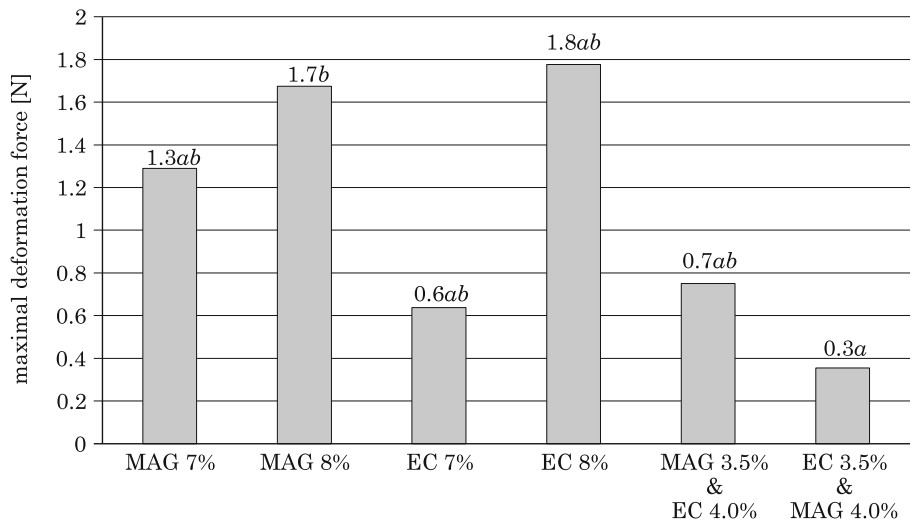

Fig. 1. Hardness of oleogels [N]; *a, b – different letters indicate mean values being statistically significant, $p \leq 0.05$

GRAVELLE et al. (2014) proved that as the percentage of EC in rapeseed oil increases, the hardness of the gel increases. Similar relationships were found in the tested ethyl cellulose oleogels, produced with the participation of rapeseed oil (Figure 1). SI et al. (2016) also obtained such a link, but for two variants of monoacylglycerol oleogels, containing 3% and 6% MAG. The solvent for mentioned gelling agent was soybean oil. The hardness of oleogels tested by SI et al. (2016), increased with increasing MAG concentration and was successively: 0.1 N and 0.4 N. These values are lower than obtained in organogels containing rapeseed oil as a solvent, which additionally confirms the dependence of force from monoacylglycerol concentration in the penetration test.

The Figure 2 presents the value of area, under the force-time penetration curve, interpreted as the spreadability. It is a parameter that charac-

terizes the texture of food e.x. margarines, creams, peanut butter. The greater its value, the worse the possibility of spreading them (JAKUBCZYK et al. 2014). The obtained data indicate an upward trend in the spreadability values of oleogels containing a single gelling substance (MAG or EC), along with their increasing percentage in organogels.

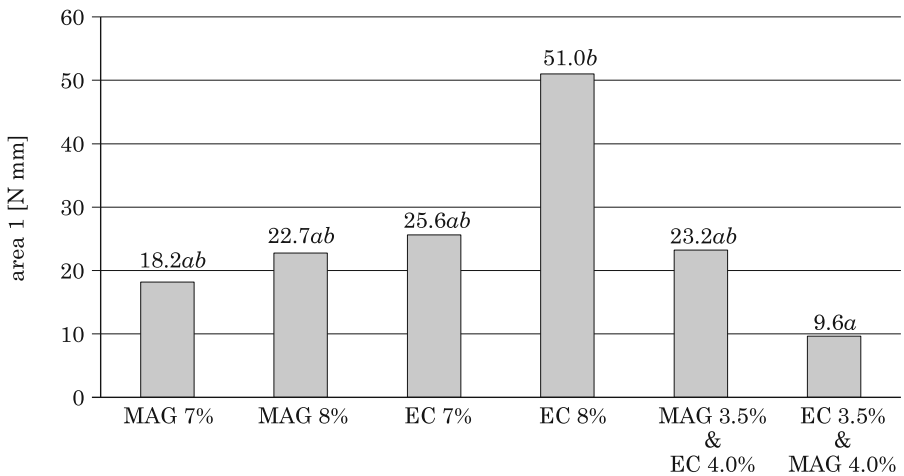

Fig. 2. Spreadability of oleogels [N mm]; *a, b – different letters indicate mean values being statistically significant, $p \leq 0.05$

The highest value of the tested parameter – 51.0 N mm, was obtained by the variant containing 8% concentration of ethyl cellulose in rapeseed oil. Oleogel with an 8% addition of the lipid gelator MAG had more than twice the lower spreadability value (22.7 N mm). The results indicate the effect of ethyl cellulose on the increase in the value of this parameter. Among mixed oleogels, the variant with higher concentration of ethyl cellulose – 3.5% MAG & 4.0% EC obtained a more-than-twice-higher spreadability value, which also confirms a stronger structural effect of the polysaccharide gelling agent (EC).

Stability of the tested oleogels

For examination the oil binding capacity of gelling agents, a stability test was carried out using the centrifuge method. The results present a relatively high durability of the structure of the all organogels (Figure 3). The mean values did not differ significantly and ranged from 97.9% for the variant containing 8% ethyl cellulose to 96.7%, for the oleogel with 3.5% MAG & 4.0% EC. This proves that both mentioned gelators created a stable oleogel network, resistant to centrifugal force.

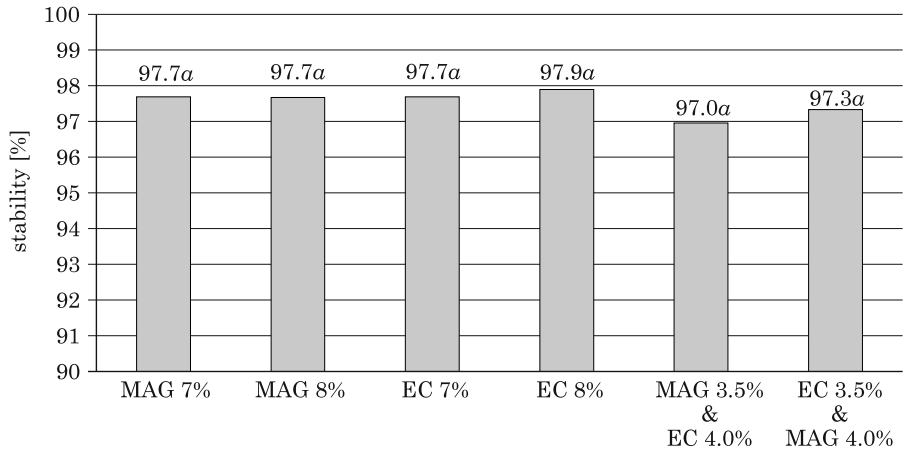

Fig. 3. Stability of oleogels; *a – homogenity group

YILMAZ and ÖĞÜTCÜ (2014) received 99.8% stability for oleogel with a 7% share of monoacylglycerol in hazelnut oil. This is a 2.1% higher parameter value, compared to an organogel with the same content of gelling substance in rapeseed oil (Figure 3). The difference in the stability of the mentioned oleogels indicates a more stable bonding of hazelnut oil by monoacylglycerol. This also presents, that the oil binding capacity is affected not only by the concentration of the gelling substance (MAG), but also by the type of lipid solvent.

Determination of stability by filtration was aimed to present the durability of the structure of oleogels at room temperature depending on time. The weight of the oil phase was measured. The stability of the systems was expressed as a percentage of the effluent relative to the initial weight of the filtered oleogel.

All organogels have retained their structure within 1 hour (Figure 4). The first spills were observed after 1.5 hours in variant with MAG 3.5% & EC 4.0%, which proved to be the least stable system. Oleogels with the most stable structure turned out to be those of variants that contained only monoacylglycerol – the first leakage was recorded in MAG 7% conical flasks after 24 hours and after 48 hours in MAG 8% (Figure 4). Also the variant with an 8% concentration of the polysaccharide gelator obtained a longer time of the first drop of oil and a smaller loss of mass throughout the entire test. These results show the dependence that the higher the concentration of a single gelling substance, the longer the separation time of the oleogels and thus their greater durability.

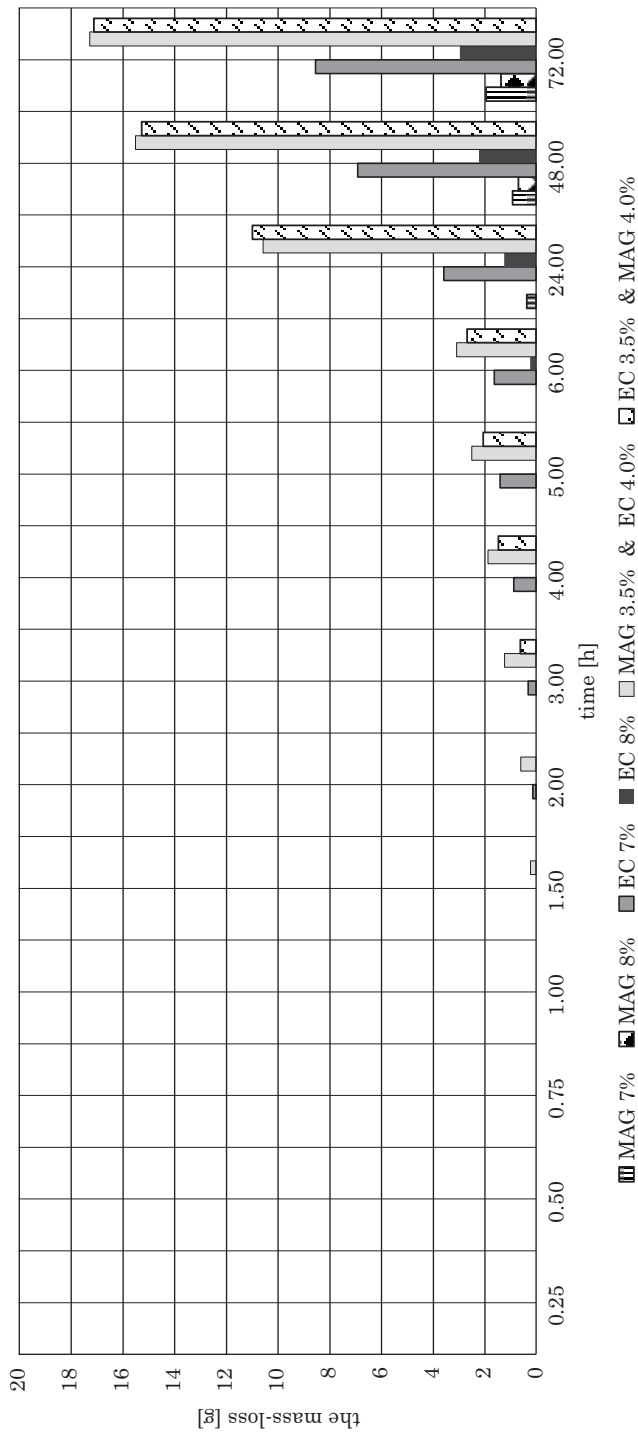

Fig. 4. Oil-binding capacity depending on time [h]

Both variants of ethyl cellulose-monoacylglycerol oleogels were characterized by very similar values of mass loss after 72 hours, higher, compared to organogels with a single gelling agent. However, the first filtrates were observed at an uneven time: after 1.5 h in MAG 3.5% & EC 4.0% flasks and after 3 h in EC 3.5% & 4.0% MAG variant (Figure 4). Determination of oleogels stability by filtration presents that lipid-type gelator had better binding capabilities of rapeseed oil than polysaccharide-type.

After the regression analysis, different models of the equation were obtained, depending on the type and amount of gelator additive – Table 2

Table 2

Correlation between loss mass (rapeseed oil leakage) and test duration

Gelator	Correlation	Model equation	R^2
MAG 7%	0.83	$Y = - 0.92 + 0.44x$	68.4
MAG 8%	0.96	$Y = - 0.11 + 0.02x$	91.6
EC 7%	0.99	$Y = 0.34 + 0.13x$	97.3
EC 8%	0.99	$Y = - 0.08 + 0.04x$	98.6
MAG 3,5% & EC 4,0%	0.97	$Y = 1.14 + 0.26x$	93.4
EC 3,5% & MAG 4,0%	0.96	$Y + 0.75 + 0.26x$	92.2

(R^2 = 68.4 for MAG 7% to R^2 = 98.6 for EC 8%). Stability of all oleogels, regardless of the variant, decreased statistically significantly over time (the mass of the liquid fraction in the oleogel increased statistically). The size of the leakage significantly increased (p <0.05). The weakest correlation was found between a lipid gelling substance (MAG 7% and MAG 8%) and the time of measurement (Table 2). The strong (R^2 > 0.9) model was obtained during regression analysis: leakage mass = - 0.11 + 0.02x (Table 2) – for oleogel with a 8% share of MAG. In the other variants, stronger models were obtained, i.e. the systems were less stable over time.

Conclusion

Ethyl cellulose as a sample of polysaccharide gelator and monoacylglycerol as a lipid gelling agent are able to convert rapeseed oil into semi-solid oleogel structure, also as gelling mixtures. Increase in concentration of single mentioned gelling agents by 1% influenced the increase of hardness and spreadability of oleogels, as well as their stability in room temperature depending on time (especially variants with EC). The ethyl cellulose and monoacylglycerol belonging to different groups of chemicals do not show any synergy in the structuring of rapeseed oil.

References

Animal and vegetable fats and oils. Analysis by gas chromatography of trans fatty acids. ISO 15304:2003. International Organization for Standardization, Geneva, Switzerland.

Animal and vegetable fats and oils. Analysis by gas chromatography of methyl esters of fatty acids. ISO 5508:1996. International Organization for Standardization, Geneva, Switzerland.

Animal and vegetable fats and oils. Preparation of methyl esters of fatty acids. ISO 5509:2001. International. Organization for Standardization, Geneva, Switzerland.

ARGAW A. 2016. *Effectiveness of Rhizobium inoculation on common bean productivity as determined by inherent soil fertility status*. J. Crop Sci. Biotechnol., 19(4): 311–322.

BLAKE A.I., CO E.D., MARANGONI A.G. 2014. *Structure and physical properties of plant wax crystal networks and their relationship to oil binding capacity*. J. Am. Oil Chem. Soc., 91: 885–903.

CHEN C.H. TERENTJEV E.M. 2015. *Monoglycerides in Oils.* In: *Edible oleogels: structure and health implications.* Eds. A.G. Marangoni, N. Garti. Elsevier, Aocs Press Illinois, pp. 173–202.

DA PIEVE S., CALLIGARIS S., CO E.D., NICOLI M.C., MARANGONI A.G. 2010. *Shear nanostructuring of monoglyceride organogels.* Food Biophysics., 5(3): 211–217.

DAVIDOVICH-PINHAS M., BARBUT S., MARANGONI A.G. 2016. *Development, characterization, and utilization of food-grade polymer oleogels,* Annu. Rev. Food Sci. Technol., 7: 65–91.

DOAN C.D., TAVERNIER I., OKURO P.K., DEWENTTINCK K. 2017. *Internal and external factors affecting the crystallization, gelation and applicability of wax-based oleogels in food industry.* IFSET, 45: 42–52.

EICHMANN T.O., KNITTELFELDER O.L. 2015. *Glycerolipids: tri-, di-, and monoacylglycerols. Encyclopedia of Lipidomics.* Netherlands, pp. 1–4.

GOLDSTEIN A., MARANGONI A., SEETHARAMAN K. 2012. *Monoglyceride stabilized oil in water emulsions: an investigation of structuring and shear history on phase.* Behaviour, Springer, 7(3): 227–235.

GRAVELLE A.J., BARBUT S., QUINTON M., MARANGONI A.G. 2014. *Towards the development of a predictive model of the formulation-dependent mechanical behaviour of edible oil-based ethyl cellulose oleogels.* J. Food Eng., 144: 114–122.

HUGHES N.E., MARANGONI A.G., WRIGHT A. J., ROGERS M. A., RUSH J.W. 2009. *Potential food applications of edible oil organogels.* Trend Food Sci. Technol., 20(10): 470–480.

HWANK H.S., SINGH M., LEE S. 2016. *Properties of cookies made with natural wax-vegetable oil organogels.* J. Food Sci., 81(5): C1045–1054.

JAKUBCZYK E., GONDEK E., SAMBORSKA K. 2014. *Charakterystyka tekstury wybranych miksów tłuszczowych*. Zeszyty Problemowe Postępów Nauk Rolniczych, 579: 17–26.

JANG A., BAE W., HWANG H.S., LEE H.G., LEE S. 2015. *Evaluation of canola oil oleogels with candelilla wax as an alternative to shortening in baked goods.* Food Chem., 187: 525–529.

LIN L., ALLEMEKINDERS H., DANSBY A., CAMPBELL L., DURANCE-TOD S., BERGER A., JONES P.J.H. 2013. *Evidence of health benefits of canola oil*. Inter Life Sci. Institute, Nutr. Rev., 71(6): 370–385.

MARANGONI A.G. 2012. *Organogels: an alternative edible oil-structuring method.* J. Am. Oil Chem. Soc., 89(5): 749–780.

MARANGONI A.G., GARTI N. 2015. *An overview of the past, present, and the future of organogels*. In: *Edible organogels: structure and health implications*. Eds. A.G. Marangoni, N. Garti. AOCS Press, Illinois, pp. 1–19.

MENG Z., QI K., GUO Y., WANG Y., LIU Y. 2018. *Effects of thickening agents on the formation and properties of edible oleogels based on hydroxypropyl methyl cellulose.* Food Chem., 246: 137–149.

PATEL A.R. 2015. *Alternative routes to oil structuring*. Springer, pp. 29–62.

PATEL A.R., DEWETTINCK K. 2016. *Edible oil structuring: an overview and recent updates*. Food Funct., 7: 20–29.

Regulation of the Minister of Health regarding the permitted additional substances from November 22, 2010, Poland (J.L.No 232, v. 1525) based on Regulation No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives.

ROGERS M.A., STROBER T., BOT A., TORO-VAZQUEZ J.F., STORTZ T., MARANGONI A.G. 2014. *Edible oleogels in molecular gastronomy*. Int. J. Gas. Food Sci., 2: 22–31.

SAGIRI S.S., SAMATEH M., JOHN G. 2017. *Fat for the future: designing multifunctional molecular oleogels*. Inform., 28(10): 19–22.

SI H., CHEONG L.Z., HUANG J., WANG X., ZHANG H. 2016. *Physical properties of soybean oleogels and oil migration evaluation in model praline system*. J. Am. Oil. Chem. Soc., 93: 1075–1084.

STORTZ T.A., MARANGONI A.G. 2013. *Ethyl cellulose solvent substitution method of preparing heat resistant chocolate*. Food Res. Int., 51(2): 797–803.

WRIGHT A.J., MARANGONI A.G. 2007. *Time, temperature and concentration dependence of ricinoelaidic acid-canola oil organogelation*. J. Am. Oil Chem. Soc., 84: 3–9.

YILMAZ E., ÖĞÜTCÜ M. 2014. *Properties and stability of hazelnut oil organogels with beeswax and monoglyceride*. J. Am. Oil Chem. Soc., 91: 1007–1017.

ZETZL A.K., MARANGONI A.G., BARBUT S. 2012. *Mechanical properties of ethyl cellulose oleogels and their potential for saturated fat reduction in frankfurters*. Food Func., 3: 327–337.

ŻBIKOWSKA A., RUTKOWSKA J., KOWALSKA M. 2015. *Consumption safety of pastries, confectionery and potato products as related to fat content*. J. Am. Coll. Nutr. 34(6): 507–514.

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THE IMPACT OF DIETARY INCLUSION OF AMARANTH MEAL ON HEMATOLOGICAL AND BIOCHEMICAL PARAMETERS OF BLOOD AND HISTOPATHOLOGICAL CHANGES IN LIVER OF RAINBOW TROUT*

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Key words: *Oncorhynchus mykiss*, *Amaranthus cruentus*, liver histology, hematology, blood biochemistry, anti-nutritional factors.

Abstract

The effect of dietary inclusion of amaranth meal on hematology and blood biochemistry, of rainbow trout *Oncorhynchus mykiss* was examined. The fish (mean length 35.2 ± 0.6 cm; mean weight 524.8 ± 28.5 g) were divided into three groups: a reference group (RF) fed commercial trout pellet and two experimental groups (EF5 and EF10) fed feeds containing 5.0% and 10.0% of amaranth meal respectively. Determined indices covered: packed cell volume, red blood cell count, hemoglobin concentration, mean cell volume, mean cell hemoglobin concentration, mean hemoglobin content, inorganic phosphates, total proteins, albumins, globulins, ammonia, triacylglycerols, glucose, and the activity of creatine kinase, alkaline phosphatase and aspartate aminotransferase. Supplementation of feed with amaranth meal significantly raised levels of blood glucose, cholesterol, total protein, ammonia, creatinine and aspartate aminotransferase activity in trout blood.

These results indicate that inclusion of amaranth meal in extruded diets for rainbow trout can get negative effect on liver and blood biochemistry profile.

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Introduction

The replacement of fishmeal as a major protein source with plant origin components is challenging the sustainability of aquaculture industry (VILHELMSSON et al. 2004). There is a general interest in increasing the level of plant protein ingredients in feeds for farmed fish. (GATLIN et al. 2007). Many researchers in recent years investigated influence of vegetable components on fish growth, liver and intestinal histology (HANSEN et al. 2006, 2007, ESCAFFRE et al. 2007, LANSARD et al. 2009, BORQUEZ et al. 2010, MURASHITA et al. 2013, RANDALL et al. 2013), hematological and blood biochemical profile (YAMAMOTO et al. 2010, MURASHITA et al. 2013, TUSCHE et al. 2013, LÓPEZ et al. 2015).

The main problem with plant components is anti-nutritional factors (ANFs) impacting salmonid fish yield, by means of decreased digestion and reduced utilization of proteins followed by decreased growth rates (MOYANO et al. 1991, KROGDAHL et al. 1994), feed intake and decreased nutrient absorption (FRANCIS et al. 2001). Some researchers reported impact of ANFs on fish hematology and blood biochemistry (SHAFAEIPOUR et al. 2008, IWASHITA et al. 2009, KUMAR et al. 2010). Protease inhibitors, phytic acid, lectins, gossypol, glucosinolates and saponins are some of the most common ANFs in plant ingredients used in fish feeds (TACON 1997, KROGDAHL et al. 2010). The effectiveness of common processing techniques such as dry and wet heating, solvent extraction, and enzyme treatment in removing ANFs from vegetable raw materials was discussed in many papers (FRANCIS et al. 2001, KUMAR et al. 2010). However, processing is usually expansive and/or decreases nutritional value of raw materials.

Balanced amino acid profile with high level of lysine makes amaranth (*Amaranthus cruentus*) seeds attractive protein source for fish (PEDERSEN et al. 1987). However, chemical analysis of amaranth showed low level of ANFs: phytic acid and saponins (ESCUDERO et al. 2004). Previous studies have shown, high level of amaranth meal (level at 5–10%) summplentation induced negative influence on the growth performance and liver condition. (NIEWIADOMSKI et al. 2016).

We suppose that the addition of amaranth flour may have a negative effect on the health status of rainbow trout. Current research was at the aim of the study was the assessment of effect of dietary inclusion of amaranth meal on health status – hematology, blood biochemistry and liver histology of rainbow trout *Oncorhynchus mykiss*.

Materials and Methods

Fish, feeding, experimental system, diet preparation

Rainbow trout with initial mean length of 35.2 ± 0.6 cm and mean body weight of 524.8 ± 28.5 g were used in the experiment. Fish (n = 144) were randomly divided into three groups: a reference group (RF) fed commercial trout pellet and two experimental groups (EF5 and EF10) fed pellets that contain 5.0% and 10.0% of amaranth meal respectively. Fish were distributed in 9 tanks (3 groups in triplicates; n = 16 for each replicate). Experimental conditions have been described by NIEWIADOMSKI et al. (2016). Fish were sampled after 21 days of feeding. Ingredients and nutrients composition of the experimental diets are presented in Table 1.

Table 1
Ingredients and chemical composition of experimental diets (following NIEWIADOMSKI et al. 2016)

Ingrediens [%]	EF5	EF10	RF*
Fishmeal	44.25	44.25	NA
Wheat flour	20.31	20.31	NA
Soybean meal	15.00	10.00	NA
Amaranth meal	5.00	10.00	NA
Fish oil	6.24	6.24	NA
Soybean oil	6.00	6.00	NA
Vitamin premix[1]	2.00	2.00	NA
Mineral premix[2]	0.10	0.10	NA
Choline	0.50	0.50	NA
Ascorbic acid	0.50	0.50	NA
Chromic oxide	1.00	1.00	1.00
Chemical composition [%]			
Dry matter	94.27	95.02	93.51
Crude protein	41.01	40.62	42.26
Crude fat	11.95	13.45	13.70
Crude ash	10.44	10.43	8.52
Crude fibre	3.27	3.25	3.22
Chromic oxide	1.00	1.00	1.00
Gross energy [MJ kg^{-1}]	18.04	15.41	17.42

* Data not available

[1] Witamin premix (IU kg^{-1} or mg kg^{-1} dry diet): witamin A – 15 000 UI kg^{-1}; witamin D – 6000 UI kg^{-1}; witamin E – 15; witamin C – 70; witamin B_1 – 0.8; witamin B_2 – 3.0; witamin B_6 – 1.50; witamin B_{12} – $8 \cdot 10^{-3}$; witamin K – 1.5; biotin – 2.5.

[2] Mineral premix (mg kg^{-1} dry diet): calcium – $25 \cdot 10^3$; phosphorus – $27 \cdot 10^3$; sodium – $18 \cdot 10^3$; magnesium – $2 \cdot 10^3$; mangan – 720; iron (II) – 400; copper – 127; zinc – 800; iodine – 23.

Experimental feeds were extruded with a co-rotating twin screw extruder (METALCHEM, Poland) equipped with a Ø 4.5 mm pellet stencil.

Chemical analysis

The content of the basic chemical components in feed (dry matter, crude protein, crude fat, ash) was determined in accordance to standard methods (AOAC 2016). Dry matter was determined by drying in an oven at 105°C for 24 h. Total protein was determined by Kjeldahl's method and crude fat by Soxhlet's method.

Hematology

Before the blood sampling, fish were caught, immediately anaesthetized with propofol – 7 mg dm^{-3} (GOMUŁKA et al. 2014). Blood was sampled with a syringe covered with heparin lithium salt from caudal vessels. Hematological indices were determined according to SVOBODOVA et al. (1991) and covered: hematocrit (PCV), hemoglobin concentration (HB), red blood cell count (RBC), mean cell volume (MCV), mean cell hemoglobin concentration (MCHC), mean hemoglobin content (MHC).

Biochemistry indices

Blood samples were centrifuged at 12 000 g for 30 s and frozen. Plasma samples were analyzed with a Catalyst Dx Chemistry Analyzer (Idexx Lab; USA) using dedicated test slides (custom panels). The following biochemical measurements were performed: albumins (ALB), globulins (GLOB), total protein (TP), ammonia (NH_3), glucose (GLU), triacylglycerols (TAG), cholesterol (CHOL), creatinine (CR) and the activity of amylase (AMS), lipase (LIP), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). Each plasma sample was thawed only once at room temperature and all of the above measurements were performed at once to eliminate multiple freezing/thawing cycles.

Liver histology

Randomly collected liver samples (n = 9 per group) were fixed in Bouin's fluid, dehydrated in ethanol, cleared in xylene, embedded in paraffin blocks, and then sliced into 4–5 µm sections with a RM 2155 rotational microtome (LEICA Microsystems, Wetzlar, Germany). Cross-sections of tissues were stained with haematoxylin and eosin (ZAWISTOWSKI 1986). The number of hepatocytes was established in a field with a surface area of 2500 μm^2 (50×50 µm). These measurements were performed with a LEICA DM 3000 light microscope and LEICA QWin Pro micro image

analysis software (LEICA Microsystems AG, Heerbrugg, Switzerland). For each fish, the diameters of 100 hepatocytes (HD) and their nuclei (HND) were measured under the light microscope equipped with computer analysis software. Nuclear-cytoplasmic index (NCPI) was calculated as follow: $NCPI = HND \cdot HD^{-1}$.

Statistical analysis

Normality of data distribution was tested by Shapiro-Wilk test and variance homogeneity by Leven's test. When above assumptions were met, differences between means were analysed using ANOVA and *post hoc* Tuckey's test (TT). For the others, Kruskal-Wallis ANOVA and Dunn's test (DT) were used. Results were analysed with Statistica 12.0 (Statsoft, USA) software at significance level $P \leq 0.05$.

Results

Haematology

No significant differences were found between experimental groups in RBC, Hb, PCV, MCV and MHC (TT, $P > 0.05$). Significantly higher values of MCHC were determined in EF10 group (TT, $P < 0.05$) when compared to the reference group (Table 2).

Table 2

Hematological indices of *Oncorhynchus mykiss* fed experimental diets formulated with amaranth meal

Specification	RF	EF5	EF10
RBC [T l^{-1}]	$0.74^a \pm 0.15$ (0.44–1.01)	$0.66^a \pm 0.15$ (0.36–0.97)	$0.67^a \pm 0.18$ (0.34–0.99)
Hb [g l^{-1}]	$97.2^a \pm 8.9$ (74.6–115.8)	$98.9^a \pm 10.5$ (77.7–117.5)	$102.1^a \pm 10.0$ (84.5–119.5)
PCV [1]	$0.34^a \pm 0.06$ (0.22–0.47)	$0.35^a \pm 0.03$ (0.27–0.41)	$0.33^a \pm 0.04$ (0.25–0.39)
MCV [fl]	$449^a \pm 74$ (333–554)	$565^a \pm 157$ (378–958)	$518^a \pm 175$ (253–1072)
MHC [pg]	$138^a \pm 37$ (93–251)	$159^a \pm 47$ (95–276)	$163^a \pm 52$ (101–303)
MCHC [pg fl^{-1}]	$285^a \pm 33$ (222–331)	$284^a \pm 34$ (214–348)	$319^b \pm 50$ (251–427)

Results are presented as mean ± SD (range). Number indexes show columns with significantly different results ($P \leq 0.05$)

Blood biochemistry profile

Results of biochemical blood analysis are presented in detail in Table 3. No significant differences were found between experimental groups in the case of TAG, ALB, GLOB, AMS, LIP, ALP level (TT, $P > 0.05$). In the other

biochemical indicators not mentioned above, a statistically significant increase was observed in relation to the reference group (TT, $P < 0.05$).

Table 3

Blood biochemical parameters of *Oncorhynchus mykiss* fed experimental diets formulated with amaranth meal

Specification	RF	EF5	EF10
GLU [mmol dm^{-3}]	5.73^{a} ± 2.06 (2.97–9.72)	8.43^{b} ± 3.51 (3.01–17.35)	7.38^{ab} ± 2.60 (2.26–13.86)
TAG [µmol dm^{-3}]	2.47^{a} ± 0.86 (1.31–4.11)	2.94^{a} ± 0.85 (1.38–4.24)	2.72^{a} ± 0.97 (1.04–4.24)
TP [g dm^{-3}]	45.0^{a} ± 7.3 (34–60)	51.5^{b} ± 9.0 (37–74)	50.1^{ab} ± 8.7 (37–66)
ALB [g dm^{-3}]	18.5^{a} ± 2.9 (15–25)	19.0^{a} ± 2.4 (12–23)	19.1^{a} ± 2.2 (16–23)
GLOB [g dm^{-3}]	26.5^{a} ± 5.0 (19–55)	33.0^{a} ± 8.8 (22–55)	31.9^{a} ± 7.6 (21–47)
CHOL [mmol dm^{-3}]	4.85^{a} ± 1.02 (3.34–7.39)	5.88^{b} ± 1.62 (3.73–8.76)	5.86^{b} ± 1.32 (3.82–7.76)
NH_3 [µmol dm^{-3}]	126^{a} ± 54 (37–220)	184^{a} ± 80 (49–333)	207^{b} ± 79 (71–373)
CR [mmol dm^{-3}]	13^{a} ± 4 (9–19)	89^{b} ± 36 (35–164)	123^{b} ± 83 (33–330)
AMS [U l^{-1}]	1279^{a} ± 369 (506–2019)	1240^{a} ± 377 (490–1841)	1176^{a} ± 341(641–1736)
ALP [U l^{-1}]	193^{a} ± 86	254^{a} ± 100	243^{a} ± 123
LIP [U l^{-1}]	153^{a} ± 38 (84–232)	176^{a} ± 110 (95–647)	154^{a} ± 30 (106–203)
AST [U l^{-1}]	39.9^{a} ± 16.7	58.3^{b} ± 20.1	56.3^{b} ± 18.6

Results are presented as mean ± SD (range). Number indexes show columns with significantly different results ($P \leq 0.05$)

Liver histology

The addition of amaranth meal affects the size of hepatocytes and the NCPI is statistically significant increase (TT, $P < 0.05$) in experimental groups (Table 4). The representative histological picture of liver sections from the fish fed with experimental and reference diet are shown on Figure 1.

Table 4

Measurements of hepatocytes of *Oncorhynchus mykiss* fed experimental diets formulated with amaranth meal

Specification	RF	EF5	EF10
HD [µm]	14.60^{a} ± 41.99	16.88^{b} ± 0.99	17.63^{b} ± 1.44
HND [µm]	5.86^{a} ± 0,59	5.81^{a} ± 0.21	6.35^{a} ± 0.06
INCP [1]	0.41^{a} ± 0.03	0.34^{b} ± 0.01	0.36^{b} ± 0.03

Results are presented as mean ± SD. Different superscripts means significantly different results in rows ($P \leq 0.05$). HD – hepatocyte diameter; HND – hepatocyte nucleus diameter; NCPI – nucleo-cytoplasmatic index

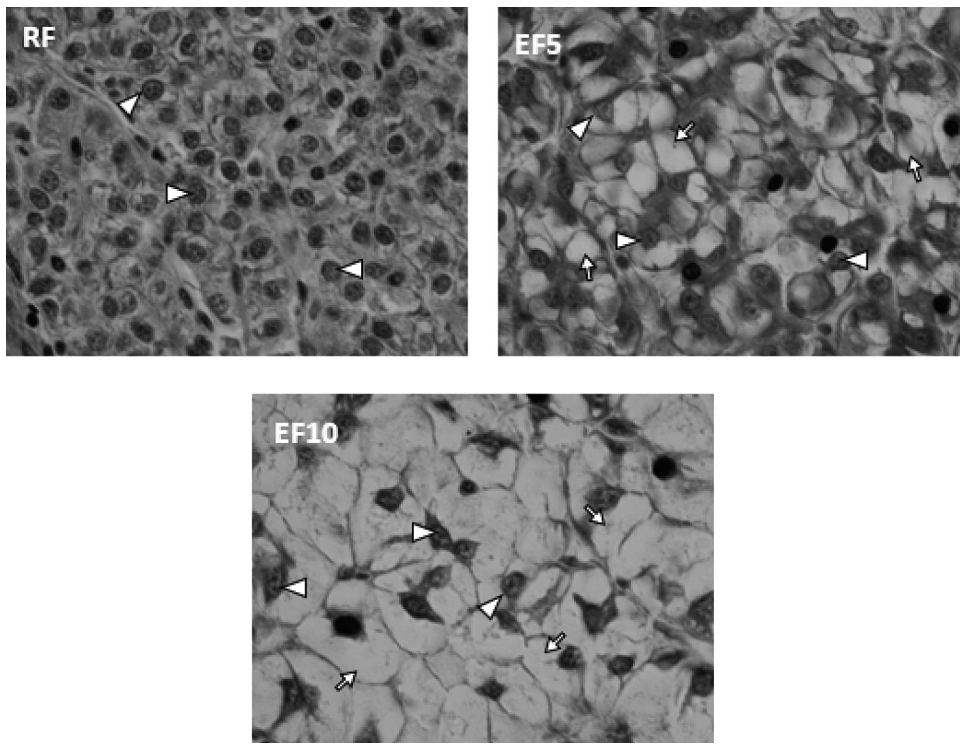

Fig. 1. Representative picture of liver sections of *Oncorhynchus mykiss* fed reference and experimental diets formulated with amaranth meal content. H & E. Reference feed (RF), experimental feeds (EF5 and EF10), hepatocyte nuclei (arrow heads), vacuole (arrows). Photographs were taken at approximately 40x.

Discussion

The hepatosomatic index (HSI) is among the most popular indices used to identify possible liver disorders (VAN DER OOST et al. 2003). The HSI in experimental fish (1.96 and 2.08 for EF5 and EF10 feeds respectively) was much higher compared to values reported for rainbow trout fed with soya meal based diet (1.18) (ØVERLAND et al. 2009) and lupine meal based diet (1.1) (BORQUEZ et al. 2011). This increase was probably caused by fat and/or glycogen accumulation inside hepatocytes (Figure 1), what was followed by the increase of mean hepatocyte diameter and resulted in growth of liver mass. GÜMÜŞ AND İKIZ (2009) found that increasing NFE level in feeds caused the HSI increase in rainbow trout. Although the NFE levels in reference and both experimental diets were similar (25,81% and 27.60% and 27.70% respectively), our results seems to be in agree with above finding as apparent digestibility of NFE was much lower in refer-

ence diet comparing to EF5 and EF10 feeds (47.4% and 75.5% and 78.4% respectively) (NIEWIADOMSKI et al. 2016). Higher digestibility of amaranth meal NFE can be probably addressed to small-particle size and easy to digest amaranth starch (ECKART et al. 2002). However, MURASHITA et al. (2013) recorded similar histopathological changes in liver of trout fed feed with soya meal-based diets.

High digestibility of NFE was probably the cause of hyperglycaemia found in the experimental fish. Usually, glucose level in blood plasma of rainbow trout ranged between 4.25 and 5.73 mmol dm^{-3} (FIGUEROA et al. 2000, SANTIN et al. 2013). In our experiment, glucose level reached 7.38 and 8.43 mmol dm^{-3} in EF5 and EF10 respectively. These level is much higher than results reported for fermented soya meal (5.7–6.6 and 6.2–6.6 mmol dm^{-3}) and soya protein concentrate (3.34–5.67 mmol dm^{-3}) diets respectively (YAMAMOTO et al. 2010, 2012, KUMAR et al. 2010, and MURASHITA et al. 2013). YAMAMOTO et al. (2007) and TUSCHE et al. (2013) reported similar results for trout fed soya protein concentrate (7.2–7.6 mmol dm^{-3}) and potato protein concentrate based diets (7.4–9.9 mmol dm^{-3}) respectively. However, we are aware that the very high glucose level could be the result of our experiment design. We did not stop feeding 24 hours before blood sampling because we examined apparent digestibility of nutrients in the same experiment (results are reported in NIEWIADOMSKI et al. 2016). From the other hand, blood glucose level in the reference group (5.73 mmol dm^{-3}) was not altered.

Morphological changes in the liver were accompanied by 1.5-fold increase of AST serum activity in experimental groups. AST similarly to ALP and ALT belongs to cytoplasmic intracellular enzymes and it appears in blood plasma due to a damage to cell membranes. Such increase of activity can be a result of parenchymal organs damage or breakdown of red blood cells (RACICOT et al. 1975). However, as we did not record any differences in RBC or PCV between reference and experimental groups, we believed that the AST activity increase is the sequel of pathological processes observed in fish liver. Moreover, increase of ALP activity, although not significant, suggest increased metabolic burden of the liver (KUMAR et al. 2010) and support above statement.

From the other hand, 7.9 to 9.5-fold higher level of CR in fish fed with experimental diets (89 and 123 μmol dm^{-3} for EFD5 and EF10 respectively) when compared to reference group (13 μmol dm^{-3}) suggest that some kidney failure also occurred. According to CHAROO et al. (2013) high CR level is an indicator of impaired renal function. Moreover, mean CR level was correlated to mean NH_3 level ($r = 0.9996$; $p < 0.05$) which was also significantly higher in experimental fish blood.

Total protein level in trout blood in experimental groups (50.1–51.5 g l^{-1}) was higher than those reported by YAMAMOTO et al. (2007, 2010) for fermented soybean meal (30–36 g l^{-1}), KUMAR et al. (2010) for jatropha flour (38–41 g l^{-1}), MURASHITA et al. (2013) for soybean meal (33–36 g l^{-1}) and TUSCHE et al. (2013) for potato concentrate (33.3–36.8 g l^{-1}). According to BANAEE et al. (2011) there is a close relationship between the rate of protein synthesis in the liver and the concentration of total blood protein. However, such high results suggest some pathological reasons. SHAMOUSHAKI et al. (2012) studied the EDTA toxicity to rainbow trout. They found similar TP levels in trout exposed to 1.4 g l^{-1} to 2.1 g l^{-1} of EDTA (52.3 g l^{-1} to 58.3 g l^{-1} respectively). YILMAZ et al. (2015) found TP levels as high as 100.2 g l^{-1} to 106.3 gl^{-1} in trout orally exposed to carvacrol. These authors addressed the increased level of TP to the increase in innate immune response of fish. However, in their study TP level in the control group was also extremally high (87.2 g l^{-1}).

Many authors found lowered CHOL level in fish fed feeds based on plant origin raw material (ROMARHEIM et al. 2006, IWASHITA et al. 2008, YAMAMOTO et al. 2007, 2010, KUMAR et al. 2010). Values of CHOL recorded in experimental fish blood plasma (5.86–5.88 mmol dm^{-3}) in our experiment were much higher than those reported by SHAFAEIPOUR et al. (2008), IWASHITA et al. (2008), YAMAMOTO et al. (2010, 2012), MURASHITA et al. (2013) for rape (2.10–2.83 mmol dm^{-3}), soybean (2.04–2.32 mmol dm^{-3}), fermented soybean (2.74– 2.96, 2.93–3.41 mmol dm^{-3}) and modified soybean (3.22–4.09 mmol dm^{-3}) meals respectively. We suppose that such high increase of blood CHOL level in experimental fish was a result of cholesterol synthesis in the liver from the squalene which is present in amaranth seeds. Squalene is the precursor of cholesterol. The squalene level in amaranth meal is about 6.23% (ESCUDERO et al. 2004). The increase of cholesterol synthesis was found in hamster fed with amaranth supplemented feed (MENDONÇA et al. 2009).

According to DENG et al. (2013) the blood cholesterol level is built by exogenous cholesterol supplied in feed and cholesterol synthetized *de novo* in the liver. The authors suggested that in case of excessive supply of cholesterol for a longer time, fish can inhibit endogenous cholesterol production. SHAFAEIPOUR et al. (2008) obtained the reduction of CHOL level in trout blood plasma after 112 days of feeding with experimental canola diets when compared to results obtained after 56 days of feeding. We can expect that longer feeding with amaranth meal feed can result in lower cholesterol blood level.

KROGDAL et al. (2010) found that despite heat treatment many ANFs (for example saponins) are not eliminated from feeds. Saponins present in

both soya and amaranth meals can impact some blood parameters including CR, TP and NH_3 causing liver and kidney (PISARIKOVA et al. 2006) or gills impairment (FRANCIS et al. 2001)

Conclusions

However, one should take into account that the experiment was relatively short. Thus, our results should be considered as a preliminary study. These results suggest that inclusion of amaranth meal in extruded diets for rainbow trout can get negative impact on functional status on liver and kidney of rainbow trout. We assume that the obtained results were influenced by ANFs derived from soybean meal and amaranth meal. Next experiment, describe to the effect of amaranth meal feeding on fish with amaranth meal as the only one source of potential ANFs.

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Reference

AOAC. 2016. *Official Methods of Analysis of the Association of Official Analytical Chemists*. Washington, DC, USA.

BANAEE M., SUREDA A., MIRVAGHEFI A.R., RAFEI G. R. 2011. *Effects of long-term silymarin oral supplementation on the blood biochemical profile of rainbow trout (Oncorhynchus mykiss).* Fish Physiol. Biochem., 37: 885–896.

BORQUEZ A., SERRANO E., DANTAGNAN P., CARRASCO J., HERNANDEZ A. 2010. *Feeding high inclusion of whole grain white lupin (Lupinus albus) to rainbow trout (Oncorhynchus mykiss): effects on growth, nutrient digestibility, liver and intestine histology and muscle fatty acid composition.* Aquacult. Res., 1: 1–12.

BÓRQUEZ A.S., HERNÁDEZ A.J., DANTAGNAN P. 2011. *Incorporation of Whole Lupin, Lupinus albus, seed meal in commercial extruded diets for rainbow trout, Oncorhynchus mykiss. Effect on growth performance, nutrient digestibility, and muscle fatty acid composition.* J. World Aquacult. Soc., 42(2): 209–221.

CHAROO S.Q., CHALKOO S.L., QURESHI T.A. 2013. *Sexual differentiation in blood biochemistry of rainbow trout (Oncorhynchus mykiss).* Int. J. Adv. Fish Aquat. Sci., 1(1): 32–38.

ECKART W., ABERLE T., BURCHARD W., LANDERS R. 2002. *Peculiarities of aqueous amaranth starch suspensions.* Biomacromolecules, 3(1): 17–26.

ESCUDERO N.L., de ARELLANO M.L., LUCO J.M., GIMÉNEZ M.S., MUCCIARELLI S.I. 2004. *Comparison of the chemical composition and nutritional value of amaranthus cruentus flour and its protein concentrate.* Plant Foods Hum. Nutr., 59: 15–21.

ESCAFFRE A.M., KAUSHIK S., MAMBRINI M. 2007. *Morphometric evaluation of changes in the digestive tract of rainbow trout (Oncorhynchus mykiss) due to fish meal replacement with soy protein concentrate.* Aquaculture, 237: 127–138.

FRANCIS G., MAKKAR H.P.S., BECKER K. 2001. *Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish.* Aquaculture, 199: 197– 227.

FIGUEROA R.I., RODRÍGUEZ-SABARÍS R., ALDEGUNDE M., SOENGAS J.L. 2000. *Effects of food deprivation on 24 h-changes in brain and liver carbohydrate and ketone body metabolism of rainbow trout.* J. Fish. Biol., 57: 631–646.

GATLIN D.M. III., BARROWS F.T., BELLIS D. et al. 2007. *Expanding the utilization of sustainable plant products in aquafeeds – a review*. Aquacult. Res., 38: 551–579.

GOMUŁKA P., WŁASOW T., SZCZEPKOWSKI M., MISIEWICZ L., ZIOMEK E. 2014. *The effect of propofol anesthesia on hematological and biochemical blood profile of European whitefish*. Tur. J. Fish. Aqua. Sci., 14: 331–337.

GÜMÜŞ E., İKIZ R. 2009. *Effect of dietary levels of lipid and carbohydrate on growth performance, chemical contents and digestibility in rainbow trout, Oncorhynchus mykiss Walbaum, 1792*. Pak. Vet. J., 29(2): 59–63.

HANSEN A.C., ROSENLUND G., KARLSEN Ø., OLSVIK P.A., HERME G.I. 2006. *The inclusion of plant protein in cod diets, its effects on macronutrient digestibility, gut and liver histology and heat shock protein transcription*. Aquacult. Res., 37: 773–784.

HANSEN A.C., ROSENLUND G., KARLSEN Ø., KOPPE W., HERME G.I. 2007. *Total replacement of fish meal with plant proteins in diets for Atlantic cod (Gadus morhua L.) I – effects on growth and protein retention*. Aquaculture, 272: 599–611.

IWASHITA Y., YAMAMOTO T., FURUITA H., SUGITA T., SUZUKI N. 2008. *Influence of certain soybean antinutritional factors supplemented to a casein-based semipurified diet on intestinal and liver morphology in fingerling rainbow trout Oncorhynchus mykiss*. Fish Sci., 74: 1075–1082.

IWASHITA Y., SUZUKI N., MATSUNARI H., SUGITA T., YAMAMOTO T. 2009. *Influence of soya saponin, soya lectin, and cholyltaurine supplemented to a casein-based semipurified diet on intestinal morphology and biliary bile status in fingerling rainbow trout Oncorhynchus mykiss*. Fish Sci., 75: 1307–1315.

KROGDAHL Å., LEA T.B., OLLI J.L. 1994. *Soybean proteinase inhibitors affect intestinal trypsin activities and amino acid digestibility's in rainbow trout (Oncorhynchus mykiss)*. Comp. Biochem. Physiol. A., 107: 215–219.

KROGDAHL Å., PENN M., THORSEN J., REFSTIE S., BAKKE A.M. 2010. *Importatnt antinutrients in plant feedstuffs for aquaculture: an update on recent findings responses in salomonids*. Aquacult. Res., 41: 333–344.

KUMAR V., MAKKAR H.P.S., BECKER K. 2010. *Nutritional, physiological and haematological responses in rainbow trout (Oncorhynchus mykiss) juveniles fed detoxified Jatropha curcas kernel meal*. Aquacult. Nutr., 17: 451–467.

LANSARD M., PANSERAT S., SEILIEZ I., POLAKOF S., PLAGNES-JUAN E., GEURDEN I., MÉDALE F., KAUSHIK S., CORRAZE G., SKIBA-CASSY S. 2009. *Hepatic protein kinase B (Akt) – target of rapamycin (TOR)-signalling pathways and intermediary metabolism in rainbow trout (Oncorhynchus mykiss) are not significantly affected by feeding plant-based diets*. Br. J. Nutr., 102: 1564–1573.

LÓPEZ L.M., FLORES-IBARRA M., BAÑUELOS-VARGAS I., GALAVIZ M.A., TRUE C.D. 2015. *Effect of fishmeal replacement by soy protein concentrate with taurine supplementation on growth performance, hematological and biochemical status, and liver histology of totoaba juveniles (Totoaba macdonaldi)*. Fish Physiol. Biochem., 41: 921–936.

MENDONÇA S., SALDIVA P.H., CRUZC R.J., ARÊAS J.A.G. 2009. *Amaranth protein presents cholesterol-lowering effect*. Food Chem., 116: 738–742.

MOYANO F.J., GARDENETE G., DE LA HIGUERA M. 1991. *Nutritive and metabolic utilization of proteins with high glutamic-acid content by the rainbow-trout (Oncorhynchus mykiss)*. Comp. Biochem. Physiol. A., 100: 759–762.

MURASHITA K., AKIMOTO A., IWASHITA Y., AMANO A., SUZUKI N., MATSUNARI H., FURUITA H., SUGITA T., YAMAMOTO T. 2013. *Effects of biotechnologically processed soybean meals in a nonfishmeal diet on growth performance, bile acid status, and morphological condition of the distal intestine and liver of rainbow trout Oncorhynchus mykiss*. Fish Sci., 79: 447–457.

NIEWIADOMSKI P., GOMUŁKA P., POCZYCZYŃSKI P., WOŹNIAK M., SZMYT M. 2016. *Dietary effect of supplementation with amaranth meal on growth performance and apparent digestibility of rainbow trout Oncorhynchus myskiss*. Pol. J. Natur. Sc., 31(3): 459–469.

ØVERLAND H., SØRENSEN M., STOREBAKKEN T., PENN M., KROGDAHL Å., SKREDE A. 2009. *Pea protein concentrate substituting fish meal or soybean meal in diets for Atlantic salmon (Salmo salar). Effect on growth performance, nutrient digestibility, carcass composition, gut health, and physical feed quality*. Aquaculture, 288: 305–311.

PEDERSEN B., KALINOUS L.S., EGGUM B.O. 1987. *The nutritive value of amaranth grain (Amaranthus caudatus)* I. *Protein and minerals of raw and processed grain (Qualitas plantarum).* Plant Food Hum. Nutr., 36: 309–324.

PISARIKOVA B., ZRALY Z., KRAČMAR S., TRČKOVA M., HERZIG I. 2006. *The use of amaranth (genus Amaranthus L.) in the diets for broiler chickens.* Vet. Med., 51: 399–407.

RACICOT J.G., GAUDET M., LERAY C. 1975. *Blood and liver enzymes in rainbow trout (Salmo gairdneri Rich.) with emphasis on their diagnostic use. Study of CCl4 toxicity and a case of Aeromonas infection.* J. Fish. Biol., 7: 825–835.

RANDALL K.M., DREW M.D., ØVERLAND M., ØSTBYE T.K., BJERKE M., VOGT G., RUYTER B. 2013. *Effects of dietary supplementation of coriander oil, in canola oil diets, on the metabolism of [1-14C] 18:3n-3 and [1-14C] 18:2n-6 in rainbow trout hepatocytes.* Comp. Biochem. Physiol. B., 166: 65–72.

ROMARHEIM O.H., SKREDE A., GAO Y.L., KROGDAHL Å., DENSTADLI V., LILLEENG E., STOREBAKKEN T. 2006. *Comparison of white flakes and toasted soybean meal partly replacing fish meal as protein source in extruded feed for rainbow trout (Oncorhynchus mykiss).* Aquaculture, 256: 354–364.

SANTIN A.E., SEARLE A.J., WINSTON V.D., POWELL M.S., HARDY R.W., RODNICK K.J. 2013. *Glycated hemoglobin is not an accurate indicator of glycemia in rainbow trout.* Comp. Biochem. Physiol. A., 165: 343–352.

SHAFAEIPOUR A., YAVARI V., FALAHATKAR B., MAREMMAZI J.G.H., GORJIPOUR E. 2008. *Effects of canola meal on physiological and biochemical parameters in rainbow trout (Oncorhynchus mykiss).* Aquacult. Nutr., 14: 110–119.

SHAMOUSHAKI M.M.N., JAHANSHAHI R., RAHMATI M., DERAKHSHAN M., MAZINI M., GHORAYSHI S. 2012. *Effect of ethylenediaminetetraacetic acid (EDTA) on some serum constituents of Oncorhynchus mykiss.* Blob. Vet., 9(3): 341–344.

SVOBODOVÁ Z., PRAVDA D., PALÁČKOVÁ J. 1991. *Unified methods of haematological examination of fish.* Research Institute of Fish Culture and Hydrobiology, Vodňany. Methods, pp. 20–31.

TACON A.G.J. 1997. *Fish meal replacers: review of anti-nutrients within oil seeds and pulses – a limiting factor for the aquafeed green revolution?* In: *Feeding tomorrow's fish, cahiers options Mediterranean's*. Eds. A. Tacon, B. Basurca. Mazarron, Spain, pp. 154–182.

TOA D.G., AFONSO L.O.B., IWAMA G.K. 2004. *Stress response of juvenile rainbow trout (Oncorhynchus mykiss) to chemical cues released from stressed conspecifics.* Fish Physiol. Biochem., 30: 103–108.

TUSCHE K., NAGEL F., ARNING S., WUERTZ S., SUSENBETH A., SCHULZ C. 2013. *Effect of different dietary levels of potato protein concentrate supplemented with feed attractants on growth performance of rainbow trout (Oncorhynchus mykiss).* Anim. Feed Sci. Technol., 183: 202– 209.

VAN DER OOST R., BEYER J., VERMEULEN N.P.E. 2003. *Fish bioaccumulation and biomarkers in environmental risk assessment: a review.* Environ. Toxicol. Phar., 13: 57–149.

VILHELMSSON O.T., MARTIN S.A.M., MÉDALE F., KAUSHIK S.J., HOULIHAN D.F. 2004. *Dietary plant-protein substitution affects hepatic metabolism in rainbow trout (Oncorhynchus mykiss).* Br. J. Nutr., 92: 71–80.

YAMAMOTO T., SUZUKI N., FURUITA H., SUGITA T., TANAKA N., GOTO T. 2007. *Supplemental effect of bile salts to soybean meal-based diet on growth and feed utilization of rainbow trout Oncorhynchus mykiss.* Fish Sci., 73: 123–131.

YAMAMOTO T., IWASHITA Y., MATSUNARI H., SUGITA T., FURUITA H., AKIMOTO A., OKAMATSU K., SUZUKI N. 2010. *Influence of fermentation conditions for soybean meal in a non-fish meal diet on the growth performance and physiological condition of rainbow trout Oncorhynchus mykiss.* Aquaculture, 309: 173–180.

YAMAMOTO T., MATSUNARI H., SUGITA T., FURUITA H., MASUMOTO T., IWASHITA Y., AMANO S., SUZUKI N. 2012. *Optimization of the supplemental essential amino acids to a fish meal-free diet based on fermented soybean meal for rainbow trout Oncorhynchus mykiss.* Fish Sci., 78: 359–366.

YILMAZ E., ERGÜN S., YILMAZ S. 2015. *Influence of carvacrol on the growth performance, hematological, non-specific immune and serum biochemistry parameters in rainbow trout (Oncorhynchus mykiss).* Food Nutr. Sci., 6: 523–531.

ZAWISTOWSKI S. 1986. *Histological techniques, histology and the foundations of histopathology.* PZWL, Warsaw.

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SELECTED CATTLE HOOF DISEASES: CHARACTERISTICS, CONSEQUENCES, CONTROL AND PREVENTION

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Key words: limb diseases, lameness, housing system, production performance, hoof trimming.

Abstract

Limb diseases are the most common ones next to the mammary gland diseases (*mastitis*) and those connected to reproduction (*metritis, endometritis*). Appearing lameness significantly decrease welfare and have an impact on the culling of cows in herds and what is more important lower milk performance and decrease its composition as well as its technological usefulness in processing. Prevention is based on the timely hoof correction and hoof baths in the 10% copper sulfate water solution or 5% formalin. The assessment of the level of lameness, which is performed on a moving cow (locomotion scoring) with the use of a 5-point Zinpro (2014) scale, is essential to the fast diagnosis. Performing timely hoof correction, should be performed twice a year in case of the all year alcove keeping system. It is advised to prefer the free-standing housing system, which considerably enhances welfare of cows.

Introduction

Systematically appearing increase in dairy performance of cows, as well as selection-achieved increase in cow body weight, especially in Holstein-Friesian breed, favors the more common appearance of metabolic diseases including locomotion diseases (GOFF and HORST 1997, KÖNIG et al. 2005). Such problem concerns most herds in Poland and in the world. Limb construction defects, lameness and soft pastern occurrence concerns from 6% to 16% of dairy herds in our country, additionally including over-

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-gaped hoof (5–7%), limbs twisted inward (5–9%) and to limbs directed under the trunk or too wide ankles (about 1%) (KOŁACZ and BODAK 1999, WINNICKI et al. 2006).

Frequency of various types of hoof diseases is different (Table 1). Lameness caused by the plantar ulcer, double sole, digital skin inflammation and the inflammation of the skin of the interdigital area most often occur in cows kept in the indoor system, without the access to pasture. In cows kept in the mix inn-outdoor system, which use pasture in the summer season the most often diseases are: toe ulcer, white line disease, hoof wall traumas, interdigital growth (limax), bruised soles and overgrown claws (GREEN et al. 2014). In 8/10 cases lameness is caused by toe diseases (HERNANDEZ-MENDO et al. 2007).

Table 1

Frequency of hoof diseases occurrence in dairy cattle based on different authors

Type of disease	Frequency of lameness occurrence [%]			
	WINKLER and MARGERISON (2012)	DE FRAIN et al. (2013)	NAVARRO et al. (2013)	GREEN et al. (2014)
Sole ulcer	17.14	21.05	6.84	38.68
Bruised sole	28.57	1.43	10.00	12.98
Digital dermatitis	22.86	47.67	3.68	10.00
White line disease	5.71	17.26	16.32	8.28
Interdigital growth	2.86	0.19	6.84	4.50
Overgrown claw	–	31.08	3.68	3.11
Toe ulcer	–	2.61	3.68	1.79
Double sole	–	–	6.84	7.42
Vertical sand cracks	–	0.33	8.42	–
Heel erosion	–	0.04	6.84	–
Other	22.86	8.34	26.86	13.24

Such diseases were observed on the 3rd place among the factors lowering milk producing breeding farm activity income, right after reproduction problems and mammary gland inflammations (*mastitis*) (GREEN et al. 2002) and reduce the healthy quality of cow's milk MICIŃSKI et al. 2012, MICIŃSKI et al. 2013). Appearing lameness significantly lower welfare and have an impact on the level of cow culling. The highest culling risk of cows appears at the beginning of lactation. Lameness, which appear during the first 30 days after calving may be connected to ovary cysts presence, which lower the calving probability (SOGSTAD et al. 2005).

In limping cows reproductive processes are elongated. Resting time i.e. time from calving to the 1st insemination attempt increases nearly

three times, whereas the service period i.e. time from the 1st insemination attempt to successful fertilization increases more than fifteen times. Such state results in an increase in insemination treatments per calving (HASKELL et al. 2006, HERNANDEZ-MENDO et al. 2007).

Lameness is considered a serious problem in high-performance dairy cattle hers. Their occurrence is matched to many factors, such as keeping system and nutrition, age, performance, condition. Lameness causes decrease of animal welfare, as well as economical losses caused by a decrease in performance and fertility of animals and by an increased cow culling (FLIS 2015).

For the breeder early (from the start of lactation) limping cow identification is essential. This way the spread of lameness in the herd is reduced allowing for hoof diseases treatment in their early stages.

The aim of this work was to present and characterize chosen hoof diseases, as well as to present their impact on performance and welfare of Holstein-Friesian cows.

The work is a review, where the scientific literature characterizing the most important limb diseases of cows was collected and main factors impacting the incidence of such diseases, as well as the decrease in morbidity level were presented.

Discussions

There is a very large diversity of limb diseases, mostly concerning hoof and the hoof gap, whereas in bedding-less cowsheds bruises as well. Those may be caused by differences in the ways of cow keeping, thus differentiated hoof exposure to environmental factors (SWALVE et al. 2005). The most common limb diseases are (BAGGOT and RUSSELL 1981, NOCEK 1997, BERGSTEN 1999, CRUZE et al. 2001, GREGORY 2004, VERMUNT and HILL 2004, SOGSTAD 2005, HOLZHAUER et al. 2006, ELLIOTT and ALT 2009, EVANS et al. 2009).

Horn rotting – mainly concerning heels. It occurs most often in cows kept in bad zoohygienic conditions i.e. standing in the so-called fecal manure. During the first period of a disease hoof correction and tar soaking may be enough. However, during the intensified stadium of disease it is essential to apply iodoform and copper sulfate dressing.

Whitlow – the disease of the hoof gap and its surroundings. It develops as a result of anaerobic bacteria activity, as well as due to inappropriate bedding, stony passes and pastures. Hard surface is a source of different types of hoof gap injuries. Treatment is based on antibiotics application at the place of injury, as well as iodoform and rivanol wraps.

Prevention means: disinfection, proper animal nutrition, obeying hoof correction schedule and keeping them clean, as well as hoof hardening.

Sprains and dislocations – these are painful and complicated diseases. Edema appears. They are hard to treat, often being untreatable.

Joints inflammation – develops during hits or sprains, as well as a result of injuries, when contamination from blood or surrounding tissues occurs. This disease is manifested by a painful and hot edema making animals lie most willingly, also decreasing their appetite and dairy performance.

Finger skin inflammation (*dermatitis digitalis*) – superficial skin inflammation at the border of the coronet edge of pads. Changes developing during the finger skin inflammation may transform into the papillary formations. The disease often appears in dairy cattle herds in Europe and South America. Favoring factors are not known. Only adult cattle get sick. Finger skin inflammation is a contagious disease. It is believed that spirochetes of the *Treponema* species are the infectious factor.

Fingerpad skin inflammation (*erosion ungulae*) – it appears as irregular cavities in the pad horn in the form of many dark holes (black color) or pockets with cavities. As time progresses, such cavities transform into oblique grooves. Such disease is widely distributed among the cattle kept in buildings during winter. Usually, symptoms disappear during the pasture period. Predispositions for the appearance of this disease are humid environment and long lasting contact with manure.

Interdigital fissure skin overgrowth (*hyperplasia interdigitalis*) – the disease is based on the expansion of the interdigital area skin of all limbs, most often posterior ones, with the tendency to keratosis and necrosis, as well as purulent inflammations. Disease etiology is complicated. One of the causes is a mechanical overload of the interdigital skin area and as a result it grows fast. Genetic factors have a major impact on occurrence of this disease, especially the low endurance for interdigital tendons stretching. The changes are located in the interdigital area in the form of growths and thickenings. The tumor is painless and hard. The disease develops into the disintegrating form manifesting itself as tumor rupture and wound contamination leading to inflammation.

Hoof box rupture – this disease is based on the mechanical damage to the hoof box. It is conditioned by the presence of obstacles on animal's way, which may lead to box injuries. The mechanical injury opens the way for pathogenic microorganisms to soft tissues of hoof and development of inflammation, thus leading to phlegmon and horn rotting.

Interdigital skin inflammation (*dermatitis interdigitalis*) – it is manifested through mild inflammatory changes in the interdigital skin, leading to moderate pain. Fissures in the pad's horn may lead to the

damage of dermis or later plantar ulcers. In such conditions the lameness may be of high degree or chronic. Such disease starts with the moment of wet, smelly skin inflammation in the interdigital area. Breeders often call this disease "smelly leg". The inflammation may expand to the heel horn of the adjacent hoof. The improper shaping of the horn area may lead to pressure on the cortex there with the evident pain or lameness.

Rusterholz ulcer – it concerns the softened hoof horn and leads to a change called the "plantar ulcer". It appears in animals of higher body weight, with faulty hoofs and improper posture. Changes in hoof called "plantar ulcers" need hoof correction with a proper cleaning, applying dressing with an antibiotic and then with iodoform. Prevention is based on a timely hoof correction and hoof bathing in formalin. Ulcers are mainly caused by a high body weight and injuries; they are very painful and usually lead to severe lameness. Rumen acidosis favors this disease. Plantar ulcer is manifested by an unwillingness to move, elongated standing phase ("kneeling" on wrists) and with both legs used – "sitting dog" posture. Sick cows avoid hard surfaces, moving better on the soft ones. Abduction relieving the outer hoof is visible and the movement may be mowing. Animals in the advanced state of the disease lose appetite. Treatment is based on the removal of the horn in places where the exudate had separated it from the laminar layer. Then the exposed dermis may be precisely cleaned. The role of the dressing under the band is to keep medicines for a longer time, put a pressure on the laminar layer, stop the bleeding and reduce overgrowth of the repair tissue. Treatment is supplemented by a proper hoof shape correction favoring the relocation of the point of support of limbs from the back to the front. It is recommended to use the relieving block (heel) in case of necrotic changes. Thanks to such a procedure the sick hoof is not used in supporting of the limb, thus it is not stimulated and it does not cause pain and is not getting dirty. The dressing should be changed in the 10-day intervals.

Exudate (*interdigital phlegma*) – it is an interdigital inflammation, thus inflammation above the interdigital skin and is connected to a hard swelling in the middle of the staple area, precisely above the interdigital area. Cow is evidently limping in this case. Fast treatment at the early stages makes the inflammation slowly disappear and not doing any persistent damage.

Laminitis – the aseptic inflammation of the laminar layer of the hoof wall; develops due to the damage of the capillaries caused by toxins and histamine, which are overproduced in severe indigestions (rumen acidosis), uterus and mammary gland inflammations and after complicated parturitions. Diffused inflammation of the hoof laminar layer is connected

to the permeation of the serous liquid from blood. The course of the process is subacute, acute or chronic. The disease attacks both posterior hoofs at the same time. Sick animals lie on the side with limbs stretched. Hoof cans are painful. Red discolorations are visible on the plantar side. Such animals are unwilling to stand up at all, taking improper postures, stretching front limbs, sometimes crossing them and consume the feed in the kneeling position. Their body temperature, heart rate and respiratory rates increase.

Most often the appearance of main limb diseases is caused by the improper cow keeping, thus it is connected to the technical equipment of farms, as well as cow keeping and nursing. It is also connected to feeding, posture of the back limbs or genetic factors (cattle breed for example). It is essential to avoid prolonged exposure of animals to wet surfaces, as well as to diagnose fast – the faster, the smoother the course.

Systematic control of hoof condition is an essential nursing element. It allows to detect posture faults, diseases and hoof deformation. Hoof nursing should be understood as taking care of proper environmental and hygienic conditions of barns and especially their state as well as material from which the ground for cattle stands is made (WEBB and CLARK 1981). Negligence of hoof nursing leads to lameness, bone and joints deformation. The most important part of nursing treatments is a timely hoof correction, which aims to reestablish physiological posture of a limb and hoof, deformed in the breeding conditions.

Decontamination and hoof hardening liquid baths are very helpful in the prevention of hoof diseases. A 10% aqueous solution of copper sulfate or 5% formalin is used for this purpose. Such prepared liquid is stored in special pools of smooth spades and shores to assure cows take a bath while moving through it. The deepness must be enough for cow to dip the hoof and the interdigital fissure. Proper feeding and animal maintenance is an equally essential prevention factor. Alternatively, spraying hoofs with disinfectant may be a good solution. Most often solutions based on copper compounds are used, however here environmental factors should be considered. Research concerning the effectiveness of aqueous copper sulfate solutions are still in progress. It has been stated that copper compounds have a significant impact on the treatment of skin processes. In some cases, depending on instructions, a proper solution of potassium permanganate is used. In cattle hoof nursing special self-adhesive bandages are used as well.

An assessment of lameness level in the herd of dairy cattle

The simplest form of on-farm lameness detection is visual locomotion scoring. For the assessment of the level of lameness in dairy cattle a 5-grade scale of locomotion review is commonly used. The assessment of a moving cow (locomotion scoring) provides basic information on the lameness level in each cow. The first people who introduced such locomotion assessment system were Americans, and then it was successfully introduced in other countries as well. It allows for a fast lameness diagnosis and use of proper treatment (ADAMS et al. 2016). Locomotion assessment is based on cow observation in the standing position, as well as in motion on the hard surface, focusing especially on the back line. The review is 5-grade, and according to Zinpro (2014) it consists of:

1^0 – normal posture. In the standing posture and while moving the back line is straight, legs and hoofs are properly aligned.

2^0 – light lameness. In the standing position the back line is straight, while moving it is slightly curved; a subclinical form of lameness.

3^0 – moderate lameness. The cow is standing and moving with the curved back, it makes short steps with one or few limbs; a subclinical form of lameness.

4^0 – lameness. The curved back in the standing position and while moving, cow is limping on one or more limbs, lameness is visible; a clinical form of hoof lameness.

5^0 – acute lameness. The back is strongly bent, the cow is unwilling to stand and load at least one of the limbs, it may have difficulties with standing up or it does not want to do it; a clinical form of lameness.

In order to keep the 1^{st} level and avoid lameness in dairy cattle a proper hoof correction must be performed at least twice a year, while in case of cows in which the subclinical form of lameness has been diagnosed (2^{nd} and 3^{rd} degree) it is essential to perform an additional correction. The 4^{th} degree is connected with the 17% decrease in milk performance, whereas the 5^{th} degree – 36%. In cows with the 2^{nd} degree or higher serious reproduction problems are diagnosed as well. The probability of culling of such cows increases 8.5 x as well. VAN NUFFEL et al. (2015) identified at least 25 different visual scoring systems for dairy cow lameness characteristics. They noted that although these methods are relatively easy to use and inexpensive to implement, the amount of time it takes to conduct scoring on an entire herd means they are not often executed.

Now, individual animal monitoring technologies have shown potential for lameness detection. Walk-over or stand-on load cells, pressure-sensitive position mats, vision techniques, accelerometers, and other already

available sensor data have all been evaluated for the possibility of automated lameness detection (VAN NUFFEL et al. 2015). Producers tend to underestimate the prevalence of lameness in their herds. They perceive lameness management to be more challenging to include in daily routines compared to other health issues, like mastitis, which can be managed in the parlor (LEACH et al. 2010). Instead, lame cows are often only identified after they become severely lame (MILL and WARD 1994), completely ignoring mildly lame cows that would benefit most from early detection.

In summary, a basic element of the hoof nursing in herds are periodic inspections of limbs. The aim of such inspections is to diagnose posture faults, as well as diseases or hoof deformations in order to perform correction.

The influence of chosen factors on lameness appearance in cows

Nutrition

In case of nutrition it is essential to focus attention on functioning of the rumen. High intake of concentrated feed (NFC fiber) with a simultaneous low volumetric feeds intake (NDF fiber) causes an increase in the pH level of rumen content, thus causing production of histamine and endotoxins, which get to capillaries in hoofs, destroy them and impair production of good quality horn tissue (BRAMLEY et al. 2008, LEAN et al. 2013). What is more, mineral i.e. S, Cu, Zn, Se, Mn and Co, as well as retinol and biotin deficiencies, so elements and vitamins taking part in keratin metabolism, decrease horn hardness. Such elements are components of antioxidative enzymes, which protect lipids in the horn tissue from free radicals. Their deficiency leads to an increase in concentration of sialic acid and malonic dialdehyde causing oxidative stress in the organism. Oxidative stress causes destruction of hoof`s capillaries, insufficiencies in oxygen and nutrients supply and as a consequence – development of low quality horn (AL-QUDAH and ISMAIL 2012, TOMLINSON et al. 2004, SEYREK et al. 2008).

Housing system

In the free standing cow keeping system a cow should spend half of the day on the bed. Interruption of that leads to limp overload and worsening of rumen digestion and blood circulation having a direct response in the decrease of milk yield. The main load of the body weight is on the back limbs (additionally loaded with the mammary gland) showed by a common appearance of hematomas on the lateral wall of the back hoofs. Standing

and moving takes the cow ⅕ of a day in average, so about 5 hours. It is important that the floor area, on which cows move or stand waiting for milking, is of a proper quality i.e. slightly roughened, non-slip and cushioning. It is important to take shape, hardness, abrasion and the ability to maintain hygiene into account (SOMERS et al. 2003). In proper conditions, when cows have an appropriate amount of motion, the balance between the horn proliferation and its abrasion appears (KOŁACZ and TRACZ 2008). The surface of the floor of high roughness leads to an excessive abrasion of hoofs, thus causing easily observable lameness. Increasing the number of cows in herds leads to building of bigger barns. In such barns cows move over higher distances in order to get to the milking hall. It also influences higher abrasion of the hoofs' horn (NAWROCKI et al. 2004).

Age

BIELFELDT et al. (2005) i HASKELL et al. (2006) during their lameness risk assessment in herds have shown that risk cofactors are higher in older cows, thus in IV, V and further lactations (Table 2).

Table 2

Cofactor of lameness appearance risk in dependence on age of cows (BIELFELDT et al. 2005, HASKELL et al. 2006)

Lactations	Risk cofactor	
	BIELFELDT et al. (2005)	HASKELL et al. (2006)
I	0.28	0.10
II	0.37	0.20
III		0.24
IV	0.80	0.23
≥ V		0.33

Whereas FLEISCHER et al. (2001), BARAŃSKI et al. (2008) and KUCZAJ et al. (2008) have shown that lameness are a cause of all cullings in herds in 13 to 23% and these indicators are higher in older cows (Table 3).

Table 3

Age-dependent cow culling in percentage (KUCZAJ et al. 2008)

Culling – reasons	Lactations				
	I	II	III	IV	≥ V
Lameness	19.15	12.68	22.37	25.00	29.17
Infertility	38.30	49.30	27.63	20.00	25.00
Low performance	12.77	14.08	11.84	2.50	–

Influence of lameness on production parameters of cows

Milk performance

Many papers show that the higher the milk yield of Holstein-Friesian cows the higher the risk of lameness appearance (Bielfeldt et al. 2005, Archer et al. 2010, Koeck et al. 2014, Alawneh et al. 2014). High-performance cows i.e. which performance per 100 kg of body weight exceeds 1000 kg of milk in the 305-day lactations are twice more often exposed for lameness than low-yield cows i.e. which performance is lower than 1000 kg of milk (Bielfeldt et al. 2005). A phase of lactation and cows age also have a significant impact. The most lameness were noted in the first three months of lactation and in older cows (Fleischer et al. 2001, Bielfeldt et al. 2005, Haskell et al. 2006, Winkler and Margerison 2012, Navarro et al. 2013).

Huxley (2013) summarized previous studies that estimated a milk yield loss of 270 to kg per lactation when lameness occurred. Evidence exists that this milk loss occurs during not only clinical lameness, but also pre-diagnosis and post-recovery depending on lameness type (Charfeddine and Pérez-Cabal 2017).

According to Hernandez (2005), Reader et al. (2011) and Van Hertem et al. (2013) the deterioration of cow locomotion causes a decrease in their daily milk yield. Such a decrease is noted even about 5 weeks before the lameness can be spotted. Simultaneously it was noted that cows with a slight lameness (3 BCS pts.) are characterized by a significant decrease in performance. The biggest yield decreases concern cows in which lameness appears at the beginning of lactation and in cows in which it persists (Archer et al. 2010). Olechnowicz and Jaśkowski (2010) noted a decrease in fat, protein, lactose yield and an increase in somatic cells content in the milk of cows, which received worse grade during locomotion assessment. Onyiro et al. (2008) have shown the negative correlation of milk yield with locomotion (-0.04±0.03) and positive concerning fat content in milk with locomotion (0.22±0.03). Gleeson et al. (2007) have shown that cows in which one of many lameness variants were found rarely approached milking robots in the VMS (voluntary milking systems). Such a situation had a negative impact on mammary gland health and caused infections through teats which were impaired by a higher pressure and swelling. Reader et al. (2011) state that in cows with treated lameness milk yield increases only after a month of milking since the moment of locomotion improvement.

In 1994 BARKEMA et al. (1994) have noticed that 1.1 x more lameness were seen in cows which yield in the last lactation increased by every 100 kg of milk in the first 100 days of lactation.

Reproduction

WALKER et al. (2008) stated that in cows with diagnosed lameness the period from calving to the first observed oestrus and the beginning of insemination is prolonged. It causes the elongation of parturition interval. In cows suffering from lameness the first successful insemination indicator decreases by 13%, whereas the calving indicator by 9% (MELENDEZ et al. 2003, KILIÇ et al. 2007). WALKER et al. (2008) present that cows of bad locomotion have a lower oestrus activity, lower progesterone and luteinizing hormone concentration in milk outside the oestrus period and higher concentration of progesterone during ovulation. MORRIS et al. (2011) have observed a decrease in the level of estradiol in the plasma of lame cows and have shown that 21% of suffering cows did not show oestral behaviordespite a properly developed ovarian follicle. What is more, authors presented that 29% of cows with improper locomotion did not develop follicles after the injection of oestrus synchronizing hormones. ONYIRO et al. (2008) have shown that the length of the calving interval is positively correlated with locomotion and susceptibility to hoof diseases.

Welfare

Nowadays the most popular are two types of barns: loose housing (*non-tethered*) and tie-up housing (*tethered*). The number of cattle influences the choice of the keeping system. In the barns amounting over 25 cows it is recommended to use the loose housing system, where the conditions are similar to the natural ones and where welfare needs are fulfilled.

The loose housing barns have many advantages, such keeping system is similar to a natural one, thus positively influencing animal performance. With the loose housing system cows tend to suffer less from the limb and mammary glands diseases and are more fertile. In the loose housing cowshed they have more movement freedom so they can fulfill their natural needs, having the possibility to move and contact with different animals in the herd. Milking is performed in the separate rooms i.e. in the milking halls or in the VMS system (voluntary milking systems) and thanks to that the milking itself is more efficient with a higher milking hygiene. Among the loose housing barns several types can be distinguished:

– with the separate standing while eating part, with deep bedding and a collective lying area,

– with the separated parts for laying and feeding,

– with boxes fulfilling the nutritional, as well as laying role at the same time (*combiboxes*).

In the loose housing barns the couching-places must allow laying down and standing up in a proper for a cow way. The number of the places must correspond to the number of animals. If this condition is not fulfilled weaker cows are phased out by the stronger ones and are not allowed to use the couching-places. The loose housing barn with a deep litter is divided into two parts: resting and feeding. The feeding part (3–3.5 m) should be separated from the resting part with the stairs. The area of the couching-place should be adjusted to the number of cows kept in the herd, for one animal there should be about 5 m^2 of a couching-place, bedding usage with such a keeping is around 8 kg per piece per day. Instead of bedding also grated floors are used, however these increase the point load of the hooves.

In the loose housing, boxed cowsheds there are special boxes corresponding to the number of the animals. These should be adjusted, so the animals can move freely. The ground may be bedded or un-bedded, where bedding is replaced by the plastic mattress. In the loose housing, boxed sheds the use of a bedding is about 1 kg per piece per day. With combiboxes feeding is performed on the couching place. On each side of the feed corridor there are combiboxes, their dimensions should be adjusted to the dimensions of the animals: 110–120 cm or 170–180 cm. The couching-place dimensions should be taken into consideration especially in the tie-up barns. Places too short or too narrow lower animal condition, restrict the movement and are unacceptable due to zootechnical reasons. The tether should allow the cow to move forward, stand up, lay down and move backwards freely. The grated couching-place must be secure from mammary gland damage. There are two types of couching-places: short and long. The short one is 180–190 cm long, used most often in the un-bedded cowsheds, the length should be adjusted in a way that the back limbs of a cow are 10 cm from its border. Too short place leads to cows standing in the manure canal, thus many diseases. If the proper distance from the border is kept, the excrements will land in the canal. The long couching-place is 210–250 cm long, such places are recommended for birth or treatment. They are bigger, so harder to keep clean. Contaminated couching-place helps to infect the mammary gland. When choosing a specific type of a barn it is essential to look on the herd's size, economic factors, animal welfare, as well as comfort and work safety. With 30 cows in a herd the cost of building of the loose housing and tie-up barns is similar, the difference is visible from

60 cows and more in favor of the loose housing system. Nowadays, in the EU countries there is a focus on the animal keeping conditions and that is why many breeders, after Poland's entrance into the EU, had to modernize their cowsheds, also in terms of welfare.

Conducted research (BERGSTEN 1999, MANSKE 2002, COOK 2003) has proven that the all-year keeping on concrete grates in the loose housing system is better for hooves if combined with pasture grazing in the summer. On the other hand, (WHAY 2002), gives proof that the least amount of lameness cases are observed during the all-year use of pasture, dominating in countries such as New Zealand, Argentina and Chile, where cows have total freedom of standing and laying on earth i.e. surface which is amortizing the pressure of body weight on limbs most efficiently.

An important factor for the occurrence of limb diseases is a surface of the milking hall anteroom, where cows sometimes spend several hours. Improper abrasive surface and ground slope predisposes for hoof horn damage appearance. Cows feel hesitant on such surface, making cautious steps, in wrong order and wrong angulation, which damages very delicate laminae of the white line of hoof. Similar effect is caused by improperly designed milking halls, where entrance and exit are set at an acute angle (HERLIN and DREVEMO 1997).

The stressed of cows often move out of the milking hall fast and when they meet the sharp turn on their way to the feeding table they perform unnatural limb twists, overloading the white line, leading to its strain or damage. Particular attention should be paid to primiparous cows, which are kept with older cows right after calving. Stress caused by relocation to a different place, change in feeding and beginning of milking is multiplied by the presence of dominating cows in the herd and may cause more seldom staying of primiparous cows on the couching place, thus overload of limbs. With the improper surface the risk of plantar surface ulcers rises (WEBB and CLARK 1981).

Research presented by TELEZHENKO and BERGSTEN (2004) concerned animal locomotion on different surfaces. Measurements of length of steps and hoof stepping angle on 5 different surfaces: grated concrete surfaces, grates covered with mattes, smooth concrete surfaces, concrete surfaces covered with mattes and compressed sand surface, as the one which is the most similar to the natural pasture surface, were performed. The abrasive ability of these surfaces was also examined. Three groups of cows were used for this research – the ones which did not show any signs of lameness, ones which showed the angulation of the back during walk, with no sign of it while standing and ones with angled back in both cases. The results showed that the most slippery surface was the grated concrete one and the

most abrasive was continuous concrete surface. The lowest movement speed was noted on the concrete surfaces. Cows were moving faster on grates covered with mattes.

Lameness prevention

Prevention means adopting a specific plan. These can be control and prevention strategies for reducing lameness incidence repetitive actions or one-time long-term investments. Examples of repetitive investments include preventive hoof trimming, footbaths, hoof health feed additives, or even genetic selection. An example of a long-term investment in lameness prevention would be the installation of rubber flooring or the redesigning of poorly constructed freestalls (FJELDAAS et al. 2006, LAVEN and HUNT 2002, BERGSTEN et al. 2003, PRITCHARD et al. 2013).

One of the most common prevention elements is a timely hoof trimmers. On-farm staff, hoof trimmers, or veterinarians most frequently treat lameness. In a survey of 184 farms across the United States, 77% of farms used a professional hoof trimmer for hoof trimming services whereas 16% used a veterinarian or on-farm staff and 7% used no hoof trimming services at all (ADAMS et al. 2016). Its frequency depends on the cow keeping system and is most often performed once a year – in the pasture-alcove system or twice a year – in the all-year alcove keeping system (MANSKE et al. 2002).

When making a decision of a treatment the abrasion of the hoof horn is taken into account assuming that the hoof's horn grows from 3 to 13 mm monthly in average. That is why there is a need to perform hoof trimmers at least twice a year. Most often the first one is performed in May i.e. before releasing animals onto the pasture or not later than 4 weeks before them leaving. In the loose housing barns it is enough to make such treatment only once, as an increased cow activity appears there. Animals are able to rub off the hoof horn only slightly during the daily activity both in the cowshed, as well as on pasture. The problem concerns cows kept in the deep-bedding system also, where the approach to the feeding table and its surroundings is most often hardened.

The lack of hoof trimmers and faulty surface makes the horn rub off unevenly or too slow. As a consequence the overgrown hoof horn, mainly in the front, causes improper body mass distribution leading to calluses, as well as an overload of the finger and joint capsule tendons. This leads to hoof joint bones inflammation and a visible lameness and unwillingness to move. Such animals stumble, slip, lose balance making injuries even bigger and deeper.

Skillfully carried out correction reestablishes a proper hoof shape, allowing to distribute body weight evenly and lowers the amount of visible joint swellings. An optimal slope angle of the frontal wall of the hoof is 50–55º, whereas the back wall 45–50º, the most optimal setting of the ankle joint viewed from the side is 145–155º.

Conclusion

Surfaces on which cows move should be clean, comfortable for moving and if possible allow to rub off the hoof. Hoof baths while moving should be regular and adjusted to herd's requirements. It is needed to observe cows in order to detect lameness early and perform a proper treatment fast. All lameness in the herd and their causes should be noted down to enhance cows welfare, for example to change cow keeping system. Hoof correction should be regular and carried out professionally. Cows with lameness of the 2º and 3º types show almost 3 times higher re-insemination per one fertilization index. Lameness lower cow performance, because they definitely eat less feed. The share of leftovers in cows reaches 36%. On farms, which do not perform limb and hoof based selection and do not maintain basic limb diseases prevention together with providing cows with welfare, more disease cases are observed with the biggest share of limb diseases. The enhancement of limb structure in dairy cattle, through selection, requires a very long time, as features connected to their structure and health are of a very low inheritance. Due to this fact it is essential to take care of the practical aspects connected to a good milk-producing environment.

In summary, it is right to say that lameness are very costly cow diseases, both in terms of herd health, as well as farm profitability. Costs include milk losses, veterinary services, decrease in milk performance and as an effect – much earlier cow culling. Research shows that the differences between farms in terms of appearance, as well as lameness degree assessment are significant, meaning that it is needed to act to improve such situation.

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References

Adams A., Lombard J., Fossler C., Román-Muñiz I., Kopral C. 2016. *Associations between housing and management practices and the prevalence of lameness, hock lesions, and thin cows on US dairy operations.* J. Dairy Sci., 100: 2119–2136.

Alawneh J.I., Stevenson M.A., Williamson N.B., Lopez-Villalobos N., Otley T. 2014. *The effects of liveweight loss and milk production on the risk of lameness in a seasonally calving pasture fed dairy herd in New Zealand.* Prev. Vet. Med., 113: 72–79.

Al-Qudah K.M., Ismail Z.B. 2012. *The relationship between serum biotic and oxidant/antioxidant activities in bovine lameness.* Res. Vet. Med., 92: 138–141.

Archer S.C., Green M.J., Huxley J.N. 2010. *Association between milk yield and serial locomotion score assessments in UK dairy cows.* J. Dairy Sci., 93: 4045–4053.

Baggot D.G., Russell A.M. 1981. *Lameness in cattle.* Brit. Vet. J., 137(1): 113–132.

Barański W., Janowski T., Zduńczyk S. 2008. *Incidence of reproduction disorders, clinical mastitis and lameness in cross-breed HF x BW cows and Jersey cows maintained in the same conditions.* Med. Weter., 64: 1201–1204.

Barkema H., Westrik J., Van Keulen K., Schukken Y., Brand A. 1994. *The effects of lameness on reproductive performance, milk production and culling in Dutch dairy farms.* Prev. Vet. Med., 20: 249–259.

Bergsten C. 1999. *Identifying diseases of the bovine foot and their causes.* Proc. Hoof. Health Conf. Modesto., CA, pp. 28–33.

Bergsten C., Greenough P., Gay J., Seymour W., Gay C. 2003. *Effects of biotin supplementation on performance and claw lesions on a commercial dairy farm.* J. Dairy Sci., 86: 3953–3962.

Bielfeldt J.C., Badertscher R., Tölle K.H., Krieter J. 2005. *Risk factors influencing lameness and claw disorders in dairy cows.* Liv. Prod. Sci., 95: 265–271.

Bramley E., Lean I.J., Fulkerson W.J., Stevenson M.A., Rabiee A.R., Costa N.D. 2008. *The definition of acidosis in dairy herds predominantly fed on pasture and concentrates.* J. Dairy Sci., 91: 308–321.

Charfeddine N. and Pérez-Cabal M.A. 2017. *Effect of claw disorders on milk production, fertility, and longevity, and their economic impact in Spanish Holstein cows.* J. Dairy Sci., 100: 653–665.

Cook N.B. 2003. *Prevalence of lameness among dairy cattle in Wisconsin as a function of housing type and stall surface.* J. Americ. Vet. Med. Assoc., 223: 1324–1328.

Cruze C., Driemeier D., Cerva C., Corbellini L.C. 2001. *Bovine digital dermatitis in southern Brazil.* Vet. Rec., 148(18): 576–577.

De Frain J.M., Socha M.T., Tomlinson D.J. 2013. *Analysis of foot health records from 17 confinements dairies.* J. Dairy Sci., 96: 7329–7339.

Elliott M.K, Alt D.P. 2009. *Bovine immune response to papillomatous digital dermatitis (PDD) – associated spirochetes is skewed in isolate reactivity and subclass elicitation.* Vet. Imm. Immunopat., 130(3–4): 256–261.

Evans N.J., Brown J.M., Demirkan I., Murray R.D., Birtles R.J., Hart C.A., Carter S.D. 2009. *Treponema pedis sp. Nov., a spirochaete isolated from bovine digital dermatitis lesions.* Int. J. Syst. Evol. Microbiol., 59(5): 987–991.

Fjeldaas T., Sogstad Å., Østerås O. 2006. *Claw trimming routines in relation to claw lesions, claw shape and lameness in Norwegian dairy herds housed in tie stalls and free stalls.* Prev. Vet. Med., 73: 255–271.

Fleischer P.M., Metzner M., Bayerbach M. 2001. *The relationship between milk yield and the incidence of some diseases in dairy cows.* J. Dairy Sci., 84: 2025–2028.

Flis E. 2015. *Problem kulawizny u krów rasy holsztyńsko-fryzyjskiej.* In: *Nauka w służbie przyrodzie – wybrane zagadnienia.* Eds. M. Olszówka, K. Maciąg. Wyd. Fundacja TYGIEL, Lublin, pp. 83–97.

Gleeson D.E., O'Brien B., Boyle L., Early B. 2007. *Effect of milking frequency and nutritional level on aspects of the health and welfare of dairy cows.* Anim., 1(1): 125–132.

GOFF J.P., HORST R.L. 1997. *Physiological change at parturition and their relationship to metabolic disorders*. J. Dairy Sci., 80: 1260–1268.

GREEN L.E., HEDGES V.J., SCHUKKEN Y.H., BLOWEY R.W., PACKINGTON A.J. 2002. *The impact of clinical lameness on the milk yield of dairy cows*. J. Dairy Sci., 85(9): 2250–2256.

GREEN L.E., HUXLEY J.N., BANKS C., GREEN M.J. 2014. *Temporal associations between low body condition, lameness and milk yield in a UK dairy herd*. Prevent. Vet. Med., 113: 63–71.

GREGORY N.G. 2004. *Swelling of cattle heel horn by urine*. A. Vet. J., 82(3): 161–163.

HASKELL M.J., RENNIE L.J., BOWELL V.A., BELL M.J., LAWRENCE A.B. 2006. *Housing system, milk production, and zero-grazing effects on lameness and leg injury in dairy cows*. J. Dairy Sci., 89: 4259–4266.

HERLIN A.H., DREVEMO S. 1997. *Investigating locomotion of dairy cows by use of high speed cinematography*. Eq. Vet. J., 23(S): 106–109.

HERNANDEZ J., SHEARER J.K., WEBB D.W. 2001. *Effect of lameness on the calving – to – conception interval in dairy cows*. J. Amer. Vet. Med. A., 218(10): 1611–1614.

HERNANDEZ J.A., GARBARINO E.J., SHEARER J.K., RISCO C.A., THATCHER W.W. 2005. *Comparison of milk yield in dairy cows with different degrees of lameness*. J. Amer. Vet. Med. A., 227: 1292–1296.

HERNANDEZ-MENDO O., KEYSERLINGK M.A.G., VEIRA D.M., WEARY M. 2007. *Effects of pasture on lameness in dairy cows*. J. Dairy Sci., 90: 1209–1214.

HOLZHAUER M., HARDENBERG C., BARTELS C.J., FRANKENA K. 2006. *Herd – and cow – level prevalence of digital dermatitis in the Netherlands and associated risk factors*. J. Dairy Sci., 89(2): 580–588.

HUXLEY J. 2013. *Impact of lameness and claw lesions in cows on health and production*. Livest. Sci., 156: 64–70.

KILIÇ N., CEYLAN A., SERIN İ., GÖKBULUT C. 2007. *Possible interaction between lameness, fertility, some minerals, and vitamin E in dairy cows*. Bull. Vet. Inst. Pulawy, 51: 425–429.

KOECK A., LOKER S., MIGLIOR F., KELTON D.F., JAMROZIK J., SCHANKELA F.S. 2014. *Genetic relationship of clinical mastitis, cystic ovaries, and lameness with milk yield and somatic cell score in first lactation Canadian Holsteins*. J. Dairy Sci., 97: 5806–5813.

KOŁACZ R., BODAK E. 1999. *Dobrostan zwierząt i kryteria jego oceny*. Med. Weter., 3: 147–154.

KOŁACZ R., TRACZ E. 2008. *Systemy utrzymania krów mlecznych i ich dobrostan*. Byd., 10: 12–16.

KÖNIG S., SHARIFI A.R., WENTROT H., LANDMANN D., EISE M., SIMIANER H. 2005. *Genetic parameters of claw and foot disorders estimated with logistic models*. J. Dairy Sci., 88: 3316–3325.

KUCZAJ M., ZIELAK A., BLICHARSKI P. 2008. *Reasons for the culling of Polish Holstein Friesian cows in a high yield herd*. Med. Weter., 64: 1205–1208.

LAVEN R., HUNT H. 2002. *Evaluation of copper sulphate, formalin and peracetic acid in footbaths for the treatment of digital dermatitis in cattle*. Vet. Rec., 151: 144–146.

LEACH K.A., WHAY H.R., MAGGS C.M., BARKER Z.E., PAUL E.S., BELL A.K., MAIN D.C. 2010. *Working towards a reduction in cattle lameness:* 1. *Understanding barriers to lameness control on dairy farms*. Res. Vet. Sci., 89: 311–317.

LEAN I.J., WESTWOOD C.T., GOLDER H.M., VERMUNT J.J. 2013. *Impact of nutrition on lameness and claw health in cattle*. Liv. Sci., 156: 71–87.

MANSKE T. 2002. *Hoof lesions and lameness in swedish dairy cattle*. Acta Univ. Agri. Sueciae. Vet., 135: 1401–6257.

MANSKE T., HULTGREN J., BERGSTEN C. 2002. *Prevalence and interrelationships of hoof lesions and lameness in Swedish dairy cows*. Prev. Vet. Med., 54: 247–263.

MELENDEZ P., BARTOLOME J., ARCHBALD L.F., DONOVAN A. 2003. *The association between lameness, ovarian cysts and fertility in lactating dairy cows*. Ther., 59: 927–937.

MICIŃSKI J., KOWALSKI I., ZWIERZCHOWSKI G., SZAREK J., PIEROŻYŃSKI J., ZABŁOCKA E. 2013. *Characteristics of cow's milk proteins including allergenic properties and methods for its reduction*. Pol. Ann. Med., 20(1): 69–76.

MICIŃSKI J., ZWIERZCHOWSKI G., KOWALSKI I. M., SZAREK J., PIEROŻYŃSKI B., RAISTENSKIS J. 2012. *The effect of bovine milk fat on human health*. Pol. Ann. Med., 19(2): 170–175.

MILL J., WARD W. 1994. *Lameness in dairy cows and farmers' knowledge, training and awareness.* Vet. Rec., 134: 162–164.

MORRIS M.J., KANEKO K., WALKER S.L., JONES D.N., ROUTLY J.E., SMITH R.F., DOBSON H. 2011. *Influence of lameness on follicular growth, ovulation, reproductive hormone concentrations and estrus behavior in dairy cows*. Ther., 76: 658–668.

NAVARRO G., GREEN L.E., TADICH N. 2013. *Effect of lameness and lesion specific causes of lameness on time budgets of dairy cows at pasture and when housed.* Vet. J., 197: 788–793.

NAWROCKI L., WINNICKI S., GŁOWICKA R., MYCZKO A., TOMALA A., KOWALSKI K., DEMBOWSKI K. 2004. *An example of a technological solution that ensures the welfare of cows*. Zesz. Nauk. Zoot. L II AR Wrocław, 505: 179–186.

NOCEK J.E. 1997. *Bovine acidosis: Implications on laminitis*. J. Dairy Sci., 80: 1005–1028.

OLECHNOWICZ J., JAŚKOWSKI J.M. 2010. *Impact of clinical lameness, calving season, parity, and month of lactation on milk, fat, protein, and lactose yields during early lactation of dairy cows.* Bull. Vet. Inst. Pulawy, 54: 605–610.

ONYIRO O.M., ANDREWS L.J., BROTHERSTONE S. 2008. *Genetic parameters for digital dermatitis and correlations with locomotion, production, fertility traits, and longevity in Holstein-Friesian dairy cows*. J. Dairy Sci., 91: 4037–4046.

PRITCHARD T., COFFEY M., MRODE R., WALL E. 2013. *Genetic parameters for production, health, fertility and longevity traits in dairy cows*. Animal, 7: 34–46.

READER J.D., GREEN M.J., KALER J., MASON S.A., GREEN L.E. 2011. *Effect of mobility score on milk yield and activity in dairy cattle.* J. Dairy Sci., 94: 5045–5052.

SEYREK K., YAYLAK E., AKŞIT H. 2008. *Serum sialic acid, malondialdehyde, retinol, zinc, and cooper concentrations in dairy cows with lameness.* Bull. Vet. Inst. Pulawy, 52: 281–284.

SOGSTAD A.M., FJELDAAS T., OSTERAS O. 2005. *Lameness and claw lesions of the Norwegian red dairy cattle housed in free stalls in relation to environment, parity and stage of lactation.* Acta Vet. Scan., 46(4): 203–217.

SOMERS J.G.C.J., FRANKENA K., NOORDHUIZEN-STASSEN E.N., METZ J.H.M. 2003. *Prevalence of claw disorders in Dutch dairy cows exposed to several floor systems.* J. Dairy Sci., 86: 2082–2093.

SWALVE H.H., PIJL R., BETHGE M., ROSNER F., WENSCH DORENDORF M. 2005. *Analysis of genetic and environmental effects on claw disorders diagnosed at hoof trimming. 56th annual meeting of the European Association for Animal Production 05–08.06.* Uppsala, Sweden, Book of abstracts, p. 323.

TELEZHENKO E., BERGSTEN CH. 2004. *Influence of floor type on the locomotion of dairy cows.* A. Anim. Beh. Sci., 93(3): 183–197.

TOMLINSON D.J., MÜLLING C.H., FAKLER T.M. 2004. *Invited review: formation of keratins in the bovine claw: roles of hormones, minerals, and vitamins in functional claw integrity*. J. Dairy Sci., 87: 797–809.

VAN HERTEM T., MALTZ E., ANTLER A., ROMANINI C.E.B., VIAZZI S., BAHR C., SCHLAGETER-TELLO A., LOKHORST C., BERCKMANS D., HALACHMI I. 2013. *Lameness detection based on multivariate continuous sensing of milk yield, rumination, and neck activity*. J. Dairy Sci., 96: 4286–4298.

VAN NUFFEL A., ZWERTVAEGHER I., PLUYM L., VAN WEYENBERG S., THORUP V.M., PASTELL M., SONCK B., SAEYS W. 2015. *Lameness detection in dairy cows:* Part 1. *How to distinguish between non-lame and lame cows based on differences in locomotion or behavior*. Animal, 5: 838–860.

VERMUNT J.J., HILL F.I. 2004. *Papillomatous digital dermatitis in a Holstein-Friesian bull.* New. Zeal. Vet. J., 52(2): 99–101.

WALKER S.L., SMITH R.F., JONES D.N., ROUTLY J.E., DOBSON H. 2008. *Chronic stress, hormone profiles and estrus intensity in dairy cattle.* Hormon. Beh., 53: 493–501.

WEBB N.G., CLARK M. 1981. *Livestock foot – floor interactions measured by force and pressure plate.* Farm Building Progress, 66: 23–36.

WHAY H.R. 2002. *Locomotion scoring and lameness detection in dairy cattle.* In Practice, 24: 444–449.

WINKLER B., MARGERISON J.K. 2012. *Mechanical properties of the bovine claw horn during lactation*. J. Dairy Sci., 95: 1714–1728.

WINNICKI S., NAWROCKI L., WĘGLARZY K. 2006. *Maintenance systems and the primiparous purity and hygienic quality of milk*. Inż. Rol., 4(79): 341–346.

ZINPRO 2014. *Cattle lameness claw lesions: identify, prevent, control*. Zinpro Corporation, Eden Prairie, MN.

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POLYETHYLENE GLYCOL – MODIFIED NANOCARRIER ENCAPSULATION OF DIMINAZENE ACETURATE IMPROVED HAEMATOBIOCHEMICAL RECOVERY IN *TRYPANOSOMA BRUCEI BRUCEI* INFECTED RATS

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Abstract

The enhanced safe drug delivery properties of polyethylene glycol (PEG) – modified Diaminazene aceturate nanocomposite against *Trypanosoma brucei* infected rats were evaluated. The drug conjugation was carried out by adding gold nanoparticle to PEG and diminazene aceturate. A group of infected rats were treated with free diaminazene aceturate, while other groups were treated with conjugated drug at different drug release time interval. Results revealed complete parasite clearance on day 4 and 6 post infection for rats treated with free drug and nano-formulated drug at 3–15 minutes release time. Rats treated with three minutes drug release shows satisfactory serum and liver enzyme activities as well as high RBC counts when compared ($p < 0.05$) with infected untreated and infected treated with free drug. The hematatological indices of non treated rats were lower than those treated with free and nano-formulated drug. Nanoencapsulation of diminazene aceturate therefore improved drug delivery and reduced toxicity in the treatment of African *trypamosomiasis.*

Introduction

Most drugs are limited by their pharmacodynamics properties, cytotoxicity and aggregation due to poor solubility, nonspecific delivery and short circulating half-lives (LIANG et al. 2008, PARVEEN et al. 2012). Nanoparticles emerge as the future of drug delivery technology as they might be

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future crucial diagnostic and therapeutic tools (BHARALI et al. 2009). Additionally, one of the major benefits of nanotechnology is the targeted drug delivery at the site of the disease by passive targeting of drugs to the site of action or by selective active targeting of the active pharmaceutic agent (ADEYEMI et al. 2014). Drug delivery nanomediated systems are based on biocompatible nanocarriers, such as gold nanoparticles, carbon nanotubes, nanovesicles, micellar systems and dendrimers (TING et al. 2009). Of these, gold nanoparticles with their unique chemical and physical properties have emerged as promising carrier for delivery of various molecules with therapeutic properties (GILJOHANN et al. 2010, SHITTU et al. 2018).

Trypanosomiasis, a disease of major importance in human and animals has continued to threaten human health and economic development (MANN et al. 2011). Natural products and chemotherapy are the most widely used means of controlling trypanosomiasis (BASHIR et al. 2015a). The few registered trypanocides are often associated with severe side effects and require lengthy parenteral administration and lack efficacy (LEGROS et et al. 2002). Therefore, novel drug-delivery systems which will offer better efficacy, safety as well as enhance the pharmacokinetics of easily degradable compounds and havea short half-life in vivo are urgently needed (MOCAN et et al. 2010).

Biodegradable polymeric nanoparticles, particularly those coated with hydrophilic polymer such as poly ethylene glycol have been used as carriers of DNA in gene therapy and as a potential drug delivery devices because of their ability to target an organ and circulate for a prolonged period of time (LEE et al. 2005). In this study, the use of *Hyptis suaveolens* aqueous leaf extract in the biosynthesis of gold nanoparticles and its capability as drug carrier using polyethylene glycol as a coat in the formulation of nano drug was investigated in the treatment of *Trypanosoma brucei*.

Materials and Methods

Plant sample. Fresh leaves of *Hyptis suaveolens* was obtained from Minna, Niger State Nigeria. The collected fresh leaves of *Hyptis suaveolens* were destalked, washed with clean-water, dried at room temperature and finally grounded using a grinder mill.

Experimental animals. Healthy albino rats of average weight 120–150 g were obtained from Small Animal Holding Unit, of Federal University of Technology, Minna, Niger State Nigeria. The study was carried out per the Guide for the Care and the Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council, USA (CCAC, 1997).

Reagent and assay kits. The gold chloride ($HAuCl_4$) was a product of Sigma Aldrich. The assay kits for AST, ALT and ALP were products of Randox Laboratories Ltd, United Kingdom. Diminazene aceturate (Berenil®) was a product of NGL Fine-Chem Ltd, India.

Parasite strain. The parasite *Trypanosoma brucei brucei* was obtained from the Nigeria Institute for Trypanosomiasis Research (NITR) Vom Jos, Plateau State.

Synthesis of Gold Nanoparticles. Gold Nanoparticles was synthesis as described previously (SHITTU and STEPHEN 2016a, JAE et al. 2010). Briefly, 100 ml of distilled water were added to 5 g of milled plant in an Erlenmeyer flask and then boiled for 5mins after which it was filtered, and then 0.5 ml of the plant extract was added to 9.5 ml of 1 mM aqueous $HAuCl_4$ solution for the reduction of Au^{3+} ions.

Characterization of biosynthesized Gold Nanoparticle. The UV-spectroscopy measurements of the $HAuCl_4$ – Plant extract solution were carried out using UV-1800 Shimadzu spectrophotometer with various peaks present over a range of 300–800 nm. The hydrodynamic diameter of the nanoparticles in solution was determined by dynamic light scattering (DLS) with the help of a Zetasizer 3000 (Malvern Instruments, UK) using an argon ion laser beam at a wavelength of 550 nm and a scattering angle of 90º. The levels of functional groups were evaluated using Fourier transform infrared (FTIR) spectroscopy. The biosynthesized gold nanoparticles were freeze-dried into pellets and washed with deionized water to get rid of the free proteins/enzymes that were not capped on the gold nanoparticles. Thereafter, the samples were dried and ground with KBr pellets and analyzed on a thermo Nicolet model 6700 spectrum instrument. The images were studied using Scanning Electron Microscope (SEM), HITACHI (model: S-3400N) with secondary electron detectors at an operating voltage of 30 kV. Energy Dispersive X-ray Spectroscopy (EDAX) of the reduced gold nanoparticle was done on S-3400N, Hitachi instrument as described previously (SHITTU and STEPHEN 2016a, 2016b).

Drug conjugation and release. Drug conjugation was done by adding standard drug (Diminazene aceturate (Berenil®) to Polyethylene Glycol (molecular weight 3000 M) functionalized gold nanoparticles in aqueous phase and stirred for 30 minutes at room temperature. The diameter and height of the formulated tablet was taken and the tablet was dissolved in a beaker containing 3 ml of sterile deionized water at a release time of 3 min. this was done until the tablet finally dissolves. After the release, the absorbance of each release was determined spectrophotometrically. Each release was kept in a sterile bottle and stored in a refrigerator.

In vivo antitrypanosomal study

Inoculation of experimental animals

The blood of the heavily infected rat was collected from the tail and immediately diluted with physiological saline (0.9%) to serve as the inoculums (EKANEM and YUSUF 2008, SHITTU et al. 2013). Twelve (12) healthy rats were infected intraperitoneally with 0.2 ml of the inoculum containing about $1x10^3$ *T. brucei brucei* parasites per 0.2 ml of blood (EKANEM and YUSUF 2005). Infection was monitored after 72 hours for the appearance of parasites in the newly infected animals. This was done on daily basis for microscopic examination of blood sample taken from the tail of the infected animals and viewed under microscope at x 40 magnification (EKANEM and YUSUF 2005).

Experimental design

Thirty five (35) albino rats were group into 7 groups (*A*–*G*). Groups *A* to *E* were treated with 3 (IT3), 6 (IT6), 9 (IT9), 15 (IT15) and 21 (IT21) minutes released formulated drug, while Groups Fand *G* were treated with free standard drug and distilled water (untreated) respectively. Parasite count was done on daily basis, by preparing a wet film that wasviewed under a microscope at x 40 magnification (YUSUF et al. 2012).

Collection of blood and isolation of liver

The procedure described by YUSUF et al. (2018) was adopted for the preparation of serum and tissue homogenates. Briefly, the animals were anaesthetized with diethyl ether and the blood was collected through cardiac puncture. Blood was collected into EDTA bottlesand EDTA free bottles. The rats were dissected to reveal the internal organs and the liver was removed and placed in sample bottle containing sucrose solution (0.25 M) to maintain a normal physiological environment. The liver was homogenized and the supernatant of the homogenized liver and serum were stored at -4^{o}C for further studies.

Haematological studies

Automated Haematologic Analyzer (Sysmex Haematology Systems, Sysmex America Inc., model no. KX-21N, Kobe, Japan) was used to determine the levels of haemoglobin (Hb), packed cell volume (PCV), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular haemo-

globin (MCH), mean corpuscular haemoglobin concentration (MCHC), white blood cells (WBC), neutrophils, lymphocytes and platelets as described by DACIE and LEWIS (1995).

Determination of biochemical parameters

The biochemical parameters were determined for alkaline phosphatase (ALP) based on methods of TIETZ (1995), Aspartate transaminase (AST) and alanine transaminase (ALT) as described by REITMAN and FRANKEL (1957). The serum catalase activities was determined as described by BEERS and SIZER (1952).

Statistical analysis

Data analyses were performed using SPSS software (SPSS 10.0 for Windows, SPSS Inc, Chicago, IL). All data are expressed as mean ± SEM. Analysis of variance (ANOVA) was used to test for differences between the groups. Duncan's multiple range tests was used to determine the significance of differences among the mean values at the level of $p < 0.05$ (MAHAJAN 1997).

Results

Characteristic of the Biosynthesis of Gold Nanoparticles from *Hyptissuaveolens* leaf extract

The synthesis of gold nanoparticles was confirmed by color changes from yellow to rubyred. Tyndall effect of the synthesized gold nanoparticle (Figure 1) shows that the light rays are only scattered by the solution of the synthesized gold nanoparticle when a direct light from laser pointer was pass through a solution of gold chloride and biosynthesized gold nanoparticle.

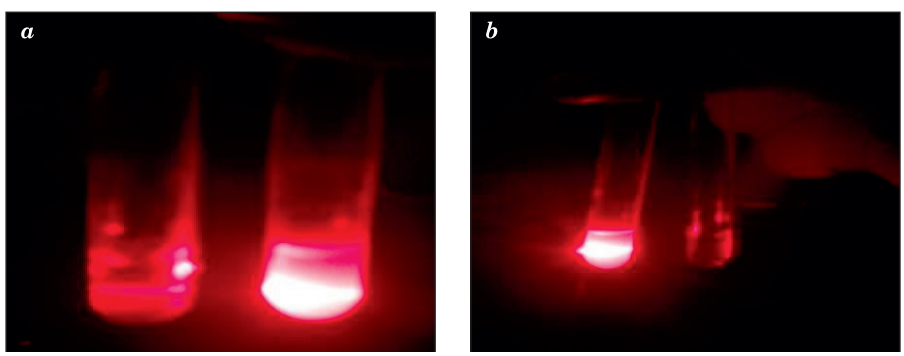

Fig. 1. Tyndall effect of gold nanoparticle and gold chloride solution

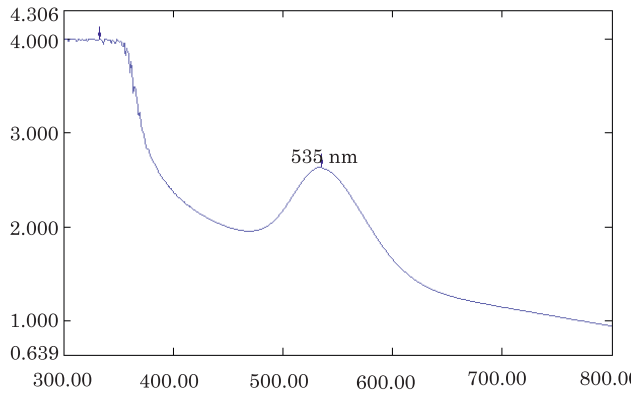

Fig. 2. Absorption spectra of gold chloride before and after reduction from *Hyptis suaveolens*

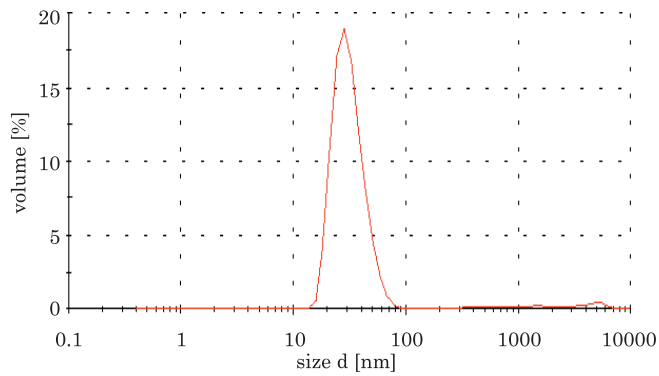

Fig. 3. Particle size of gold nanoparticle from aqueous leaf extract of *Hyptis suaveolens*

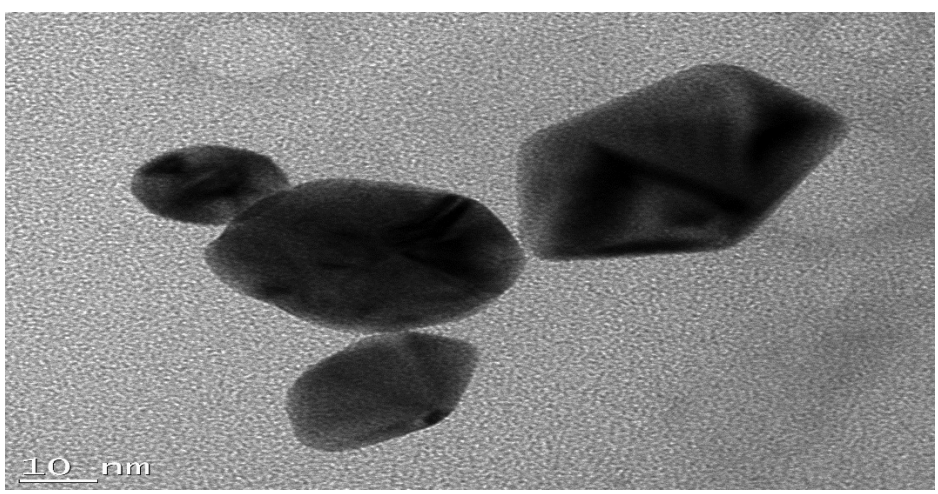

Fig. 4. TEM micrograph result of synthesized gold nanoparticle

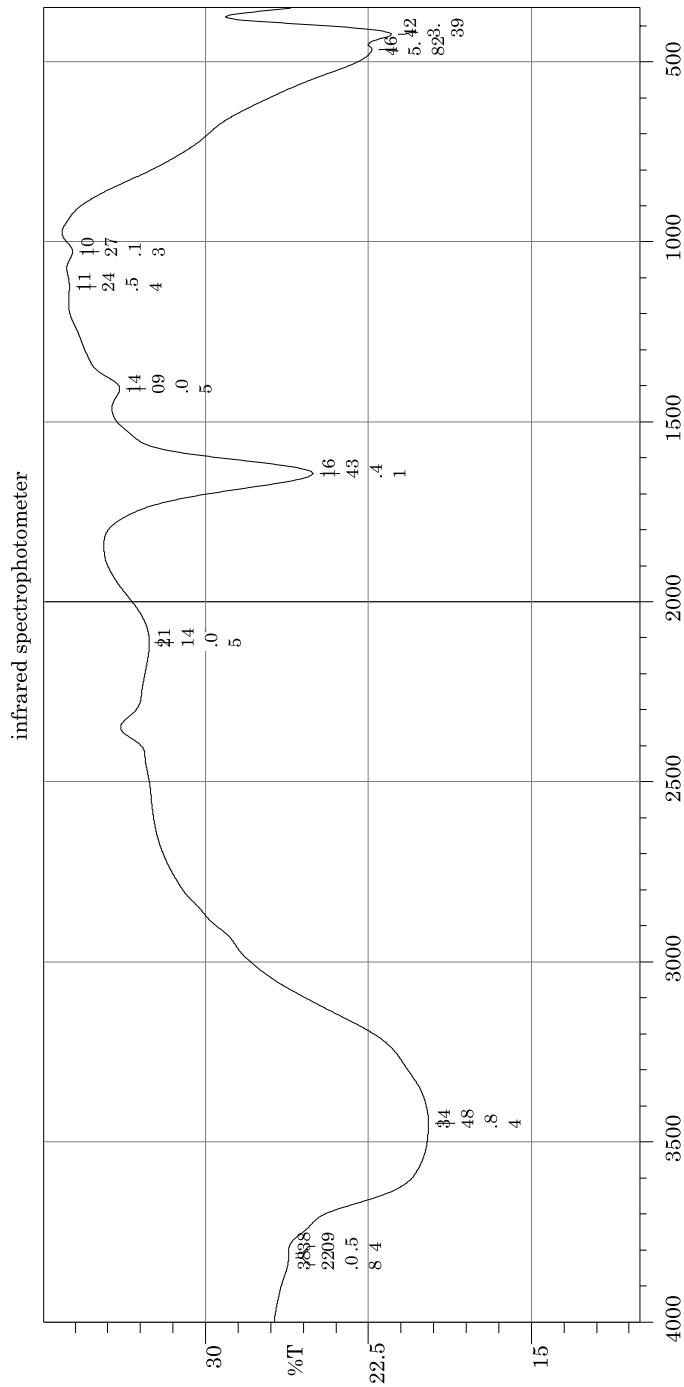

Fig. 5. FTIR spectra of gold nanoparticle synthesized from leaf extract of *Hyptis suaveolens*

UV-Visible spectrophotometric analysis of the gold nanoparticles shows a surface resonance with visible peak of 535 nm with an absorbance of 2.641 (Figure 2). Figure 3 shows the average particle size of biosynthesized gold nanoparticles to peak size 50 nm with intensity of 18%. The TEM micrograph result of synthesized gold nanoparticle is shown in Figure 4. The fourier transmission infrared FTIR spectroscopy of gold nanoparticles synthesized under optimized condition showed strong band at 34 488.84 $cm^{-1}$and a weak band at 1643.41 cm^{-1} (Figure 5). The energyDispersive Xray Spectroscopy of the gold nanoparticle are shown in Figure 6, while the drug release capability of nano formulated drug (diminazene aceturate) [mg ml^{-1}] over time (minutes) are shown in Figure 7.

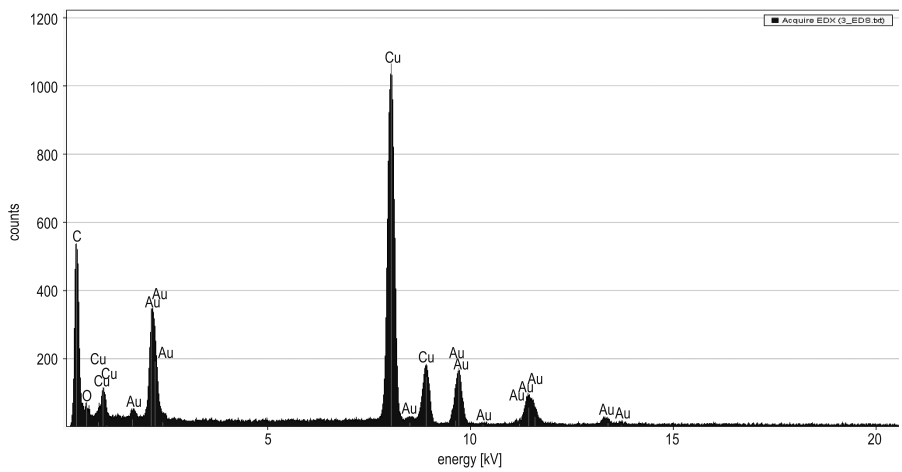

Fig. 6. The result of EDS of gold nanoparticle from aqueous leaf extract of *Hyptis suaveolens*

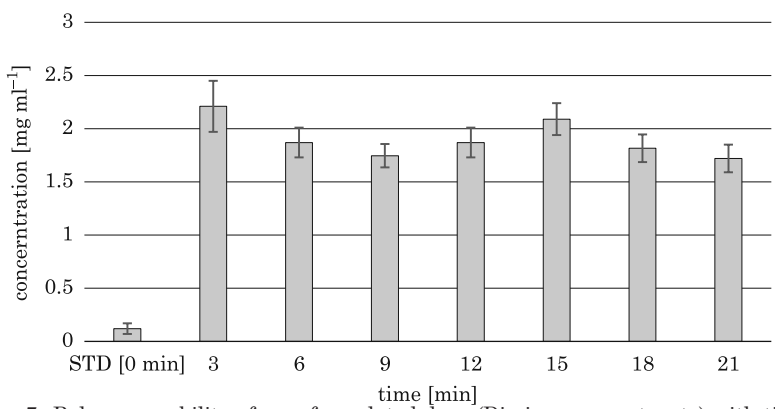

Fig. 7. Release capability of nanoformulated drug (Diminazene aceturate) with time

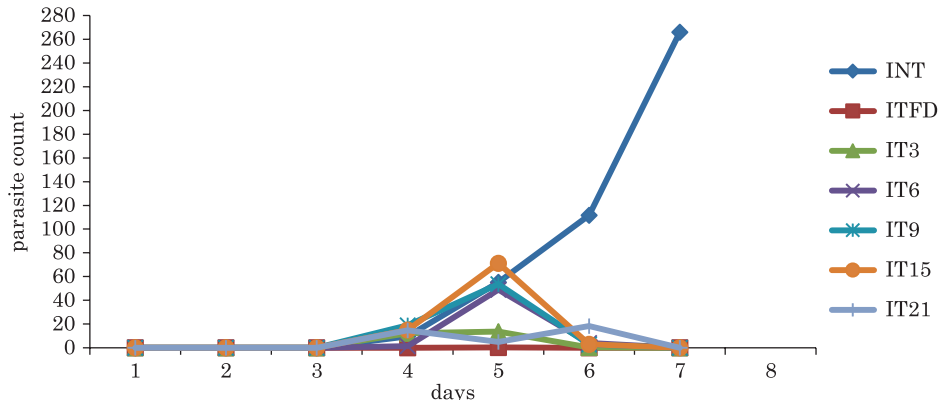

Fig. 8. Effect of Nano formulated drug on parasite count in *T. brucei* infected rat
INT: infected not treated, ITFD: infected treated with free drug, IT3: infected treated with three minutes of formulated drug release: IT6 – infected treated with six minutes of formulated drug release; IT9 – infected treated with nine minutes of formulated drug release; IT15 – infected treated with fifteen minutes of formulated drug release; IT21 – infected treated with twenty one minutes of formulated drug release

Antitrypanosoma activities

The effect of nano formulated drug on parasite count in *T. brucei* infected rat are presented in Figure 8. The parasitaemia of infected untreated group increased infinitely while infected rat treated with free drug and those treated with poly ethylene glycol modified drug-nanocomposite at at 3–15 minutes release time produce a complete parasite clearance on day 4 and 6 post infection respectively. However, the 21 minutes release polyethylene glycol modified diminazene aceturate nanocomposite exhibited complete parasite clearance on day 7 post infections.

Biochemical parameters

The serum ALT activities shows a significant increases in the infected un-treated, infected treated with free drug andinfected treated with IT6 (nanoformulated drug) when compared with the rats treated with IT3 and other nanoformulated groups (Table 1). Similarly, the liver ALT activities in rats treated with free drug and IT6 (nanoformulated drug) were significantly raised when compared with other experimental groups.The serum AST activities showed significant increases in the infected un-treated, infected treated with free drug and infected treated with IT6–IT15 (nanoformulated drug) when compared with the rats treated with IT3 and IT21 (Table 1). The liver AST activities in rats treated with IT21 were significantly lower than

Table 1

Serum and liver biomarker enzymes in *T. brucei* infected rat treated with nanoformulated drug (Diminazene aceturate)

Parameters	Aspartate transaminase [UL^{-1}]		Alanine transminase [UL^{-1}]		Alkaline phosphatase [UL^{-1}]		Catalase [UL^{-1}]	
–	serum	liver	serum	liver	serum	liver	serum	liver
INT	237 ± 0.00^{c}	187 ± 0.00^{b}	159 ± 0.00^{b}	79.20 ± 0.00^{a}	717 ± 0.00^{b}	220 ± 0.00^{a}	7.56 ± 0.00^{a}	6.34 ± 0.00^{a}
ITFD	210 ± 1.33^{b}	193 ± 6.66^{b}	137 ± 2.00^{b}	147 ± 2.00^{b}	450 ± 36.8^{a}	808 ± 22.0^{c}	7.28 ± 0.26^{a}	6.88 ± 0.33^{a}
IT3	137 ± 5.77^{a}	213 ± 29.6^{b}	65.20 ± 5.03^{a}	108 ± 17.1^{a}	305 ± 14.8^{a}	119 ± 51.2^{a}	7.60 ± 0.33^{a}	6.16 ± 0.80^{a}
IT6	227 ± 0.00^{c}	213 ± 6.66^{b}	122 ± 1.93^{b}	125 ± 8.00^{ab}	616 ± 33.1^{b}	645 ± 14.3^{b}	7.86 ± 0.55^{a}	7.66 ± 0.50^{a}
IT9	193 ± 27.2^{b}	207 ± 7.09^{b}	64.53 ± 1.33^{a}	87.20 ± 3.05^{a}	662 ± 57.4^{b}	423 ± 13.9^{ab}	8.28 ± 0.43^{a}	6.82 ± 0.74^{a}
IT15	207 ± 10.5^{b}	107 ± 52.9^{a}	63.20 ± 3.05^{a}	99.86 ± 13.2^{a}	874 ± 64.4^{b}	184 ± 75.3^{a}	7.40 ± 0.15^{a}	7.33 ± 0.52^{a}
IT21	167 ± 5.77^{ab}	157 ± 40.0^{a}	60.53 ± 2.90^{a}	89.20 ± 11.3^{a}	616 ± 15.31^{b}	289 ± 15.1^{a}	8.69 ± 0.99^{a}	6.20 ± 0.09^{a}

Values are mean ± SEM ($n = 3$)

INT – infected not treated; ITFD – infected treated with free (standard) drug; IT3 – infected treated with three minutes of formulated drug release; IT6 – infected treated with six minutes of formulated drug release; IT9 – infected treated with nine minutes of formulated drug release; IT15 – infected treated with fifteen minutes of formulated drug release; IT21 – infected treated with six minutes of formulated drug release

Table 2

Hematological parameters of *Trypanosoma brucei* infected rats treated with nanoformulated Diminazene aceturate

Parameters	Hb [g dL^{-1}]	PCV [%]	MCV [fl]	MCH [pg]	MCHC [g dl^{-1}]	RBC [$\cdot 10^9$ l^{-1}]	PLC [$\cdot 10^9$ l^{-1}]	WBC [$\cdot 10^9$ l^{-1}]
INT	6.30±1.52^a	19.00±4.58^a	8.40±2.39^a	2.80±0.80^a	33.00±0.00^a	3.33±1.76^a	355±7.81^a	12.00±2.51^b
ITFD	10.20±0.23^b	44.66±0.88^b	52.66±0.67^b	17.00±0.57^b	32.00±0.00^a	3.03±0.23^a	385±1.45^a	11.13±1.93^b
IT3	10.93±1.16^b	34.66±2.33^b	57.33±1.45^b	17.00±0.57^b	30.33±1.20^a	6.30±0.56^b	365±1.41^a	12.53±1.68^b
IT6	11.46±0.46^b	34.00±2.30^b	55.66±0.88^b	18.33±0.88^b	33.33±1.33^a	5.80±0.05^b	365±2.23^a	7.26±0.59^a
IT9	11.60±0.40^b	38.33±1.33^b	59.00±2.51^c	17.33±0.66^b	30.00±0.00^a	6.93±0.49^b	417±4.94^a	10.63±4.29^a
IT21	13.00±0.37^b	42.33±0.88^b	57.00±0.57^b	18.00±0.00^b	31.00±0.00^a	7.46±0.12^b	443±1.51^a	19.16±2.47^c

Hb – haemoglobin; PCV – packed cell volume; MCV – mean cell volume; MCH – mean cell haemoglobin; MCHC – mean cell haemoglobin concentration; RED – red blood cell; PLC – platelet count; WBC – white blood cell values are mean ± SEM (n = 3). Values along the same rows with different superscript alphabet are significantly different ($p \cdot 0.05$); INT– infected not treated; ITFD – infected treated with free (standard) drug; IT3 – infected treated with three minutes of formulated drug release; IT6 – infected treated with six minutes of formulated drug release; IT9 – infected treated with nine minutes of formulated drug release; IT15 – infected treated with fifteen minutes of formulated drug release; IT21 – infected treated with six minutes of formulated drug release

all other experimental groups. The serum ALP activities shows a significant increases in the infected un-treated, infected treated with IT6-IT21 (nanoformulated drug) when compared with the rats treated with IT3. The liver ALP activities in rats treated with free drug were significantly raised when compared with other experimental groups.

Hematological parameters

The haemoglobin concentration, PCV, MCV and MCH of infected not treated rats were significantly lowered ($p < 0.05$) when compared with rats treated with free drugs and formulated drug. The RBC was significantly lowered in the infected un-treated and infected treated with free drug when compared to the rats treated with nanoformulated drug. However, the WBC was significantly raised in the infected treated with IT21 nanoformulated drug (Table 2).

Discussion

Synthesis of nanoparticles from various materials has been reported, but among all, biosynthesis of nanoparticles from plants is considered the most suitable method. In this present research, *Hyptis suaveolens* aqueous leaf extract successfully reduced gold chloride to nano size. Previous studies have also reported a rapid synthesis of gold nanoparticles with neem (*Azadirachta indica*) leaves and sundried *C. campphor*a leaves, they attributed reduction and stabilitzation of gold nanoparticles to water soluble heterocyclic compound found in the plants (SHANKAR et al. 2004, HUANG et al. 2007). The tyndall effect of light scattering by synthesized gold nanoparticle solution indicated that the solution is colloidal. Gold ion is also present in reduced forms. This was confirmed by the UV-visible spectra. The nanoparticles showed an absorption band at 535 nm but gold chloride shows no absorption band. An absorption band of range 500–600 nm of gold nanoparticle has been reported (UMESH et al. 2011).

The biosynthesized gold nanoparticle was further characterized using nanosizer confirming the gold nanoparticle to have a peak size of 50 nm which is within the range of 1–100 nm. The TEM result from Figure 4, shows that the the biosynthesized gold nanoparticle is hexagonal in shape. FTIR analysis used in the characterization of the biosynthesized gold nanoparticle shows a strong band at 3448.84 cm^{-1} and a weak band at 1693.41 cm^{-1} (Figure 5).

During the in vivo studies, *T. brucei* parasite produced a severe acute infection in rats. The parasitaemia of untreated infected rats continued to

rise steadily and mortality of some rats were recorded 6th day post-infection. The parasitaemia count obtained from treatment with diminazene aceturate showed the drug produce satisfactory efficiency in clearing the parasite from circulation. This is in agreement with pervious work of EKANEM and YUSUF (2015). However, the unwanted deleterious effect of this drug is the major concern (SHITTU et al. 2018). The delay in parasite clearance of the nanoformulated drug could be attribute to controlled release of the active ingredient, a property confer by the nanoparticle

The biochemical indices monitored in the liver and serum areuseful 'markers' for assessing tissue damage (SHITTU et al. 2015). The liver which plays a vital role in the intermediary metabolism of biomolecules and drugs, couldalso be affected by the toxic side effects of these drugs and diseases (LAWAL et al. 2016). ALT and AST are markers of liver damage and can be used to assess liver cytolysis (YUSUF et al. 2018b). The increase in ALT and AST activities in the infected un-treated, infected treated with free drug, infected treated with IT6 (nanoformulated drug) may be related to liver inflammation and is an indication of abnormal function of the liver. The elevation of these enzyme levels recorded here agrees with earlier reports from natural and experimental infected animals (UMAR et al. 2007, SHITTU et al. 2017). The results suggest probable infiltration of vital body organs and inflammation particularly of liver, muscles, and kidneys by *T. b. brucei.* Elevated enzyme levels may also result from effect of trypanosome lyses resulting from the host's defense mechanisms (KENNEDY et al. 2004).

Alkaline phosphatases are often used to assess the integrity of plasma membrane and endoplasmic reticulum (LAWAL et al. 2016b). The alteration in serum and liver ALP activities in rat treated with free drug andthe infected un-treated groups suggested that the integrity and functionality of endoplasmic reticulum and plasma membrane have been comprised by the trypanosome infection as well as the administration of free drug (YUSUF et al. 2018a). Although some alteration in the activities of these biomarker enzymes in infected treated with IT6–IT21 (nanoformulated drug) were comparable to those observed with free drugs. Rats treated with IT3 nanoformulated drug shows satisfactory activities of biomarker enzymes which were an indication of preserved organ integrity.

Oxidative stress play important etiologic role in the pathogenesis of African trypanosomiasis (OGUNSANMI et al. 2007) Superoxide dismutases reduce the concentration of highly reactive superoxide radicals by converting it to H_2O_2 whereas catalase convert H_2O_2 into H_2O and O_2 and protect the tissue from a highly reactive hydroxyl radical (LAWAL et al. 2016). In a disease condition, catalase activity in serum and organ is reduced. In the

present study, there was no significant difference ($P \cdot 0.05$) in both the serum and tissue catalase activity, in all groups. Contrary to these findings, SHITTU et al. (2014), reported that there was a significant difference ($p \cdot 0.05$) in serum catalase activities in rat treated with diminazene aceturate compared with untreated rats.

Measurement of anaemia gives an indication of the severity of the disease (EKANEM and YUSUF, 2008). The increases in RBC observed for infected rats treated with nanoformulated drug in comparison with infected un-treated and infected treated with free drug suggest that nano-encapsulation of drug reduces the severity of *T. brucei* infection as well as toxicity of diminazene aceturate in rats. WBC and its differentials are known for their defensive role against foreign body and infectious agents through the production, transportation and distribution of antibodies in immune response (BERINYUY et al. 2015). The increased WBC counts in infected treated with IT_{21} nanoformulated drug are also indicative of the increased host action in the presence of nanoparticles against the infection as these will contribute to the development of phagocytes and antibodies against the recognizable antigens of parasite origin.

Conclusion

From these findings, *Hyptis* plant shows the ability to reduce gold chloride to gold nanoparticle which confirms the potential value of the plant in nano particle synthesis. The nano-encapsulation of diminazene aceturate reduced the toxicity on hematological and biochemical indices that are associated with the drug in the treatments of trypanosomiasis.

Ethical approval

The principles governing the use of laboratory animals as laid out by the Federal University of Technology, Minna Committee on Ethics for Medical and Scientific Research and also existing internationally accepted principles for laboratory animal use and care as contained in the Canadian Council on Animal Care Guidelines and Protocol Review were duly observed.

References

ADEYEMI O.S., FANIYAN T.O., ADEYEMI O.S. 2014. *Antioxidant status of rats administered silver nanoparticles orally.* J. Taibah Univ. Med. Sci., 9: 182–186.

BASHIR L., SHITTU O.K., SANI S., BUSARI M.B., ADENNIYI K.A. 2015. *African natural products with potential antitrypanosomaproperties: A review*. Int. J. Biochem. Res. Rev., 7(2): 45–79.

BEERS R.F. JR., SIZER I.W. 1952. *Aspectrophotometrical method for measuring the breakdown of hydrogen peroxide by catalase*. J. Biol. Chem., 195(1): 133–195.

BERINYUY E.B., LAWAL B., OLALEKAN A.A., OLALEKAN A.A., YUSUF A.A., SAKPE S., OSSAI P.C. 2015. *Hematologicalstatus and organs/body-weight parameters in Wister rats during chronic administration of Cassia occidentalis*. Inter. Blood Res. Rev., 4(3): 1–7.

BHARALI D.J., KHALIL M., GURBUZ M., SIMONE T.M., MOUSA S.A. 2009. *Nanoparticles and cancer therapy: A concise review with emphasis on dendrimers*. Inter. J. Nanomed., 4: 1–7.

CCAC guidelines on: animal use protocol review. Ottawa, Canadian Council on Animal Care. 1997, http://www.ccac.ca/Documents/ Standards/Guidelines/Protocol_Review.pdf, Access: 1.12.2016.

DACIE J.V., LEWIS S.M. 1991. *Practical hematology*, 7th edition. Churchill Livingston, Edinburgh, pp. 1–22.

EKANEM J.T., YUSUF O.K. 2015. *Activities of alkaline phosphatase, glutamate oxaloacetate transaminase and glutamate pyruvate transaminase in liver and serum of Trypanosoma brucei – infected rats treated with honey*. Biokemistri, 17: 185–191.

EKANEM J.T., YUSUF O.K. 2008. *Some biochemical and haematological effects of black seed (Nigella sativa) oil on T. brucei – infected rats*. Afr. J. Biomed. Res., 4(11): 79–85.

GILJOHANN D.A., SEFEROS D.S., DANIEL W.L., MASSICH M.D., PATEL P.C., MIRKIN C.A. 2010. *Gold nanoparticles for biology and medicine*. Angewandte Chemie International Edition, 49: 3280–3294.

HUANG J., Li Q., SUN D., LU Y., Su Y., YANG X., WANG H. 2007. *Biosynthesis of silver and gold nanoparticles by novel sundried Cinnamomum camphora leaf*. Nanotechnology, 18(10): 10 5104–10 5115.

JAE Y.S., BEOM S.K. 2010. *Biological synthesis of gold nanoparticles using Magnolia kobus and Diopyros kaki leaf extracts*. Process Biochem., 44: 1133–1138.

KENNEDY G.E.P. 2004. *Human African trypanosomiasis of the CNS: current issues and challenges*. J. Clin. Invest., 113: 496–504.

LAWAL B., SHITTU O.K., INJE O.F., BERINYUY E.B., MUHAMMED H. 2016. *African natural products with potential antioxidants and hepatoprotectives properities. A review*. Clin. Phytosci., 2(23): 1–66.

LAWAL B., SHITTU O.K., INJE O.F., MOHAMMED H., UMAR S.I., HARUNA G.M. 2016. *Antimicrobial evaluation, acute and sub-acute toxicity studies of Allium sativum*. J. Acute Dis., 5(4): 296–301.

LAWAL B., SHITTU O.K., BLESSING UCHENNA A.B., HARUNA G.M., ABUBAKARA. N., BERINYUY E.B. 2015. *Alteration in biochemical indices following chronic administration of methanolic extract of Nigeria bee propolis in wister rats*. Asian Pac. J. Trop. Dis., 5(8): 654–657.

LEE M., KIM S.W. 2005. *Polyethylene glycol-conjugated copolymers for plasmid DNA delivery*. Pharma. Res., 22: 1–10.

LEGROS D., OLLIVIER G., GASTELLU-ETCHEGORRY M., PAQUET C., BURRI C., JANNIN J., BUSCHER P. 2002. *Treatment of human African trypanosomiasis – present situation and needs for research and development*. Lancet, 2: 437–440.

LIANG X.J., CHEN C., ZHAO Y., JIA L., WANG P.C. 2008. *Biopharmaceutics and therapeutic potential of engineered nanomaterials*. Curr. Drug Metabol., 9: 697–709.

MAHAJAN B.K. 1997. *Significance of difference in means*. In: *Methods in biostatistics for medical and research workers*. 6th edition, JAYEET Brothers Medical Publishers, New Delhi, pp.130–155.

MANN M., OLUWASEYI R.I., ABDULFATAI T.A., CHIDIEBERE U., EKPENYONG E.U., ISAAC I.O., MOHAMMED S.S., DAUDA R.I., YUSUF A.Y., KABIR A.Y., OGBADOYI E.O. 2011. *In vivo antitrypanosomal effects of some ethnomedicinal plants from Nupeland of North Central Nigeria*. J. Tradit. Complement. Altern. Med., 8(1): 15–21.

MOCAN T., CLICHICI S., AGOŞTON-COLDEA L., MOCAN L., ŞIMON Ş., ILIE I.R. 2010. *Implications of oxidative stress mechanisms in toxicity of nanoparticles (review)*. Acta Physiol., 97: 247–255.

OGUNSANMI A.O., TAIWO V.O. 2007. *Pathobiochemical mechanism involved in the control of the disease caused by Trypanosoma congolense* in *African grey duiker (Sylvicapra grimmia)*. Vet. Parasitol., 96: 51–63.

Parveen S., Misra R., Sahoo S.K. 2012. *Nanoparticles: a boon to drug delivery, therapeutics, diagnostics and imaging. Nanomedicine*. Nanotech. Biol. Med., 8: 147–166.

Reitman S., Frankel S. 1957. *A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases*. Am. J. Clin. Pathol., 28: 56–63.

Shankar S.S., Rai A., Ahmad A., Sastry M. 2004. *Rapid synthesis of Au, Ag, and bimetallic Au core Ag shell nanoparticles using neem (Azadirzchta indica) leaf broth*. J. Coll. Inter. Sci., 275: 496–502.

Shittu O.K., Lawal B., Oluyomi O.I. 2014. *Effects of methanol extract of Musca domestica larvae on antioxidants enzymes in T. brucei infected rats*. Nig. J. Biochem. Mol. Biol., 29(2): 1–10.

Shittu O.K., Musa F., Gbadamosi D.F. 2013.Tr*ypanocidal activity and heamatological changes in T. brucei infected rats treated with methanolic leaf extract of Thymus vulgaris*. Inter. J. Appl. Biol. Res., 5(1 and 2): 109–114.

Shittu O.K., Stephen D.I. 2016a. *Cytotoxicity property of biologically synthesized Gold Nanoparticles from aqueous leaf extract of Calotropis procera (Apple of Sodom) on MCF-7 Cell Line*. Brit. J. Med. Med. Res., 15(12): 1–8.

Shittu O.K., Stephen D.I. 2016b. *In vitro membranous activity of biosynthesized Gold Nanoparticle from aqueous leave Extract of Nelsonia canescens*. Europ. J. Med. Plan., 15(2): 1–8.

Shittu O.K., Aaron S.Y., Oladuntoye M.D. Lawal, B. 2018. *Diminazene aceturate modified nanocomposite for improved efficacy in acute trypanosome infection*. J. Acute Dis., 7(1): 36–42.

Shittu O.K., Lawal B., Adeniyi A.K., Kilani L.T., Saka R.B. 2017. *Effect of Methanol extract of Musca domestica larva on some enzymes and haematological parameters in Trypanosoma brucei brucei – infected rats*. Nig. J. Basic Appl. Sci., 25(2): 66–74.

Tietz N.W. 1995. *Clinical guide to laboratory tests*. 3rd ed. Philadelphia, PA, WB Saunders Company, pp. 286–288.

Ting G., Chang C.H., Wang H.E. 2009.*Cancer nanotargeted radiopharmaceuticals for tumor imaging and therapy*. Anticancer Res., 29: 4107–4118.

Umar I.A., Ogenyi E., Okodaso D., Kimeng E., Stancheva G.I., Omage J.J., Isah S., Ibrahim M.A. 2007. *Amelioration of anaemia and organ damage by combined intraperitoneal administration of vitamins A and C to Trypanosoma brucei brucei – infected rats*. Afr. J. Biotechnol., 6: 2083–2086.

Umesh K.P., Birendra K.B., Padmalochan N. 2011. *Green synthesis and characterization of gold nanoparticles using onion (Allium cepa) extract*. World J. Nanosci. Engineer., 1: 93–98.

Yusuf A.B., Umar I.A, Nok A.J. 2012. *Effects of methanol extract of Vernonia amygdalina leaf on survival and some biochemical parameters in acute Trypanosoma brucei brucei infection*. Afric. J. Biochem. Res., 6(12): 150–158.

Yusuf A.A., Lawal B., Abubakar A.N., Berinyuy E.B., Omonije Y.O., Umar S.I., Shebe M.N., Alhaji Y.M. 2018a. *In-vitro antioxidants, antimicrobial and toxicological evaluation of Nigerian Zingiber officinale*. Clin. Phytosci., 4(12): 1–8.

Yusuf A.A., Lawal B., Yusuf M.A., Omonije Y.O., Adejoke A.A., Raji F.H., Wenawo D.L. 2018b. *Free radical scavenging, antimicrobial activities and effect of sub-acute exposure to Nigerian Xylopia Aethiopica seed extract on liver and kidney functional indices of Albino Rat*. Iran J. Toxicol., 12(3): 51–58.