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EFFECT OF DIGESTATE, LIQUID AND SOLID MANURE APPLICATION ON CHEMICAL PROPERTIES OF SOIL*

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Key words: digestate, liquid cattle manure, solid cattle manure, nitrogen, phosphorus.

Abstract

The growing number of biogas plants results in increasing digestate volume used as fertilizer on arable land. This study compared the influence of digestate addition on soil chemical properties with traditional organic fertilizers such as liquid and solid cattle manure and with mineral fertilizer. The digestate supplied soil with a significant amount of NH_4-N, whose nitrification was slower comparing to soils treated with mineral fertilizer and liquid cattle manure. Digestate also slightly increased concentration of water-soluble phosphorus in soil and added high amounts of plant-available potassium and dissolved organic carbon. Therefore, the application of digestate should follow the same rules as traditional liquid fertilizers; however, its agronomic use should be based not only on N, but also on P and K content.

Introduction

Nitrogen (N) fertilizers consumption in the European Union is high and rises, with the levels per hectare of agricultural area increasing from 67.4 kg ha^{-1} in 2006 to 74.4 kg ha^{-1} in 2015. On the same time-scale, application rate of phosphorus (P) fertilizers decreased from 7.5 kg ha^{-1} in 2006 to 6.3 kg in 2015 (*Agri-environmental*... 2017). In Poland, N fertilizers consumption also increased and in 2017 was equal to 78.7 kg ha^{-1} and a similar trend was observed for P fertilizers, i.e. reduction to 9.1 kg ha^{-1} in 2015 followed by a rise to 10.2 kg ha^{-1} in 2017 (SO 2018). Poland is the

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fifth country in the EU-28 in terms of P fertilizers application rate and 14 in the case of N fertilizers consumption per 1 ha (*Agri-environmental*... 2017). Although N and P fertilizers enhance crop production, their losses from agricultural system contribute to environmental contamination (VELTHOF et al. 2009). The most important among these are ground and surface water contamination leading to worsening of drinking water quality and eutrophication. Application of N fertilizers also results in high N_2O emissions and therefore contributes to global climate change (IPCC 2013). Prices of N fertilizers are rather unstable, because ammonia, urea and ammonium nitrate are produced from natural gas, the price of which is linked to oil prices. The prices of P fertilizers produced from phosphate rock which is a non-renewable resource mined outside the EU are also high as a result of high production and transportation costs (*Agri-environmental*... 2017). Therefore, the increasing application of organic fertilizers may decrease the use of mineral fertilizers and benefit both economy and environment. The new promising organic fertilizer is a post-fermentation sludge (digestate), which is a by-product of an anaerobic digestion (AD) from biogas plants. The digestate is characterized by high ammonium-N (NH_4-N) content (RIGBY and SMITH 2013), the total amount of phosphorus and potassium at a level similar to that of the substrate used for biogas production (INSAM et al. 2015) and high organic matter content (TAMBONE et al. 2010). The AD of P organic compounds results in partial mineralization of organic-P, which is rapidly associated with particulate-bound solids. The water-soluble phosphorus fraction (P_w) is also reduced substantially after AD process (GÜNGÖR and KARTHIKEYAN 2008). The addition of digestate to soil has no serious ecotoxicological effect on plants and soil biota (PIVATO et al. 2016), but rather enhances microbial activity (ODLARE et al. 2008). Fertilization with digestate improves overall carbon balance in soil (IOCOLI et al. 2019) as a consequence of low CO_2 emissions and results in higher macroelements content in harvested plants (KOSZEL and LORENCOWICZ 2015).

Between 2009 and 2016 the number of biogas plants in Europe has grown from 6,227 to 17,662 installations (*EBA*... 2018). This growth resulted in increase of digestate volume used as fertilizer in European agriculture. However, the variability of digestate composition and unknown stability may generate problems during its storage and may cause unfavourable impacts on soil (ALBURQUERQUE et al. 2012, INSAM et al. 2015). Although digestate is perceived as a valuable fertilizer (SZYMAŃSKA et al. 2018), there is a growing concern about its potential negative environmental impact, especially in case of nitrogen, which may be leached or volatilized. Therefore N dynamics in soil after its fertilization with digestate has been subjected to numerous research, both incubation experiments (GRIGATTI et al. 2011, GÓMEZ-BRANDÓN et al. 2016, CAVALLI et al. 2017) and field studies (CAVALLI et al. 2016, SIGURNJAK et al. 2017). The N dynamics in soil fertilized with digestate is quite well recognized and depends mainly on digestate and soil properties, as well as on the rate and time of application (RIGBY and SMITH 2013, MARTIN et al. 2015, TAMBONE and ADANI 2017). High amount of ammonium-N added to soil triggers several processes in the soil, such as nitrification, immobilisation and emission. Rapid oxidation of NH_4-N added with digestate to soil is observed in first two weeks after soil fertilization, with nitrification conversion of total nitrogen (TN) added in a range of 41–84%, depending on the digestate (ALBURQUERQUE et al. 2012, DE LA FUENTE et al. 2013).

The addition of digestate do not influence the total phosphorus (TP) content in soil, however, increases the water-soluble phosphorus (P_w) content (HUPFAUF et al. 2016) and extractable P concentration (BACHMANN et al. 2011). The increase in extractable P is slower in soils amended with digestate than in soil fertilized with mineral fertilizer due to differences in the composition of organic and mineral amendments. Digestate as other organic fertilizers contains a variety of inorganic and organic P compounds, which are mineralized at different rates. Inorganic P fertilizers may contain P only in form of orthophosphates which are immediately available for soil chemical reactions and therefore increase the extractable P content in soil immediately after application (MÓRTOLA et al. 2019).

The aim of the study was the comparative analysis of chemical properties in soil treated with organic fertilizers, digestate and mineral fertilizer.

Materials and Methods

Soil and fertilizers characteristics

The incubation experiment was conducted on five treatments: unfertilized soil (S_1, S_2), soil fertilized with digestate (D), soil fertilized with liquid cattle manure (LCM), soil fertilized with solid cattle manure (SCM) and soil fertilized with commercial mineral fertilizer containing N and P (MF). The soil selected for an incubation experiment was a loamy sand consisted of 83% sand (2–0.05 mm), 16% silt (0.05–0.002 mm) and 1% clay (< 0.002 mm) and was classified as light soil according to agronomic categories (SOIL SCIENCE SOCIETY OF POLAND 2008). The soil was sampled from arable field at the organic farm near Karczmisko village located 19 km north of Białystok (53°17'N, 23°11' E, 147 m a.s.l.). The soil was collected from the plough horizon (0–20 cm) twice: in April 2016 (S_1) and in November 2017 (S_2). Soil collected in April 2016 was used for incubation experiment conducted on soil treated with D which was a part of first set of incubation experiment. Soil sampled in November 2017 was used for experiment conducted on soil amended with MF, LCM and SCM, which was performed in the second set. The soil was air-dried and sieved through 2 mm mesh prior to incubation and then re-moistened and pre-incubated for a week at 25°C. The main soil properties were as follows: pH in 1 M KCl 4.75±0.12, total organic carbon (TOC) 15.46±1.08 g kg^{-1}, total nitrogen (TN) 0.90±0.02 g kg^{-1}, total phosphorus (TP) 0.860±0.06 g kg^{-1}, total potassium (TK) 1.01±0.04 g kg^{-1}, exchangeable acidity (EA) 3.45±0.52 $cmol_{(+)}$ kg^{-1}, total exchangeable cations (TEB) 4.09±0.30 $cmol_{(+)}$ kg^{-1}, cation exchange capacity (CEC) 7.54±0.34 $cmol_{(+)}$ kg^{-1} and base saturation (BS) 45.63±5.15%. Digestate was obtained from commercial mesophilic biogas plant, where maize silage (80%), chicken droppings (10%) and potato pulp (10%) were co-digested. The liquid cattle manure and solid cattle manure were collected from a dairy farm. The liquid cattle manure containing a mixture of water, urine and soluble fecal components was sampled from the channel below the litter-bedded floor. The solid manure consisting of feces and straw was collected from the manure heap outside the building. Chemical properties of the organic material are shown in Table 1. A commercially available fertilizer contained 18% of the nitrogen in form of ammonium and 46% of phosphorus in form of monoamonium and diammonium phosphate (37% of P was soluble in water).

Table 1

Characteristics of organic fertilizers

Parameter	Digestate (D)	Liquid cattle manure (LCM)	Solid cattle manure (SCM)
pH	7.58±0.03*a*	7.99±0.04*b*	8.03±0.06*b*
Total solids (TS) [%]	5.87±0.05*a*	1.16±0.01*b*	16.73±0.21*c*
Volatile solids (VS) [%]	77.53±0.36*a*	71.78±1.08*b*	83.17±0.52*b*
Total Kjeldahl nitrogen (TKN) [g kg^{-1}]	71.02±2.28*a*	67.64±0.38*a*	24.87±2.71*b*
Total organic carbon (TOC) [g kg^{-1}]	395.17±41.92*a*	406.64±9.74*a*	396.46±18.94*a*
Total phosphorus (TP) [g kg^{-1}]	12.54±0.55*a*	10.21±0.38*b*	5.03±0.24*c*
Total potassium (TK) [g kg^{-1}]	58.21±1.01*a*	80.53±0.57*b*	21.28±1.34*c*
C/N ratio	6	6	16

Values are given on a dry weight basis. Values in the same row with the same letters are not statistically different according to Tukey's test ($p < 0.05$)

Incubation

After a week of pre-incubation, in every experimental microcosm, soil (equal to 100 g dry weight) was mixed with organic or mineral fertilizer in amount equal to 170 kg N ha^{-1}, which is the N application rate permissible per year in case of organic fertilizers (Act of 10 July 2007... Journal of Laws of 2018 item 1259). The exact amount of organic material and mineral fertilizer was calculated assuming the plough depth of 0.2 m. The bulk density of soil was 1.51 g cm^{-3}. The unfertilized soil (S_1, S_2) was treated as a control. Each treatment and control was run in triplicate for each day of sampling. The aerobic incubation was carried out in darkness, at the temperature of 25±1°C, and constant soil moisture at 60% of water-holding capacity (WHC). Soils were kept in 100 ml plastic vessels covered with Parafilm, the breathable material, which ensured the gas exchange and stable moisture at the same time (De La Fuente et al. 2010). The moisture was checked every 3–4 days by weighing samples and WHC was adjusted by adding distilled water drop by drop. For analyses of inorganic N (NO_3-N, NH_4-N), water-extractable phosphorus (P_w) and pH, soils were sampled at day 0, 2, 7, 14, 21, 28, 42 and 56. The analysis of dissolved organic carbon (DOC) was performed on samples taken at day 0, while the measurements of TN, TP, TK, TOC content as well as concentration of plant-available potassium (K_{dl}) were performed just after soil fertilization (day 0) and at the end of incubation (day 56).

Chemical analyses

Particle-size distribution was analyzed according to Bouyoucos method modyfied by Casagrande and Prószyński (*Gleby i utwory...* PN-R-04032:1998), bulk density was determined in undisturbed soil samples in a steel cylinder with a volume of 100 cm^3, pH in 1M KCl (soil to potassium chloride ratio of 1:2.5) was measured with HQ40D meter (Hach, USA). The EA was determined by Kappen method, exchangeable bases were extracted with 1 M ammonium acetate (Ostrowska et al. 1991), magnesium and calcium were measured by flame AAS (Avanta PM, GBC Scientific Equipment Pty Ltd, Australia), Na and K were determined using flame photometer (BWB Technology, UK). The results were used to calculate TEB, CEC and BS. Total solids (TS) and volatile solids (VS) in organic fertilizers were measured according to standard methods (*Standard methods...* 1999). The soil moisture was determined on 10 g of a sample by drying in 105±2°C until constant weight. Soil inorganic-N (exchangeable and soluble) was extracted with a solution of 1% K_2SO_4 (soil to solution ratio 1:10) for 24 h (*Analiza chemiczno-rolnicza...* PN-R-04028:1997).

The NO_3^--N and NH_4^+-N content in filtrates was determined by UV-1800 spectrophotometer (Shimadzu, Japan). The P_w was extracted with distilled water (soil to water ratio 1:10) by rotary-shaking for 1 h at 10 reciprocations per minute (SHARPLEY et al. 2006) and it was measured in filtrates with UV-1800 spectrophotometer (Shimadzu, Japan). The K_{dl} content, after extraction with calcium lactate solution, was analyzed using flame photometer (BWB Technology, USA). Organic carbon (TOC) in soil and organic amendments was determined in TOC-L analyzer with SSM-5000A Solid Sample Combustion Unit (Shimadzu, Japan). Total Kjeldahl nitrogen (TKN) was determined by the Kjeldahl method in Vapodest 50 s analyzer (Gerhardt, Germany). After nitric acid/hydrogen peroxide microwave digestion in ETHOS One (Milestone s.r.l., Italy) the content of P was determined with ammonium metavanadate method using UV-1800 spectrophotometer (Shimadzu, Japan) and the K content was measured using flame photometry (BWB Technology, USA). The analyses were run in triplicate.

Calculations and statistical analysis

The quantitative recovery of inorganic N from fertilizers at day 0 was calculated by subtracting inorganic N (as a sum of NO_3-N and NH_4-N content) in control soil from those measured in treated soils. The recovery was also expressed as percentage of TN added to the soil. The quantitative availability of inorganic N at the end of incubation was calculated in similar way i.e. the content of inorganic N in control soil at day 56 was subtracted from inorganic N content of treated soil at day 56. The availability was also expressed as percentage of TN added to the soils with fertilizers. The two-way Anova was used to test the statistical effects of fertilizer, time and their interactions on the following variables: NO_3-N, NH_4^+-N, P_w content and pH. The non-parametric one-way Anova (Welch test) was used on following variables: K_{dl}, TN, TOC, TP, TK, TOC and DOC at the level of accepted statistical significance of $p < 0.05$. The homogeneity of variance and normality were checked prior to ANOVA using Levene and Shapiro-Wilk tests, respectively. All the statistical analyses of data were performed using STATISTICA 12 software (StatSoft, Poland).

Results

Characteristics of organic fertilizers

All three amendments differed in almost all parameters, except the TOC content (Table 1). The highest TS and VS values characterized SCM (16.73±0.21 % and 83.17±0.52 %TS, respectively) and the lowest values were found in LCM (1.16±0.01 % and 71.78±1.08 %TS, respectively). The pH of both manures was similar and amounted to 7.99±0.03 for LCM and 8.03±0.06 for SCM (Table 1). Digestate (D) characterized with pH equal to 7.58±0.03. TKN content was the lowest in SCM (24.87±2.71 g N kg^{-1}), while both D and LCM were characterized with much higher amount of N. TP content in studied organic fertilizers followed the sequence: D > LCM > SCM, while TK content was the highest in LCM (80.53±0.57 g K kg^{-1}) and the lowest in SCM (21.28±1.34 g K kg^{-1}).

Nitrogen transformation in soil

The addition of organic and mineral fertilizers to the soil increased NH_4-N concentration at day 0 in the order: MF > LCM > D > SCM. The highest inorganic-N recovery at the first day of incubation was observed in soil fertilized with MF (100%) and LCM (82.5%). Much lower inorganic-N recovery was found for soils amended with D and SCM, equal to 59.5% and 42.3%, respectively (Table 2). The NH_4-N content was affected by fertilizer and sampling date and their interaction (Table 3). Its content decreased in all fertilized soils and both control soils throughout the course of incubation; however, the dynamics differed among the incubated soils. In soils amended with LCM and SCM, regardless the difference in initial NH_4-N concentration (51.79±2.88 and 26.72±0.08 mg N kg^{-1} for LCM and SCM, respectively), the NH_4-N content rapidly decreased in first 7 days of incubation and reached values ca. 2 mg N kg^{-1}. In soil treated with MF, the NH_4-N concentration dropped from 65.35±7.29 mg N kg^{-1} (day 0) to 14.59±0.57 mg N kg^{-1} at day 7 and to 0.63±0.04 mg N kg^{-1} at day 14. Slightly different NH_4-N dynamics was observed in soil fertilized with D, where NH_4-N content increased from 49.15±2.89 mg N kg^{-1} (day 0) to 77.03±3.81 mg N kg^{-1} at day 2 and then ammonium concentration decreased rapidly through next 12 days to 5.11±0.79 mg N kg^{-1}.

The NO_3-N content in all soils (except controls) ranged between 20.73±3.20 and 26.66±0.06 mg N kg^{-1} at day 0. In soil fertilized with SCM the NO_3-N content exceeded the NH_4-N concentration after first 12 hours and rose slowly from 20.73±3.20 mg N kg^{-1} (day 0) to 55.50±2.97 mg N kg^{-1} at the end of incubation. In soils receiving LCM and MF, changes in NO_3-N

Table 2

Summary of mineral N recoveries and availabilities

Specification	S_1	Soil + D	Soil + LCM	Soil + SCM	Soil + MF	S_2
Day 0						
pH in 1M KCl	4.57±0.02	4.78±0.01	5.97±0.03	5.91±0.04	5.78±0.02	5.73±0.01
NO_3-N [mg kg^{-1}]	22.01±0.36	22.85±1.60	18.42±1.94	20.73±3.20	26.66±0.07	21.05±1.00
NH_4-N [mg kg^{-1}]	15.38±0.88	49.15±2.89	51.79±2.88	26.72±0.08	65.35±7.29	2.40±0.54
Inorganic-N recovery [mg kg^{-1}]	–	33.73	46.75	23.99	68.56	–
Inorganic-N recovery [% of TN added]	–	59.5	82.5	42.3	100	–
Day 56						
pH in 1M KCl	4.46±0.02	4.42±0.02	5.72±0.01	5.80±0.10	5.35±0.04	5.57±0.06
NO_3-N [mg kg^{-1}]	44.99±3.45	90.62±1.93	65.25±10.53	55.50±2.97	98.69±3.27	38.22±1.34
NH_4-N [mg kg^{-1}]	0.41±0.07	0.99±0.17	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Inorganic-N availability [mg kg^{-1}]	–	41.22	27.03	17.28	60.47	–
Inorganic-N availability [% of TN added]	–	72.7	47.7	30.5	100	–

S_1 – a control for D treatment; soil + D – soil treated with digestate (D); soil + LCM – soil treated with liquid cattle manure (LCM); soil + SCM – soil treated with solid cattle manure (SCM); soil + MF – soil treated with mineral fertilizer (MF); S_2 – a control for LCM, SCM and MF treatments

Table 3

Two-way ANOVA for the chemical parameters measured throughout the incubation

Parameter	Treatment		Time		Treatment x time	
	F	*p*	F	*p*	F	*p*
NO_3-N	494.18	< 0.0001	343.86	< 0.0001	24.21	< 0.0001
NH_4-N	639.94	< 0.0001	1766.84	< 0.0001	196.32	< 0.0001
P_w	1347.25	< 0.0001	1136.46	< 0.0001	394.72	< 0.0001
pH	11591.00	< 0.0001	153.00	< 0.0001	14.00	< 0.0001

concentration were characterized with similar pattern. The NO_3-N content increased rapidly from 18.42±1.94 and 26.66±0.06 mg N kg^{-1} (for LCM and MF, respectively) at day 0 to 62.61±1.15 and 81.66±3.39 mg N kg^{-1} (for LCM and MF, respectively) at day 7 and exceeded the NH_4-N concentration at day 3. In next weeks of incubation the NO_3-N content increased very slowly to 65.25±10.53 and 98.69±3.27 NO_3-N mg N kg^{-1}, for LCM and MF, respectively. Although in soil treated with D, the dynamics of NO_3-N content had similar overall pattern, the nitrification rate was slower and the highest concentration of NO_3-N was observed after 21 days of incubation (Figure 1).

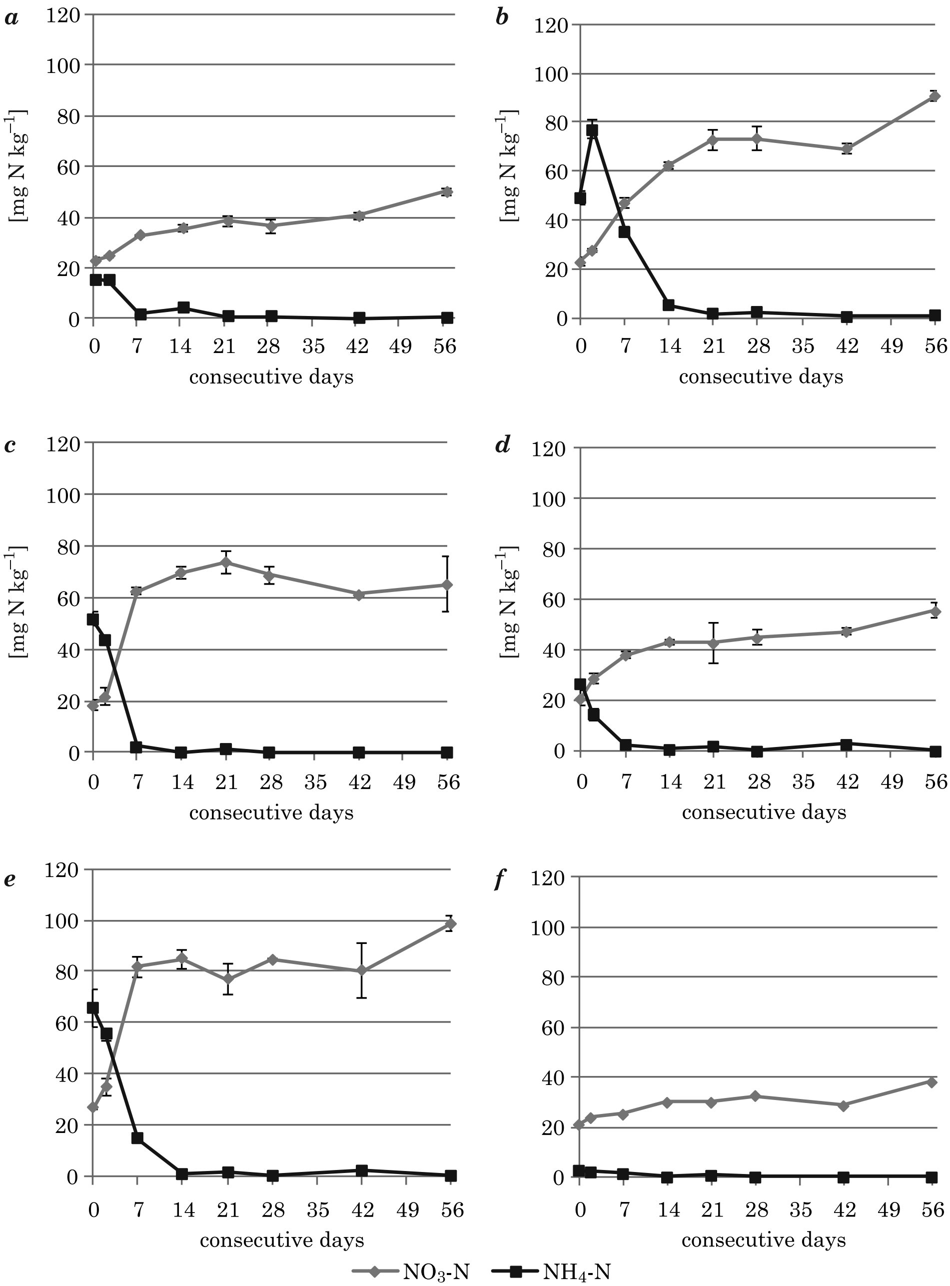

Fig. 1. Inorganic N (NO_3-N and NH_4-N) dynamics (mean value ± standard deviation; where absent, bars fall within symbols) in studied soils: *a* – control (S_1); *b* – soil + digestate (D); *c* – soil + liquid cattle slurry (LCM); *d* – soil + soild cattle manure (SCM); *e* – soil + mineral fertylizer (MF); *f* – control (S_2). Soil S_1 is a control for D treatment, soil S_2 is a control for LCM, SCM and MF treatments

Influence of fertilization on soil properties

The soil pH was affected by fertilizer and sampling date and their interaction (Table 3). At day 0 the addition of organic fertilizers increased the pH in order (pH units): LCM (+0.24) > D (+0.22) > SCM (+0.18). The rise of soil pH resulted from high pH of all organic fertilizers (Table 1). The addition of MF did not affect the soil pH. The highest decrease in pH in two first weeks of incubation was observed for both D and MF treatments, 0.33 and 0.34 pH units, respectively while LCM addition caused a drop of 0.18 pH units. The daily rate of pH decrease in first two weeks was 0.243 pH units day^{-1} for MF, 0.236 pH units day^{-1} for D and 0.0131 pH units day^{-1} for LCM. In the period from 14 to 56 day the pH values were stable (Figure 2). In the SCM treatment, the decrease rate was slower in first 14 days (0.0017 pH units day^{-1}) and was faster thereafter (0.0021 pH units day^{-1}). Throughout 56 days of incubation the pH decreased in following order (pH units): MF (0.42) > D (0.36) > LCM (0.25) > SCM (0.11).

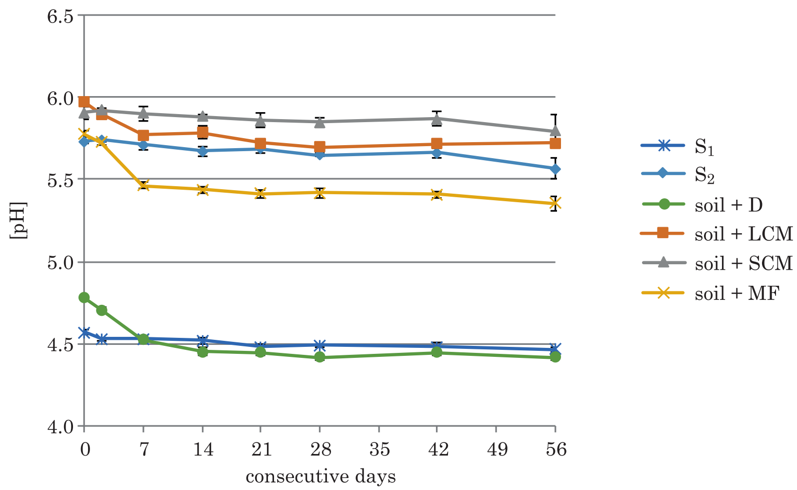

Fig. 2. Soil pH in 1 M KCl dynamics (mean value ± standard deviation; where absent, bars fall within symbols). Treatment codes: S_1 – a control for D treatment; soil + D – soil treated with digestate (D); soil + LCM – soil treated with liquid cattle manure (LCM); soil + SCM – soil treated with solid cattle manure (SCM); soil + MF – soil treated with mineral fertilizer (MF); S_2 – a control for LCM, SCM and MF treatments

Fertilization with organic materials did not influence TOC in soil, which varied within the range 13.27±0.46 – 15.06±0.96 g C kg^{-1} at day 0 and was at the same level at the end of incubation experiment. However, the addition of all organic fertilizers, except of SCM, increased the DOC concentration at day 0 comparing to control (Figure 3).

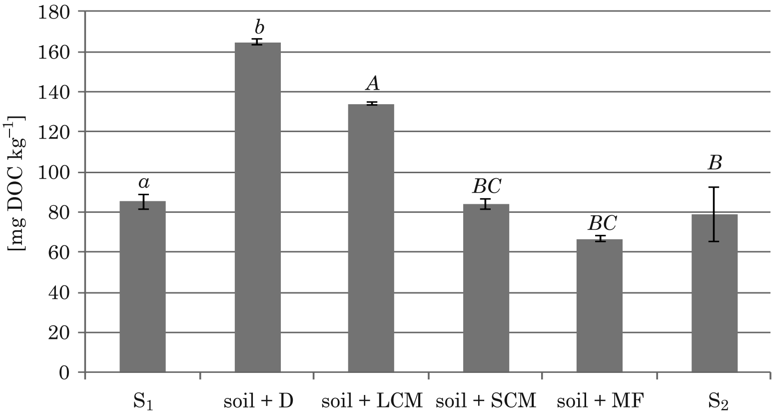

Fig. 3. Dissolved organic carbon (DOC) content (mean value ± standard deviation) at day 0. Treatment codes: S_1 – a control for D treatment; soil + D – soil treated with digestate (D); soil + LCM – soil treated with liquid cattle manure (LCM); soil + SCM – soil treated with solid cattle manure (SCM); soil + MF – soil treated with mineral fertilizer (MF); S_2 – a control for LCM, SCM and MF treatments Bars with the same letter are not significantly different according to the Tukey test (p < 0.05). Lower case letters indicate significant differences between S_1 and D treatment, upper case letters indicate significant differences among S_2 and LCM, SCM and MF treatments

The application of fertilizers slightly increased the water soluble phosphorus (P_w) content by ca. 4 mg P kg^{-1}, in case of D, LCM and SCM, while fertilization with MF supplied soil with high amount of P_w, increasing its content to 42.25±1.06 mg P kg^{-1}. In all soils fertilized with organic fertilizers the rapid decrease in P_w content was observed in the first week of incubation to the level similar to the P_w concentration in controls (Figure 4).

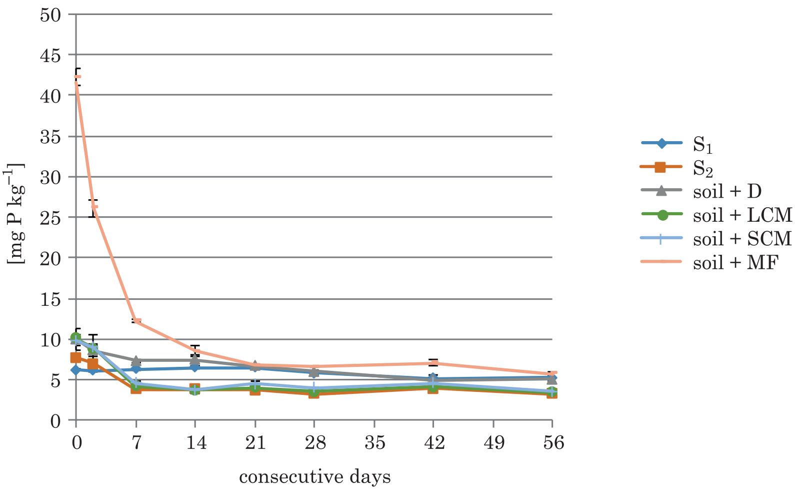

Fig. 4. Concentration of water-soluble phosphorus (Pw) in relation to time (mean value ± standard deviation; where absent, bars fall within symbols). Treatment codes: S_1 – a control for D treatment; soil + D – soil treated with digestate (D); soil + LCM – soil treated with liquid cattle manure (LCM); soil + SCM – soil treated with solid cattle manure (SCM); soil + MF – soil treated with mineral fertilizer (MF); S_2 – a control for LCM, SCM and MF treatments

Only in soil fertilized with MF, the P_w content was slightly higher than in control until day 56. The incorporation of organic and mineral fertilizers in soil increased the TP content comparing to S_1 and S_2, although this increase was not statistically significant. The TP content at day 0 was the highest in soil fertilized with MF (0.522±0.028 g P kg^{-1}) and the lowest in soil amended with D (0.410±0.048 g P kg^{-1}).

The incorporation of fertilizers to the soil had also impact on potassium forms, however only organic fertilizers supplied the soil with K_{dl} and increased significantly ($p < 0.05$) its content (Figure 5). The amounts of K_{dl}

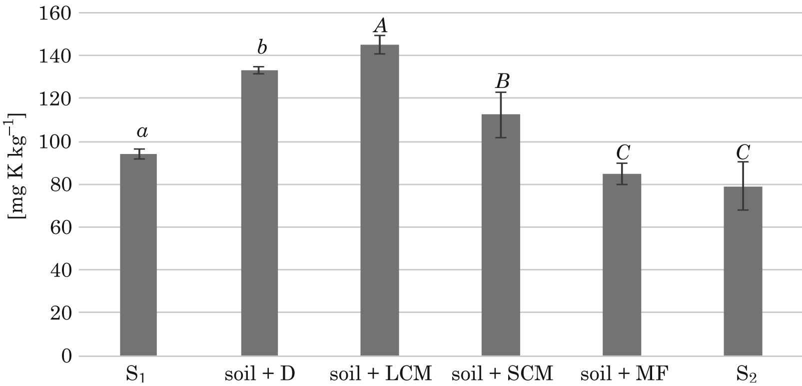

Fig. 5. Plant available K (Kdl) content (mean value ± standard deviation) at day 0. Treatment codes: S_1 – a control for D treatment; soil + D – soil treated with digestate (D); soil + LCM – soil treated with liquid cattle manure (LCM); soil + SCM – soil treated with solid cattle manure (SCM); soil + MF – soil treated with mineral fertilizer (MF); S_2 – a control for LCM, SCM and MF treatments Bars with the same letter are not significantly different according to the Tukey test ($p < 0.05$). Lower case letters indicate significant differences between S_1 and D treatment, upper case letters indicate significant differences among S_2 and CS, CM and MF treatments

at the end of incubation were similar to those from the day 0 and still they were significantly higher ($p < 0.05$) comparing to control, except for MF treatment. However, the fertilization did not increase the TK content in soil which was in the range of 1.35±0.03 to 1.69±0.06 g K kg^{-1} at the end of incubation. In both days the lowest TK content was observed for soil amended with SCM and the highest for soil fertilized with D.

Discussion

The nutrient composition differed in all 3 organic materials, with highest total Kjeldahl nitrogen (TKN) and total phosphorus (TP) content in digestate (D) and the lowest in solid cattle manure (SCM). The total solids (TS) decreased in order: SCM > D >LCM, while the highest volatile solids (VS) were found in SCM. Only total organic carbon (TOC) content was similar in all studied organic fertilizers. The chemical composition, TS and VS of D was typical for digestate produced in commercial biogas plants fed with maize silage alone or with some co-substrate constituting less than 10% of feedstock (WESTPHAL et al. 2016, NABEL et al. 2017, PROVENZANO et al. 2018). The SCM chemical properties were in a good agreement with typical ranges for this type of organic fertilizer (GRABOWSKI 2009), with rather low TS value resulting from very wet weather conditions preceding the SCM sampling from uncovered manure storage heap, which was very moist even in deeper parts. The chemical composition of LCM was characterized with much lower TS and content of nutrients than found in literature (GRABOWSKI 2009). This low level of nutrients may be a result of sampling LCM from the channel below the floor instead of storage tank. It must be emphasized that TS, VS and nutrient composition variability within every type of studied organic materials is very high (RISBERG et al. 2017) and depends on the fodder type and rate, on housing type, animal age and waste management in case of animal solid and liquid manure (HJORTH et al. 2010) while digestate characteristics depends on feedstock composition (TAMBONE et al. 2010) and quality of anaerobic digestion (AD) process.

The addition of easily-available N in the form of NH_4^+ and soluble organic C influenced the microbial activity in soil and triggered N transformation. In liquid cattle manure (LCM) nitrogen is in NH_4^+ form in 90% (GRZEŚKOWIAK 2013) and in used in this study MF nitrogen is only in NH_4^+ form therefore these two fertilizers supplied soils with the highest amounts of NH_4-N. This form of N is readily available for plants but it may be also fixed in the interlayer of clay minerals (CAVALLI et al. 2017). Lower amount of NH_4-N supplied with SCM and D resulted from higher content of N organic compounds, up to 60% in manure and 15-40% in digestate (RISBERG et al. 2017). However, high DOC content in D influenced microbial activity and in consequence the mineralization of easily-decomposable organic N compounds (ZHAO et al. 2007) resulting in the increase of NH_4-N content in soil in two days after fertilization. The important decrease of NH_4-N content occurred in all soils, except soil fertilized with D, in the first week of incubation. In soil fertilized with D, this drop in NH_4-N content from highest level to amount close to 0 took place in two weeks. In soil treated with MF the most rapid decrease of NH_4-N content was observed in first week of incubation and slower drop took place in following 7 days. This pattern of NH_4-N dynamics has been previously reported for incubation experiments (GRIGATTI et al. 2011, ALBURQUERQUE et al. 2012, DE LA FUENTE et al. 2013). The significant decrease of NH_4-N content in all fertilized soils with a concomitant rise of NO_3-N occurred through the nitrification of applied NH_4-N to the soil (GRIGATTI et al. 2011, ALBURQUERQUE et al. 2012, DE LA FUENTE et al. 2013). The nitrification was greatly stimulated by organic and mineral fertilizers inputs; however, its dynamics differed among the treatments. The most rapid nitrification was observed for soils treated with LCM and MF due to high initial NH_4-N inputs. Slower nitrification occurred in soil fertilized with SCM due to high amount of N in organic compounds. The D treatment also resulted in slower nitrification, what is contradictory to other studies on N dynamics in soils fertilized with digestates. In most incubation experiments, rapid nitrification took place in 1 or 2 weeks of incubation (GRIGATTI et al. 2011, ALBURQUERQUE et al. 2012, DE LA FUENTE et al. 2013). This slower nitrification may be the result of initial increase in NH_4-N in soil after fertilization.

All organic and mineral fertilizers provided a source of plant available N, in the beginning of the experiment mainly as NH_4^+ form and after 56 days only in NO_3^- form. The MF produced the largest recovery of inorganic N equivalent to N input and this is in a good agreement with studies of RIGBY and SMITH (2013). Nitrogen availabilities at the end of incubation were decreasing in the order: D (72.7% of TN added) > LCM (47.7% of TN added) > SCM (30.5% of TN added). Similar findings were reported by CAVALLI et al. (2017).

The addition of organic fertilizers increased the pH in all soils at the beginning of experiment due to organic fertilizers alkalinity (Table 1, Figure 2). Such rise of pH in soils treated with digestate and manures was reported by CAVALLI et al. (2017). In following days, nitrification of ammonium applied with mineral fertilizer decreased soil pH rapidly in MF treatment. The nitrification of applied NH_4-N together with those mineralized from easily-decomposed organic N compounds also influenced the soil pH in all organic treatments (CAVALLI et al. 2016). Strong relation between nitrification and soil pH dynamics (CAVALLI et al. 2017) results from acidifying effect of nitrification due to proton formation (GOULDING 2016).

The total amount of organic carbon (TOC) in soil results from net balance of all carbon fluxes entering and leaving the soil over a time period. The decomposition and mineralization of organic matter depends on the microbial activity and chemical nature of organic material and leads to the release of carbon dioxide (CO_2) and other trace gases, such as methane (CH_4) and carbon monoxide (CO) form soil (RODEGHIERO et al. 2012). The variability in soil organic carbon and the level of detection, under the regime of incubation experiment, did not allow detecting the statistically significant difference between TOC content in control and treated soil.

However, the addition of soluble C in liquid organic fertilizers such as D and LCM resulted in significantly higher DOC content in fertilized soils comparing to unfertilized controls. The supply of readily metabolizable C in organic material influences the microbial activity and biomass and therefore enhances organic matter degradation and nitrification (GÓMEZ-BRANDÓN et al. 2016).

The addition of P in the form of PO_4^{3-} in MF treatment significantly enriched soil in P_w, which was significantly higher than in soils treated with organic fertilizers; however, there was also slight increase in P_w content in these soils. Higher P_w content in MF treatment is in a good agreement with BACHMANN et al. (2011), who reported P_w content slightly higher in sandy soils amended with organic fertilizers and lower in loamy soils, comparing to mineral P treatment. In pot experiment with amaranth and sorghum, the P_w content also increased after organic fertilizer application comparing to mineral fertilization with N and K only, however the P_w in amaranth soils was not clearly affected by the amendment while in sorghum soils organic fertilizers supplied higher amount of P_w than mineral NPK addition. This was due to low P_w content in the soil and rapid fixation of easily soluble P in NPK treatment (HUPFAUF et al. 2016). Soil fertilization with organic amendments, both digested and undigested, increases the P_w content in longer time period comparing to mineral control treatment (BACHMANN et al. 2014). The P_w decrease in fertilized soils was due to the enhancement of microbial activity and growth of microbial biomass (HUPFAUF et al. 2016). Microbial P in soil is affected by fertilizer type and microbial-bound P is higher in soils amended with organic material than in mineral fertilizer treatments (BACHMANN et al. 2011). The incorporation of organic and mineral fertilizers slightly increased the total phosphorus (TP) content. Its increase in soils amended with digested and undigested dairy slurry was also reported by BACHMANN et al. (2011).

The addition of mineral fertilizer (MF) to the soil did not affect the K_{dl} and total potassium (TK) content due to mineral fertilizer chemical characteristics. This fertilizer consisted only of N and P and therefore did not supply with any K. The liquid cattle manure (LCM) treatment supplied soil with the highest amount of K, due to the highest K content, while solid cattle manure (SCM) treatment added the lowest amount of K because manure had much lower K content. According to GRZEŚKOWIAK (2013), liquid cattle manure is typical N and K organic fertilizer with K_2O content equal to 7 kg m^{-3}.

Conclusions

The digestate provided a significant amount of NH_4-N to soil, however less than that supplied in mineral fertilizer or liquid cattle manure. The nitrification rate of NH_4-N was slower for soil treated with digestate comparing to soils treated with liquid cattle manure and mineral fertilizers. This may be the advantage of digestate, because slower nitrification can limit losses of N *via* leaching. Although digestate application to soil initially increased pH, the nitrification causes pH decrease with the time. Digestate provided also water-soluble P at the same level as other organic fertilizers, but supplied soil with high amount of plant-available K and dissolved organic carbon. Therefore, the application of digestate should follow the same rules as traditional liquid fertilizers; however, its agronomic use should be based on N, P and K content. It also must be emphasized that digestate, even though is a valuable fertilizer which influences soil chemical properties similarly to liquid cattle manure, may only partially replace mineral fertilizers in Poland.

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THE MORTALITY OF THE LESSER GRAIN BORER *RHYZOPERTHA DOMINICA* (FABRICIUS 1792) INDUCED BY PLANT POWDERS

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Key words: insecticidal activity, lesser grain borer, mortality, plant powders, stored pests.

Abstract

The aim of this study was to investigate the effect of powdered plants: *Salvia officinalis* L., *Artemisia absinthium* L., *Mentha piperita* L., *Allium sativum* L. and *Pimenta dioica* L. on the mortality and development of the progeny of *Rhyzopertha dominica* F. Powdered plants were used at the following weight concentrations: 1.23%, 3.61%, and 5.88%. Experiments were conducted in a laboratory at 28°C and RH 60±5%. It has been found that the effect of the tested plants depends on their concentration. Among the five species of powdered plants: *S. officinalis*, *A. absinthium*, *M. piperita*, *A. sativum*, and *P. dioica*, the highest mortality in *R. dominica* was caused by *P. dioica* at all tested concentrations and as early as after the first day of experiments. Of the tested plants, the one that may find application in the protection of stored products from *R. dominica* is *P. dioica*, which has shown insecticidal activity against this insect species.

Introduction

The loss of food produced on our planet is colossal. According to the Global Food Reports published by GUSTAVSSON (2011) and FOX (2013), it is estimated that 30–50% (1.2–2 billion tonnes) of annual world food production is lost. These figures do not include the usage of energy, water, and fertilizers in food production. Food wastage occurs throughout different stages of food production and forms a great chain of losses stretching from harvest to consumption, through transportation, storage, processing,

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packaging, finally reaching sale and food intake. Each of these stages involves losses, e.g., via spilling, dampening, and pests. Reducing this wastage, especially that of grain soon after harvest, is cheaper and more cost-effective than increasing food production. Food losses that occur during storage resulting from foraging by pests, mainly insects and mites, are difficult to evaluate, and additionally are – very diversified depending on crops, country, climatic region, as well as on the duration of the storage period of certain products. Furthermore, there is no universal method for measuring these losses. It is generally assumed that, every year, world-wide losses caused by insect and mite pests in the storage of products amount to ca. 10%. Integrated methods (Integrated Pest Management – IPM), which are considered an alternative to chemical methods, are the currently recommended and applied methods for combating pests. The former consists of preventing the occurrence of insect pests and combating them with mechanical, physical, and biological methods, coupled with limiting the use of chemical pesticides to the lowest possible level through using them only when absolutely necessary (Cox 2004, Phillips and Throne 2010, Olejarski and Ignatowicz 2011).

Since 1 January 2014, Regulation 1107/2009 and Directive 2000/29/EC on the safe use of pesticides have been in force in EU member states. Strong emphasis is placed on the widest possible use of non-chemical methods, especially biological ones. In recent years, the studies concentrated on the search for the various forms of plant-derived materials, e.g., powders, oils, or extracts that could be used to control insect pests of stored products. A great number of studies were published indicating the insecticidal effects against these insects (Jilani and Su 1983, Khan and Marwata 2003, Park et al. 2003, Isman 2006, Moreira et al. 2007, Kim et al. 2011, Padini et al. 2013). Moreover, the natural plant products are biodegradable; therefore, they are safe for the environment. Therefore, research into the activity of herbal preparations as bio-insecticides therefore seems highly relevant. Plants contain many substances that may change insect behaviour when added to food, and they also affect food absorption and cause development disturbance in insects (Nawrot 1983, Papchristos and Stamopoulos 2002).

Rhyzopertha dominica discussed in the presented study is a dangerous and widespread grain storage pest. Both adult individuals and larvae that feed on cereal grain cause extensive damage (Irshad et al. 1988, Dowdy and Mcgaughey 1992). *R. dominica* infects numerous plant products including wheat, rice, sorghum, rye, barley, maize, beans, dried vegetables, and fruits (Nawrot 2001, Phillips and Throne 2010). The aim of the tests was to investigate the mortality inducted on this pest by the use of powdered plants, namely *Salvia officinalis* L., *Artemisia absinthium* L., *Mentha piperita* L., *Allium sativum* L., and *Pimenta dioica L.* Mer. The rate and intensity of the effects of each type of dried plant powder on the *R. dominica* population was also studied. The tests performed also had a secondary practical purpose, i.e. to indicate plant species that would show insecticidal or deterrent activity on the *R. dominica*, and could therefore prove applicable to the integrated protection of stored grain against this pest.

Materials and Methods

Mass cultures of the *R. dominica* were first established in order to obtain individuals of equal age, which were subsequently used in the experiments. According to previous research, wheat is considered the most suitable food and habitat for the development of the *R. dominica* (KŁYŚ 2006). Therefore, with the use of analytical scales, 100 g doses of wheat were placed in a number of plastic containers with a 50 cm^2 bottom and perforated lid enabling air penetration. Eighty individuals of this species were then transferred to each container with wheat. Culture containers were placed in incubators in 28°C and RH 60±5 %. After four weeks, all adult individuals were carefully removed using tweezers, while eggs, larvae, and pupae were left in the wheat. Individuals of equal age were obtained after another four weeks and used in the experiments. The experiments were carried out in plastic, 28 cm^2 bottom containers closed with perforated lids. All tests were started by transferring same-aged adult individuals (20 males and 20 females) to culture containers.

Control cultures were held, using clean wheat as a substrate, as well as a set of experimental cultures with wheat containing dried plant additives as substrate. Dried leaves of *M. piperita*, herb *S. officinalis*, bulbs of *A. sativum*, herb *A. absinthium*, and seeds of *P. dioica* were powdered with an electric grinder. Each type of dried plant material and up to 40 g of wheat was added to containers in the following amounts: 0.5 g, 1.5 g, and 2.5 g, which gave the respective percentage weight concentrations: 1.23%, 3.61%, and 5.88%. 40 g of wheat was weighed out and added to closed plastic containers, which were perforated to enable constant air inflow. The wheat was thoroughly mixed with plant powders. *R. dominica* individuals were then transferred to containers, with 40 insect individuals per each container, thus creating 15 sample variants. Six repetitions of each sample were performed. Control samples were placed in a separate incubator in the same temperature and humidity conditions as experimental samples, to prevent the effect of volatile substances on the insect mortality. The population status was monitored after 1, 2, 7, 14, and 21 days. Live and dead insects were counted. Dead insects were removed and live ones were left in the culture.

In order to investigate the effect of applied plant powders on the progeny of *R. dominica* on 21 day all beetles were removed and the cultures were left in the incubator. After 50 days the population was re-monitored.

To assess the effect of the added plants on the studied populations, a Kruskal-Wallis ANOVA test showing the statistical significance of differences was employed, with the use of PQStat ver.1.4.2.324. software assuming a significance level $\alpha = 0.05$. The Dunn test was the *post-hoc* test performed after the rejection of zero-hypothesis by the Kruskala-Wallis test.

The mortality rate, representing the percentage share of dead individuals relative to the total number of individuals in the population per controlled time unit, was calculated from the following formula (KŁYŚ 2013):

$$\frac{\overline{x}_d}{\overline{x}_d + \overline{x}_l} \cdot 100\%$$

$\overline{x}_d$ – mean number of dead insects
$\overline{x}_l$ – mean number of live insects.

Results

After the first day of testing, the highest mortality of *R. dominica* was recorded in wheat with the admixture of powdered *P. dioica*, and the lowest was found in wheat with the admixture of *M. piperita* at all concentrations (1.23%; 3.61%; 5.88%). The 1.23% concentrations of *M. piperita*, *S. officinalis* and *A. sativum* did not cause any mortality in *R. dominica* after the first day of testing. The insect mortality caused by these plants used at other concentrations did not exceed 2% in wheat with *M. piperita*, it equalled 4% in wheat with *A. sativum*, and 7.5% in wheat with *S. officinalis* (Figures 1*a–e*).

Statistical analysis of the obtained data performed using the Kruskal-Wallis test showed statistically significant differences ($H = 74.60$, $p < 0.0001$) on the first day of experiments. When analysing the results with the post-hoc Dunn test, statistically significant differences were found between the mortality of R. dominica kept in the control culture (without herbal additives) and the mortality of these insects kept with *P. dioica* at all concentrations used: 1.23% ($p = 0.0373$); 3.61% ($p = 0.0030$)

and 5.88% (p = 0.0019). When comparing the mortality of insects between cultures containing different concentrations of powdered plants, thirteen significant differences were found on the first day. In all compared pairs, statistically significant differences in the insect mortality were noted in the presence of *P. dioica* (Table 1).

Table 1

Significant differences in the mortality of *Rhyzopertha dominica* (Fabricius 1792) caused by powdered plants of different species

Kruskal-Wallis Test, a = 0.05														
Day of research														
1			**2**			**7**			**14**			**21**		
*	**	***	*	**	***	*	**	***	*	**	***	*	**	***
C- P1				C- P1		C- S3			C- S2				C- S2	
	C- P2				C- P2			C- P1		C- S3			C- S3	
	C- P3				C- P3			C- P2			C- P1			C- P1
M1- P1				M1- P1			C- P3				C- P2			C- P2
	M1- P2				M1- P2	M1- S3				C- P3				C- P3
	M1- P3				M1- P3			M1- P1		M1- S3			M1- S3	
M3- P2				Ar1- P1				M1- P2			M1- P1			M1- P1
M3- P3				Ar1- P2			M1- P3				M1- P2		M1- P2	
Ar1- P2				Ar1- P3		Ar1- S3				M1- P3			M1- P3	
Ar1- P3				S1- P1			Ar1- P1			Ar1- S3			Ar1- P1	
S1- P1					S1- P2			Ar1- P2		Ar1- P1			Ar1- P2	
	S1- P2				S1- P3		Ar1- P3			Ar1- P2		Ar1- P3		
	S1- P3					S1- S3			Ar1- P3			S1- S3		
P1- Al1							S1- P1			S1- S3				S1- P1
	P2- Al1							S1- P2			S1- P1			S1- P2
	P3- Al1						S1- P3				S1- P2		S1- P3	
							P1- Al1			S1- P3			P1- Al1	
							P2- Al1			P1- Al1			P2- Al1	
										P2- Al1		P3- Al1		

Control/Plant: C – control, M – *Mentha piperita* (Linnaeus 1753), S – *Salvia officinalis* (Linnaeus 1753), Ar – *Artemisia absinthium* (Linnaeus 1753), Al – *Allium sativum* (Linnaeus 1753), P – *Pimenta dioica* (*Linnaeus* 1753, Merrill)

Weight concentrations of powdered plant: 1–1.23%, 2–3.61%, and 3–5.88%.

Significant differences:

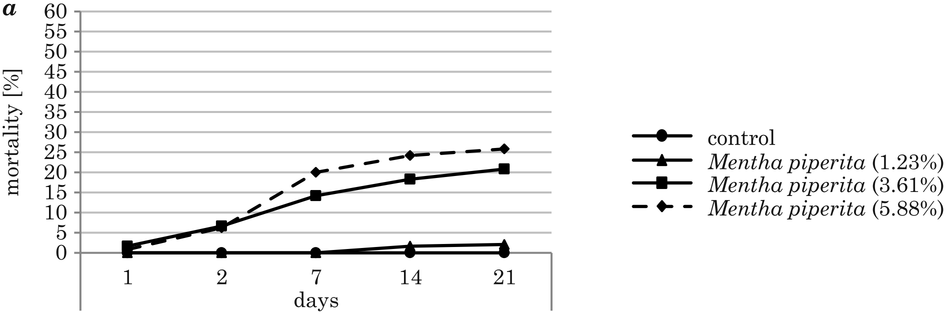
* $0.05 > p > 0.01$;

** $0.01 > p > 0.001$;

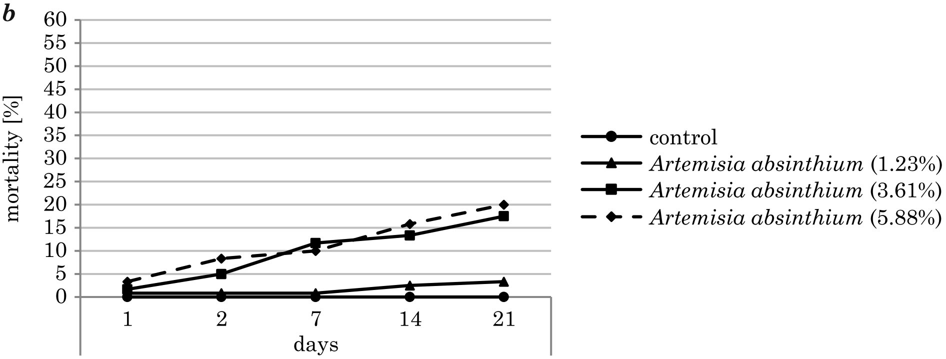
*** $p < 0.001$

In the further experimental time periods, i.e. after 2, 7, 14, and 21 days, the highest mortality was caused by *P. dioica*, followed by *S. officinalis*, *M. piperita*, *A. absinthium*, and *A. sativum*. Insects kept in the control culture containing wheat without plant additives presented a 100% survival rate in all experimental time periods (Figures 1 *a–e*). The mortality of the *R. dominica* in wheat with 5.88% admixtures of *M. piperita*, *A. absinthium*, *S. officinalis*, and *A. sativum* showed a time-dependent increase and was the highest compared to other concentrations of plant powders (Figures 1 *a–d*).

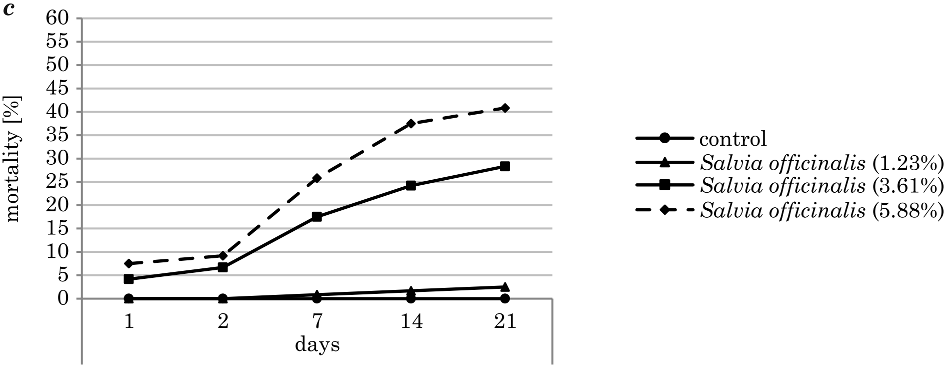
a



b



c



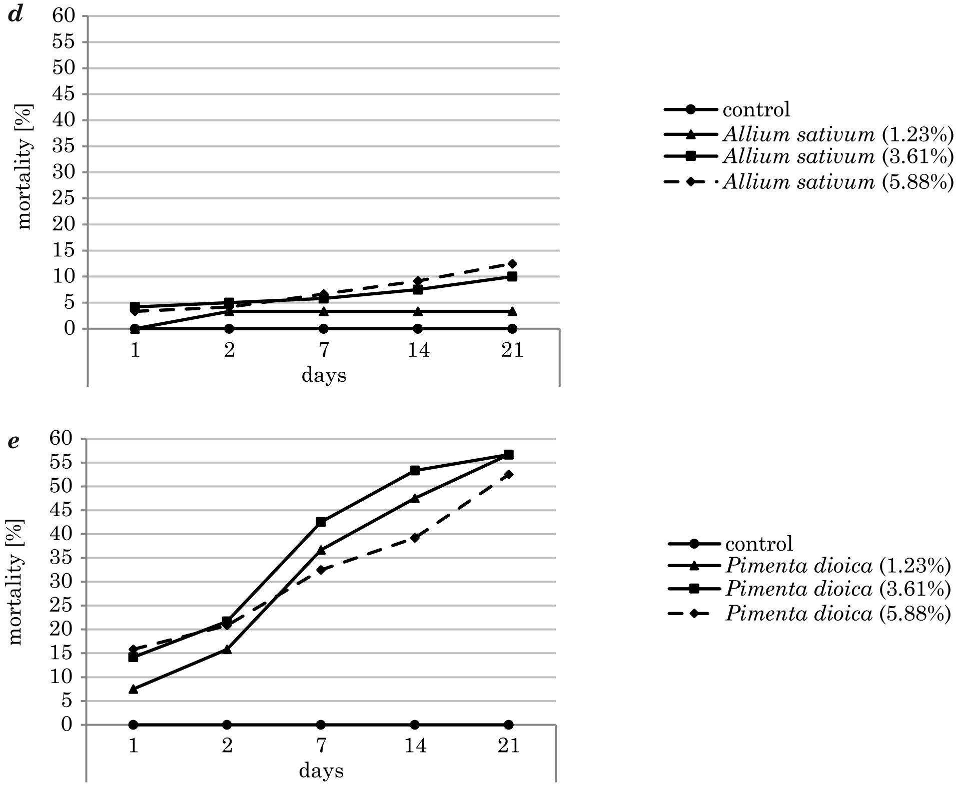


Figure 1. Mortality of *Rhyzopertha dominica* (Fabricius 1792) caused by: *a* – *Mentha piperita* (Linnaeus 1753); *b* – *Artemisia absinthum* (Linnaeus 1753); *c* – *Salvia officinalis* (Linnaeus 1753); *d* – *Allium sativum* (Linnaeus 1753); *e* – *Pimenta dioica* (Linnaeus 1753, Merrill)

It was also found that the mortality of *R. dominica* rose over time in wheat containing the admixtures of each plant at all concentrations used. Another trend discovered was the fact that the insect mortality usually increased in accordance with the concentration of plant powders. One exception was the culture in wheat with 3.61% *P. dioica*, where the mortality rate of insects was higher than that with 5.88% *P. dioica*, starting from the second day of testing, and reached from 21.67% to 56.67% (Figure 1 *e*).

While comparing mortality rates of insects in the control group (free of plant additives) with 1.23%, 3.61%, and 5.88% *P. dioica*, cultures, statistically significant differences were indicated. Significant differences were noted after 7, 14, and 21 days of testing between the control and the 5.88% *S. officinalis culture* (p = 0.0120, p = 0.0019, p = 0.0016). On the last two days of testing, significant differences were also shown between the mortality of the *R. dominica* in the control culture and the mortality in wheat containing 3.61% of *S. officinalis* ($p = 0.0380$, $p = 0.0244$). The comparison of mortality rates of insects between cultures containing powdered plants at various concentrations provided 43 significant differences, where each compared pair included *P. dioica*. Among these differences, 15 were highly significant ($p < 0.001$) – Table 1.

S. officinalis, *M. piperita* and *P. dioica* caused a 100% reduction offspring of *R. dominica*. *A. absinthium* reduced the number of progeny of *R. dominica* compared to the control culture by 35, 37 and 40 and *A. sativum* respectively by 30, 35 and 39 (depending on the concentration used) – Table 2.

Table 2

The number of progeny individuals (mean) obtained after 50 days of removal of parents

Plants powder	Dose [%]			Control
	1.23	3.61	5.88	
S. officinalis	0	0	0	45
A. absinthium	10	8	5	
M. piperita	0	0	0	
A. sativum	15	10	6	
P. dioica	0	0	0	

Discussion

Natural plant substances have become increasingly important as prophylactic measures in the protection against the pests of stored products. Numerous reports on plants that constrain the development of grain storage pests can be found in the scientific literature. Besides oils, water and alcohol extracts, or their combinations (TRIPATHI et al. 2002, MISHRA et al. 2006, ROZMAN et al. 2007, DERBALAH and AHMED 2011, MANZOOR et al. 2011), plant powders have been studied in terms of their effects on storage pests, which can be insecticidal, deterrent and/or reductive to offspring (SHARABY 1989, SRINIVASAN et al. 2003, KOONA and NJOYA 2004, KŁYŚ 2004, GOVINDAN and NELSON 2009, WAWRZYNIAK and WRZESIŃSKA 2009, CHAYENGIA et al. 2010, KŁYŚ 2011, LU and SHI 2012).

The efficiency of plant powders affecting the *R. dominica* was studied, inter alia, by KALINOVIĆ and co-authors (KALINOVIĆ et al. 2002). They demonstrated that an effective and natural insecticide for this species is *Thymus vulgaris* L., which caused the complete extinction of the population on the 11^{th} day of exposure when applied in powdered form at 7.5% concentration. Tests conducted by KŁYŚ (2013) did not confirm such a high activity of *T. vulgaris* used at lower (1%) concentration. It caused an 80%

mortality rate in the *R. dominica*, and only after 30 days of exposure. In the presented study, it was confirmed that the effectiveness of the applied herbs depended on the concentration used. The efficacy of the insecticidal effect of *S. officinalis*, *A. absinthium* and *A. sativum* increased in each time interval along with the increase of concentration.

ASHOURI and SHAYESTEH (2010) tested the effect of powdered *Capsicum annuum* L. fruit on the *Rhyzopertha dominica* and *Sitophilus granarius*. A 5% dosage of pepper fruit caused a mortality rate of approximately 60% in both species on the 14th day of testing. *Piper nigrum* L. seed powder applied at 0.5% concentration reduced the population of *S. granarius* to null as early as after five days, while the *R. dominica* was sensitive to *P. nigrum* to a lesser extent. The latter insect species showed a 100% mortality rate after 14 days *P. nigrum* of testing. In addition, *P. nigrum* significantly hindered the development of new generations.

In turn, KŁYŚ (2013) proved that powders from other plant species: *Chrysanthemum cinerariaefolium* Vis. and *Origanum majorana* L. had stronger insecticidal and deterrent effects on the *R. dominica* compared with *C. annuum* and *P. nigrum* used in the study by ASHOURI and SHAYESTEH (2010). *Ch. cinerariaefolium* (1%) caused a 100% mortality rate in the *R. dominica* already after one day of testing, and marjoram caused a 90% mortality rate during the first three months of experiments, which later reached 100%. Also at 0.5% concentration, *Ch. cinerariaefolium* inhibited reproduction in the *R. dominica* and caused near-total mortality. When used at lower concentrations (0.25%, 0.125%, and 0.06%), *Ch. cinerariaefolium* also hampered the reproduction and development of the *R. dominica* population. KŁYŚ (2004) also showed that *Salvia officinalis* L. and *Artemisia absinthium* L. exerted an antifeedant effect on *R. dominica*.

In the presented paper, a powder from *P. dioica* has been found to show strong insecticidal properties, causing a statistically significant mortality in *R. dominica* at the three concentrations used (1.23%, 3.61%, 5.88%) after only one day of experiments. However, no statistically significant effect of powders from *M. piperita*, *A. absinthium*, and *A. sativum* on the mortality of the *R. dominica* was noted, which applies to all tested time periods and all concentrations used. KALINOVIĆ and co-workers (2002) studied the influence of *Lavandula angustifolia* Mill. powder, used at a higher (7.5%) concentration than that of *Pimenta dioica* in this study, on the *R. dominica*. They found that the addition of *L. officinalis* caused a 100% mortality rate in the *R. dominica* after 11 days of exposure.

Conclusions

Out of five species of powdered plants: *S. officinalis*, *A. absinthium*, *M. piperita*, *A. sativum*, and *P. dioica*, used at three weight concentrations (1.23%, 3.61%, and 5.88%), *Pimenta dioica* caused the highest mortality of *R. dominica*. The mortality of *R. dominica* kept in wheat containing different plant species showed a time-dependent increase. The mortality of insects usually grew in accordance with the increase of herb concentrations. *S. officinalis*, *M. piperita* and *P. dioica* caused a 100% reduction offspring of *R. dominica*.

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ANTAGONISTIC EFFECTS OF BINARY MIXTURE OF TITANIUM DIOXIDE NANOPARTICLES AND LEAD ON BIOMASS AND OXIDATIVE STRESS IN EXPOSED *CHLOROIDIUM ELLIPSOIDEUM* (GERNECK)

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Key words: *Chloroidium ellipsoideum;* nanoparticles, biomass; antioxidants.

Abstract

Sub-lethal bioassay was performed on *Chloroidium ellipsoideum* to monitor the changes in the algal biomass and antioxidant activities associated with Titanium dioxide Nanoparticles (TiO_2 NPs) and Lead Pb(II). The results showed that there was significant ($p < 0.05$) decrease in biomass (density, chlorophyll *a* and *b*) of *Chloroidium ellipsoideum* as a result of adsorption of Pb(II) by TiO_2-NPs. However, the binary mixtures of the chemicals significantly increased antioxidant activities (SOD and MDA) of the alga. Furthermore, the study revealed an antagonistic (CI >1) effect of the binary chemicals on the biomass and antioxidant activities of the alga. Based on the study, it was shown that the co-exposure of TiO_2 NPs and Pb(II) decreased the Pb(II) bioavailability causing antagonistic effects on biomass and antioxidant activities in *Chloroidium ellipsoideum*.

Introduction

Increase in anthropogenic activities often leads to a direct or indirect increase in the production of waste effluents in aquatic ecosystems with harmful risks on biodiversity (JAISHANKAR et al. 2014). Mismanagement of the various effluents from industries such as mines, agriculture, phar-

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macy, medicine, and households usually leads to the accumulation of environmental pollutants and nanomaterials in aquatic ecosystems.

The use of nanomaterials is greatly increasing because of their wide application in household products, like sunscreens, cosmetics, paints and surface coating (RAY et al. 2012). In 2010, 50,400 tons of nanomaterials were produced worldwide, which increased to about 201500 tons in 2015 and worth about 2.6 trillion dollars (RAY et al. 2012). This is expected to increase further many years to come.

ZHANG et al. (2015) reported that ZnO_2, SiO_2 and TiO_2 are the most greatly used nanomaterials in the world. TiO_2 nanoparticles are commonly used because of its importance in sunscreens where the particles protect against cell damage and preventing UV light. TiO_2 (NPs) is also used in toothpastes industries, surface coatings, water treatment (CHEN and MAO 2007), and recently TiO_2 NPs bears tremendous hope in helping ease the energy crisis through effective utilization of solar energy based on photovoltaic and water-splitting devices (GRATZEL 2004). Their diversified use has led to a worldwide production of 5000 t/year within 2006–2010, 10 000 t/year within 2011–2014, and an estimated 2.5 million metric tons/year by 2025.

Thus, the large production of TiO_2 NPs with its novel properties is of critical concern because of the likelihood of their fate ending in freshwater ecosystem (ZHANG et al. 2015). However, there is an array of reports that documented the acute and chronic toxicological impacts of TiO_2 NPs in freshwater and marine organisms. Microalga *Pseudokirchneriella subcapitata* exposed to TiO_2 NPs (25–70 nm) was more toxic than bulk TiO_2 with recorded effect of aggregation on algal cells (ARUOJA et al. 2009). Reduced growth and metabolic alterations were observed in three other freshwater species of algae, *Scenedesmus quadricauda*, *Chlamydomonas moewussu* and *Chlorella vulgaris*, exposed to TiO_2 NPs (CARDINALE et al. 2012). Inhibitory effect of titania NPs (EC_{50} of 16.12 mg L^{-1} and 21.2 mg L^{-1}) was reported in both *Scenedesmus* sp. and *Chlorella* sp. (MUKHERJEE et al. 2011). WANG et al. (2012) also reported inhibition of chlorophyll *a* and cell growth as well as increased chrorophyll *b* and carotenoid in *Chlamydomonas reinhardtii* exposed to TiO_2 NPs. The crystal structure of TiO_2 NPs greatly inhibited algal growth after 6 days exposure with recorded EC 30, 30 mg L^{-1} (JI et al. 2010, HUREL et al. 2013).

Only few studies exist on the assessment of ecotoxicological effects and environmental risks of TiO_2 NPs combined with heavy metals on organisms (HARTMAN et al. 2009, MAOET al. 2012, TANG et al. 2013). Lead for instance is one of the most well-known pollutants in the environment and its bioconcentration into biological system is increasing due to anthropogenic activities (TRIPATHI et al. 2006). Lead is also known to be non-biodegradable and have harmful effects on freshwater organisms (GROSELL et al. 2006, MARTINEZ et al. 2004, SEVAKOVA et al. 2011).

However, to the best of our knowledge, studies on the effects of combined chemicals of TiO_2 NPs and Lead on microalgae has not been given due attention, hence the need for its study.

This study investigated the ecotoxicological effects of single and binary mixtures of titanium dioxide nanoparticles and lead on microalgae *Chloroidium ellipsoideum* (chlorophyta) a primary producer in the aquatic ecosystem in order to provide an insight into the impact of the presence of this nanomaterial (TiO_2 NPs) on a conventional pollutant Pb(II).

We aimed to study the effects of binary mixtures of Titanium dioxide Nanoparticles (TiO_2 NPs) and Lead Pb(II) on biomass and antioxidant activities of *Chloroidium ellipsoideum*.

Materials and Methods

Microalgal species

The microalgae *Chloroidium ellipsoideum* was obtained from the National Institute for Fisheries and Freshwater Research (NIFFR), New Bussa, Nigeria. The algae was cultured in a modified BG 11 medium consisting of 31.43 mg L^{-1} organic Nitrate (NO_3), 40.80 mg L^{-1} Phosphorus (PO_4), 170.08 mg L^{-1} Potassium (K_2O), 42.0 mg L^{-1} Magnesium (MgO), 4.66 mg L^{-1} sulfur (SO_4), 0.42 mg L^{-1}, Calcium (Ca) 2.53 mg L^{-1}, Iron (Fe), 39.27 mg L^{-1} Sodium (Na) 20.62 mg L^{-1}, Chlorine (Cl) and Zinc (Zn) 0.29 mg L^{-1}. Prior to the culture, the medium was sterilized by autoclaving at 121°C for 15 minutes. Cultures were maintained under control condition in a culture cabinet at 25 ± 2° C and 12:12 h light: dark cycle.

Nanoparticles of Titanium dioxide (TiO_2) (anatase-rutile) 21 nm and Lead nitrate ($Pb(NO_3)_2$ salt were purchased from Sigma-Aldrich (St Louis, Mo, USA). An Empyrean XRD (Panalytical, The Netherlands) was used to characterize Nano-TiO_2 particles powder by X ray diffraction. The machine was equipped with filtered Cu Kλ radiation (λ = 1.5418 Å) operated at 40 K_v and 40 mA. The XRD patterns were recorded from 10 to 80 degrees with a scanning speed of 0.526° per minute. PDF2 software was used for the analysis of peaks. Images of Nano-TiO_2 particles powder as received from the manufacturer using Scanning electron microscope (Phenom ProX SEM, Phenom-World, The Netherlands) was also obtained.

A stock solution of 100 mg L^{-1} of both chemical was prepared separately by suspension in the ionic water. TiO_2 NPs was rigorously mixed with an ultrasound water bath (100 W, 40 kHz) for 30 mins to prevent agglomeration; samples were diluted to different exposure concentrations. To analyse the interaction of Pb(II) and TiO_2 NPs, batch sorption experiment was performed. In this in-vitro assay, a stock of Pb(II) previously prepared was diluted (0.013, 0.019, 0.025, 0.031, 0.037 and 0.04) with 50 mL of culture medium. The pH of Pb(II) was adjusted to 7.00 using 0.01 M HCl and NaOH. TiO_2NPs of concentration of 0.20 μM was diluted in each flask with distilled water. The mixtures were shaken to achieve sorption equilibrium within 10h (TANG et al. 2013). The binary suspensions were centrifuged at 5000 rpm for 10 mins. The supernatants obtained from centrifugation were then measured.

Chloroidium ellipsoideum cultured at exponential growth phase (6 days) was treated with a nominal concentration of 0.01 and 0.04 μM of Pb; 0.09, 0.20 μM of TiO_2 NP_s and the four (4) couples of binary mixtures (0.01, 0.09); (0.01, 0.20); (0.04, 0.09); (0.04, 0.20) μM respectively for 72 h. In the medium, the effects of the chemicals were observed alone and in combination. Powdered Pb(II) was provided as Pb $(NO_3)_2$ because it is the most common form compared to other Pb(II) sources in aquatic ecosystems (MUTEMBEI et al. 2014).

The chosen TiO_2 NPs and Pb(II) levels were the sub-lethal concentrations. But, for Pb(II), the concentrations were 0.01 μM and 0.04 μM respectively. The concentration (0.01 μM) in this study was considered as allowable permissible concentration and the concentration (0.04 μM) is the maximum allowable limit standard of the United States environmental protection agency (USEPA) (WHO 2013). The concentrations for both chemicals were chosen after a preliminary determination of EC_{50}, where EC_{50} values of 0.190 μM and 0.86 μM were recorded for Pb(II) and TiO_2 NP_s respectively. All the experiments were carried out in triplicates.

Data collection

The suspensions collected from sorption analysis were digested using pure HNO_3 at 120°C for 2 h in a glass beaker. The Pb(II) concentrations in the digested samples were then determined according to the method described by HALTTUNEN et al. (2007). After cooling, the solution was transferred quantitatively to a 10 mL volumetric flask; Pb(II) concentrations were determined using AA-7000 Atomic Absorption spectrophotometer and read at wavelength 283.3 nm.

Sorption of Pb(II) onto TiO_2 NPs

The Langmuir isotherm model was used to fit the adsorption data with Ce/qe = Ce/q_{max} + 1/q_{max} b, where: q_{max} = maximum adsorption, b = Langmuir coefficient. The interactions of Pb (II) with TiO_2 NPs were computed by examining the sorption equilibrium. In the equilibrium isotherm experiment, a correlation between Pb(II) adsorbed on the TiO_2 NPs (qe, µg/g) and the non adsorbed Pb(II) concentration (Ce, µg L^{-1}) was calculated according to CHEN (2015).

Biomass (density, dry weight and chlorophyll content) and the biomolecular composition (protein, lipid and carbohydrate) of the alga which are the key parameters of growth and productivity assessment were determined (MATOUKE et al. 2018). Density was monitored by direct count of viable cells under the microscope (Motic Digital DMB 1 series) using a Neubauer haemacytometer (Optik labor, Qiujing, QJ1102). Dry weight was determined gravimetrically using previously oven dried (2 h, 60°C) Whatman's GF/C filters (0.45 µm pore size) with a sensitive weighing balance (Sartorius; AG Germany). Chlorophyll extraction was carried out using 80% (v/v) acetone according to the method of SHOAF and LIUM (1976) and chlorophyll *a* and *b* were determined using the equation of RITCHIE (2008).

Antioxidant activities were measured based on standard methods, Superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and malondialdehyde activities were evaluated as a measure of oxidative stress in the exposed *C. ellipsoides*.

After 72 h of exposure to the chemicals, the microalgal cells cultures were collected, centrifuged and then the pellets homogenized in 2 mL of 100 mM potassium phosphate buffer (pH 7.5), 1 mM ethylene diamine tetra acetic acid (EDTA) and 1%w/w polyvinyl polypyrrolidone (PVP) using a vortex. The homogenate was centrifuged at 12,000 rpm for 15 min at 4ºC. For enzymes activities the supernatant was separated and stored as aliquot at 80ºC.

The enzymatic activities of Glutathion peroxidase (GPx), Superoxide dismutase (SOD), Glutathione reductase (GR) and Malondialdehyde (MDA) were evaluated and described in supplementary document by the methods of ENSIBI and MOHAMMED (2017), BEAUCHAMP and FRIDOVISH (1971), SCHAEDLE and BASSHAM (1977) and HEATH and PACKER (1968) respectively.

Data Analysis

The obtained data were subjected to Levene's test for homogeneity of variance and one way ANOVA was used to determine the differences in means of the parameters (Biomass and antioxidant responses using Origin-Pro 8.5 (OriginalLab Corp., Northampton, MA, USA). Where significant differences were observed, separation of means was done using Tukey's HSD post hoc test. Values were considered significantly different when the probability was less than 0.05. The determination of interaction between chemicals or combination index used the method of Chou-Talalay (1976).

Results

Characterization of TiO_2 nanoparticles

X Ray Diffraction analysis demonstrated the crystal phases and the crystallite size of TiO_2 NPs. Figure 1*a* revealed eleven (11) peaks on the the X-ray diffractograms. SEM revealed the whitish colour of TiO_2 NPs.

The study showed the powder form of TiO_2 NPs before and after dispersion into the medium (Figures 1*a*, 1*b* and 1*c*).

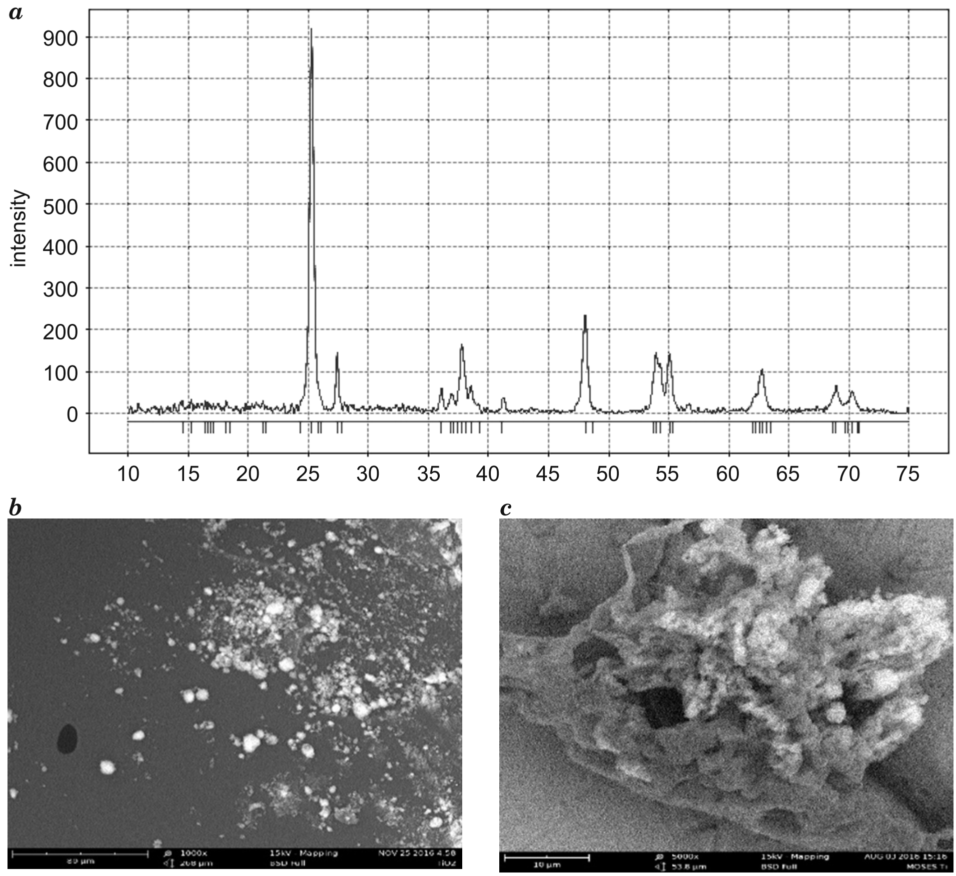


Fig. 1. Characterization of TiO_2 NPs: *a* – X-ray diffraction pattern of TiO_2 NPs; *b* – SEM image of TiO_2 NPs as received from the manufacturer; *c* – SEM image of TiO_2 NPs after dispersion in the medium

Sorption of Pb(II) onto TiO_2 NPs

The computed adsorption capacity q_{max} was 30.296 μg g^{-1}, the parameter b corresponding to the affinity of the TiO_2 NPs to Pb(II) or Langmuir constant was 33.57 μg g^{-1} and the correlation ($R^2 = 0.999$) is shown in Figure 2. The result indicated a positive and good correlation suggesting a monolayer adsorption of Pb(II) on TiO_2 NPs.

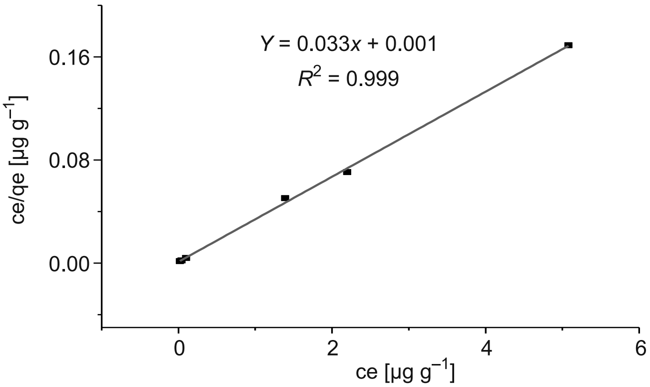


Fig. 2. Adsorption isotherms of Pb(II) on TiO_2 NPs in the culture medium; pH = 7.00; temperature = 298 K

Biomass production of *C. ellipsoideum*

The inhibitory effect of single and combined chemicals on density, chlorophyll *a* and *b* after 72 h exposure are shown in Figure 3 and Figure 4. The chemical concentrations density of Pb (0.01) and Pb (0.04) μM were significantly ($p < 0.05$) inhibited to 79%, 76%; chlorophyll *a* content to 61%, 83% and chlorophyll *b* content to 68%, 57% of the control respectively. However, TiO_{2-} NPs (0.09) and TiO_{2-} NPs (0.20) μM concentrations density was significantly ($p < 0.05$) inhibited to 58%, 63%; chlorophyll *a* content to 87%, 69% and chlorophyll *b* content to 62%, 85% of the control respectively. The combined treatment of both chemicals indicated an inhibitory effect on the density, chlorophyll *a* and *b* contents to 79%, 83%, 78% and 77%; 69%, 66%, 81%, 88%, 51,%, 44%, 70%, 66% of the control after exposure to Pb (0.01) + TiO_2 NPs (0.09), Pb (0.01)+ TiO_2 NPs (0.20), Pb (0.04) + TiO_2 NPs (0.09), Pb (0.04) + TiO_2 NPs (0.20) μM respectively. However, the decrease observed in the biomass content was not concentration dependent. The comparison between the single and the combined chemicals showed no significant ($P > 0.05$) decrease. Furthermore, the interaction between both chemicals on the biomass (density, chlorophyll *a* and *b*) showed a combination index (CI >1) which exhibited a strong antagonistic effect on the biomass.

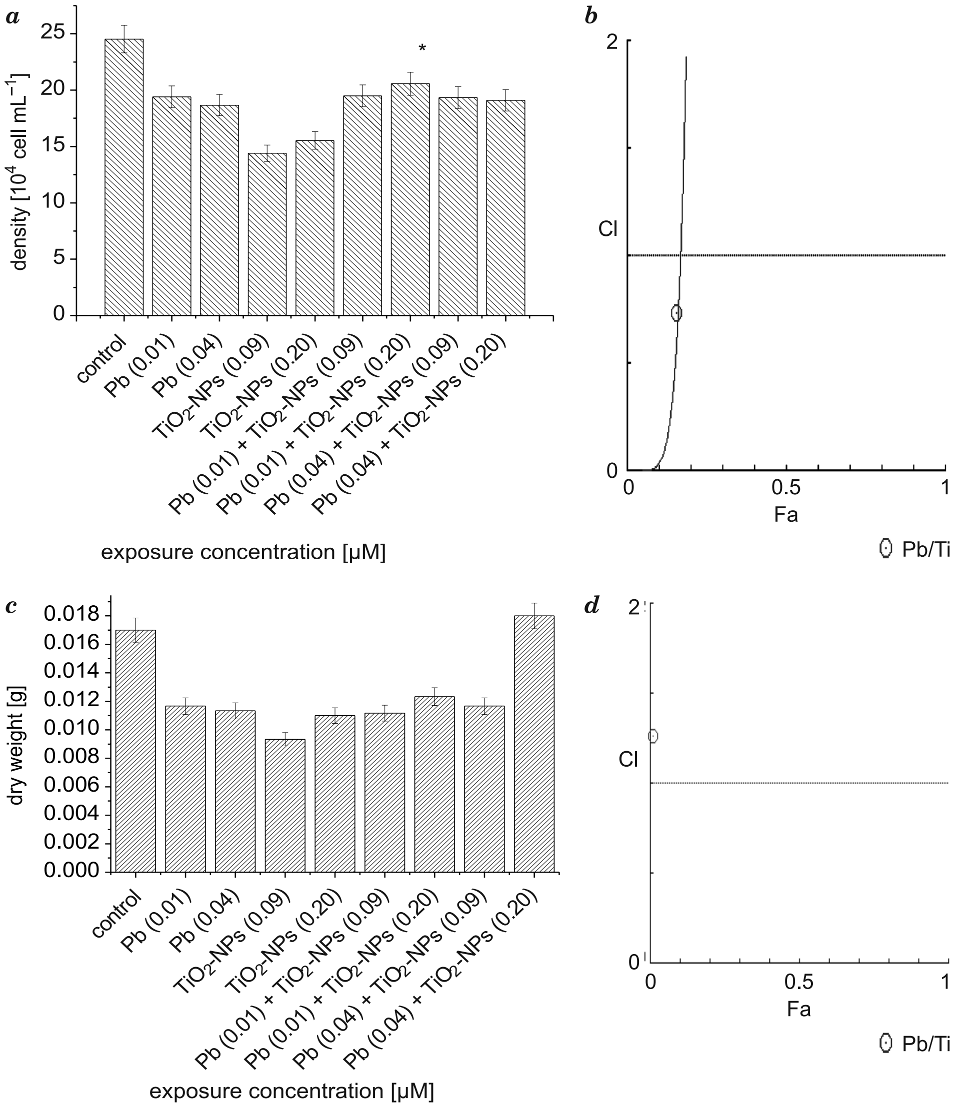


Fig. 3. *C. ellipsoideum* denisity and dry weight responses: *a* – cells density of *C. ellipsoideum* exposed to single and combined Pb and TiO_2 NPs [one-way ANOVA, F = 2.409, $p = 0.0029$; threshold replicate were included ($n = 3$); errors bars indicate; standard deviation (SE), *significant difference. Chi-square test ($p > 0.05$)]; *b* – combined effect of Pb and TiO_2 NPs (Pb/Ti) on cells density of *C. ellipsoideum* [combination index (CI); CI > 1]; c – dry weight of *C. ellipsoideum* exposed to single and combined Pb and TiO_2 NPs [one-way ANOVA, F = 0.476, $p = 0.866$; threshold replicate were included ($n = 3$); errors bars indicate Standard deviation (SE), *significant difference. Chi-square test ($p > 0.05$)]; *d* – combined effect of Pb and TiO_2 NPs (Pb/Ti) on cells dry weight of *C. ellipsoideum* [combination index (CI); CI > 1]

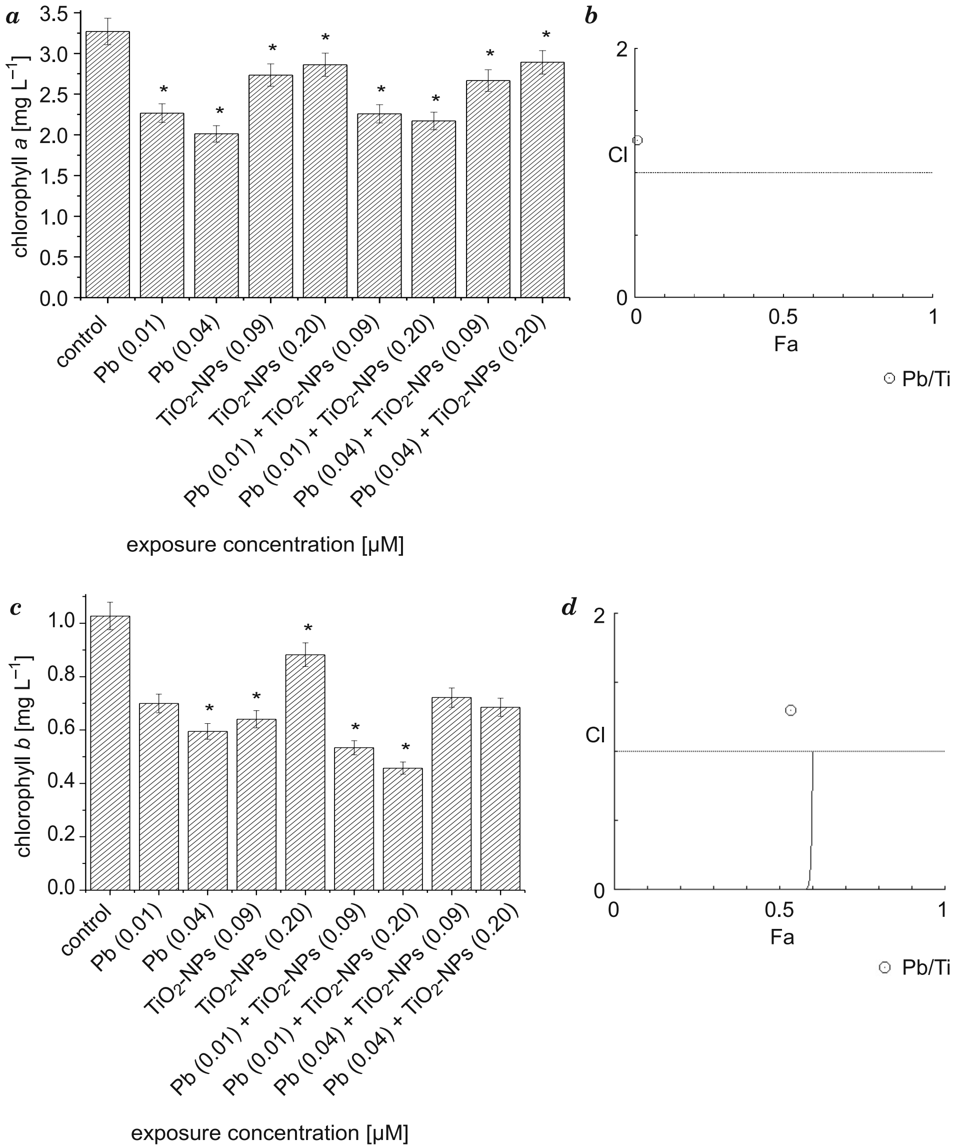
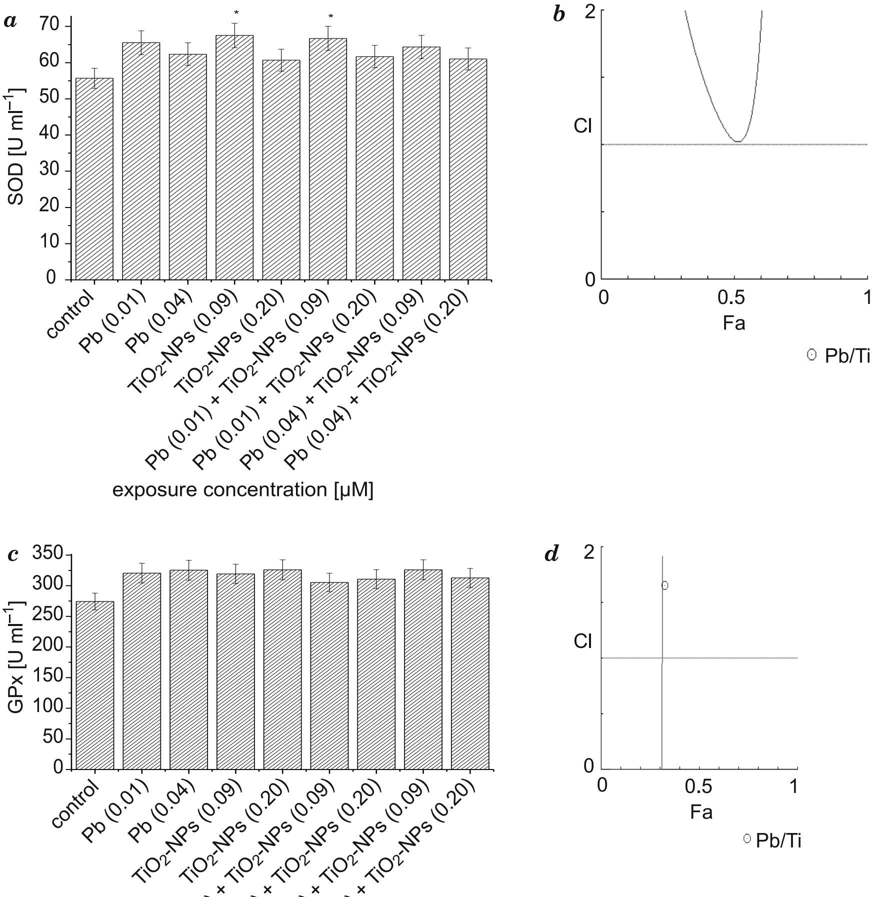


Fig. 4. Chlorophyll responses of *C. ellipsoideum*: *a* – chlorophyll a of *C. ellipsoideum* exposed to single and combined Pb and TiO_2 NPs; [one-way ANOVA, F = 6.505, p = 0.0001.73; threshold replicate were included (n = 3); errors bars indicate Standard deviation (SE), *significant difference; chi-square test (p > 0.05)]; *b* – combined effect of Pb and TiO_2 NPs (Pb/Ti) on cells chlorophyll a of *C. ellipsoideum* [combination index (CI); CI > 1]; *c* – chlorophyll b of *C. ellipsoideum* exposed to single and combined Pb and TiO_2 NPs [one-way ANOVA, F = 4.786, $p < 2.6610^{-4}$; threshold replicate were included (n = 3); errors bars indicate; standard deviation (SE), *significant difference; chi-square test (p > 0.05)]; *d* – combined effect of Pb and TiO_2 NPs (Pb/Ti) on cells chlorophyll *b* of *C. ellipsoideum* [combination index (CI); CI > 1]

Oxidative stress and antioxidant enzymes responses of *C. ellipsoideum*

The single and combined chemicals used in this study increased the antioxidant enzymes activities (SOD, GPx, GR and MDA) as shown in Figures 5*a*, 5*c*, 5*e*, and 5*g*. However, one way ANOVA showed that only SOD and MDA were increased significantly ($P < 0.05$) as compared to the control. The antioxidant enzyme activities were observed to increase irrespective of the concentration employed, indicating that responses to the activities were not concentration dependent. Chi-square showed that single and combined chemicals had no significant ($P > 0.05$) increase on antioxidant enzyme activities. Furthermore, the interaction between the



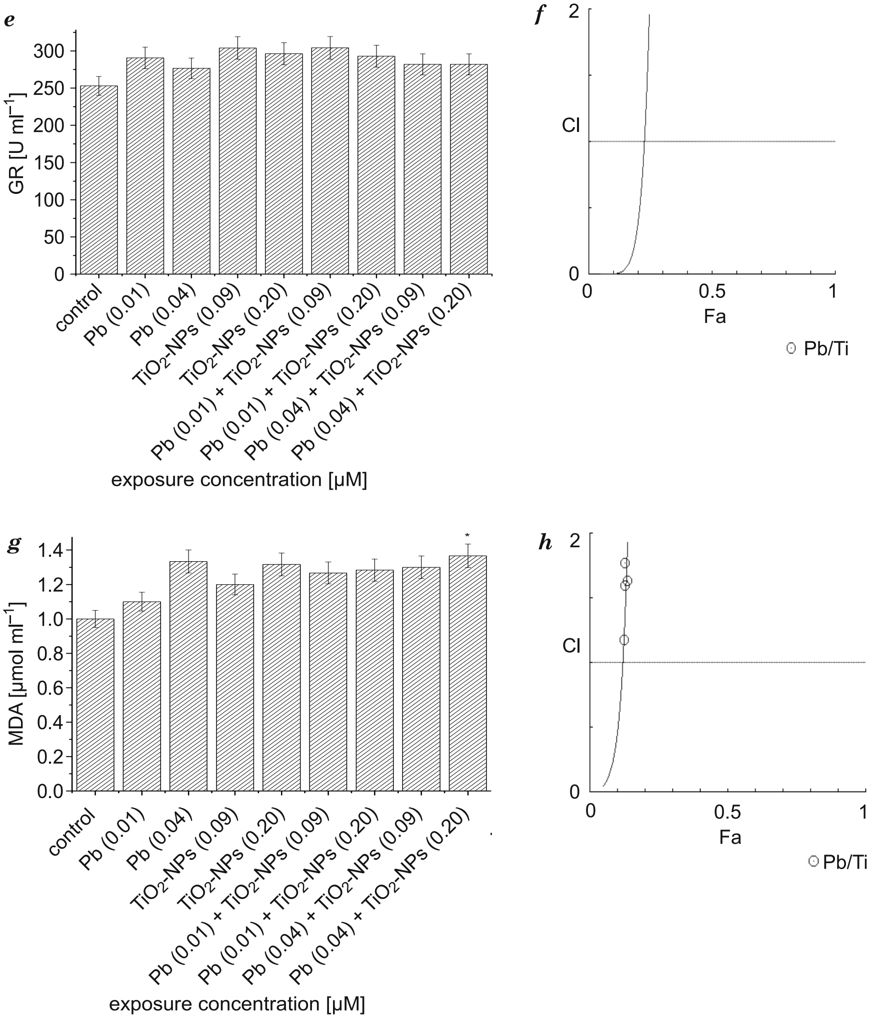


Fig. 5. Oxidative stress and antioxidant enzymes responses of *C. ellipsoideum*: *a* – SOD of *C. ellipsoideum* exposed to single and combined Pb and TiO_2 NPs [one-way ANOVA, F = 2.409, p = 0.029; threshold replicate were included (n = 3); errors bars indicate; standard deviation (SE), *significant difference; Chi-square test (p > 0.05)]; *b* – combined effect of Pb and TiO_2 NPs (Pb/Ti) on SOD of *C. ellipsoideum* [combination index (CI)]; *c* – GPx of *C. ellipsoideum* exposed to single and combined Pb and TiO_2 NPs [one-way ANOVA, F = 0.573, p = 0.793; threshold replicate were included (n = 3); errors bars indicate standard deviation (SE), *significant difference; chi-square test (p > 0.05); CI > 1]; *d* – combined effect of Pb and TiO_2 NPs (Pb/Ti) on GPx of *C. ellipsoideum* [combination index (CI); CI > 1]; *e* – GR of *C. ellipsoideum* exposed to single and combined Pb and TiO_2 NPs [one-way ANOVA, F = 0.816, p = 0.591; threshold replicate were included (n = 3); errors bars indicate standard deviation (SE), *significant difference; chi-square test (p > 0.05)]; *f* – combined effect of Pb and TiO_2 NPs (Pb/Ti) on GR of *C. ellipsoideum* [Combination index (CI); CI > 1]; *g* – MDA of *C. ellipsoideum* exposed to single and combined Pb and TiO_2 NPs [one-way ANOVA, F = 2.299, p = 0.0036; threshold replicate were included (n = 3); errors bars indicate Standard deviation (SE); *significant difference; chi-square test (p > 0.05)]; *h* – combined effect of Pb and TiO_2 NPs (Pb/Ti) on MDA of *C. ellipsoideum* [combination index (CI); CI > 1]

combined chemicals on antioxidant enzymes exhibited strong antagonistic activities with the combination index greater than one (CI > 1) shown in Figure 5*b*, 5*d*, 5*f* and 5*h*.

Discussion

The Langmuir model is a nonlinear sorption which suggests that uptake occurs on a homogeneous surface by monolayer adsorption without interaction between adsorbed molecules (DĄBROWSKI 2001). Based on the q_{max} and the Langmuir constant (b), it can be concluded that TiO_2 NPs highly adsorbed Pb(II) with higher affinity. This result also indicates the highest applicability of the model with the best coefficient $R^2 = 0.99$. The study indicated that the large surface area and pore volume generally known of nanoparticles resulted in higher and favourable adsorption capacity of titanium dioxide nanoparticles probably with consequences on the bioavailability of Pb(II). ZHANG et al. (2010) reported that titanium nanoparticles adsorption of Pb II through electrostactic interaction reduced the bioavailability of Pb II during the acute toxicity interaction of TiO_2 NPs and lead acetate exposed to mice.

Lead (Pb) and TiO_2 NPs either as individuals or in binary mixture decreased the cells density of *Chloroidium ellipsoideum*, indicating the inhibitory effect of these chemicals on the microalgae. The decrease of cells density due to Pb(II) indicated the ability of the chemicals to alter cells division. These results corroborated with the inhibitory effect of some other heavy metal (Cd, Pb) after two days exposure to *Chlorella vulgaris* as reported by BAJGUZ (2011). The decrease of cells density after treatment with TiO_2 NPs could be due to the coating ability of the metal to bind on algal cells and therefore, altering cells reproduction and their growth. A similar report by HARTMAN et al. (2009) which is in agreement with this study, demonstrated the inhibitory effect of alga exposed to TiO_2 NPs with the tendency of the nanomaterial to shade the incidence of light by coating on algal cells. The result revealed that the addition of TiO_2NPs to Pb(II) inhibit the growth of microalgae cells resulting in the reduction of cell density; however the reduction was antagonistic. This could be attributed to the adsorptive effect of TiO_2NPs in the mixture suggesting its ability to prevent the synergy of the combined compounds by hindering the Pb(II) bioavailability and thereby reducing its toxicity. Our result agreed with the findings of MIAO et al. (2012) who reported that combined $nTiO_2$ NPs and Cd (a heavy metal) had alleviating effect on alga growth.

Our result revealed a general decreased of dry weight when exposed to single and combined chemicals ($P > 0.05$). This implied that both compounds either single or mixed could lead to death and reduction of cells in the tested medium. This was in agreement with the findings obtained by SILVERBERG (1977) who reported a decrease of dry weight by 85% and 32% respectively compared to the control on freshwater green algae *Scenedesmus quadricauda* exposed to Tetramethyl lead.

The decrease in chlorophyll *a* and *b* observed in this study exposed to Pb and TiO_2 NPs demonstrated that these chemicals had prevented the photosynthetic activity indicating an absence of modulation of photosynthetic mechanism (PSII) as a result of damage of the thylakoid membranes of the chloroplast (KIRCHOFF 2014, NEELAM and RAI 2003). The interaction between Pb(II) and TiO_2 NPs concentration had an antagonistic effect on chlorophyll production. Titanium dioxide nanoparticles have the capacity to act as a carrier and entrapped nutrient that increased the growth and production of chlorophyll. The binary mixtures antagonistically decreased the chlorophyll content due to the alleviation of the inhibitory effect of Pb(II) by TiO_2 NPs. This study contradicts the result of TANG et al. (2013) who reported a synergistic response of nano-sized titanium dioxide and zinc on *Anabaena* sp.

Superoxide dismutase (SOD), GPx, GR and MDA are indicators of oxidative stress in many organisms including microalgae. Superoxide dismutase increased under the effects of individual Pb II and TiO_2 NPs respectively. This could be attributed to the absorption of these chemicals inside the cells of microalgae thereby causing the formation of reactive oxygen species (ROS). The increase of SOD activity was to quench the reactive oxygen species (ROS). Our results disagreed with that of Fan et al. (2016) who reported a decreased of SOD activity in *Scenedesmus obliquus* exposed to nanomaterials aluminium oxide ($AlO_{3)}$ and copper. The interaction of both compounds led to increase in SOD activity suggestive of the ability to protect against ROS. However, there was antagonistic response of combined compounds in binary mixtures. This result is also not in agreement with the findings of Fan et al. (2016) who reported decreased SOD activity in *Scenedesmus obliquus* exposed to nano-Al_2O_3 and copper.

Glutathione peroxidase and glutathione reductase increased in microalgae treated with single chemicals Pb (II) and TiO_2 NPs respectively, which could be due to the scavenging and inactivation of hydrogen and lipids peroxides thereby protecting the microalga against oxidative stress. Our results agreed with STOIBER et al. (2009) who reported increased GR level following exposure of *Chlamidomonas reinhardhtii* to cadmium. The combined compounds also increased GPx and GR activities in the exposed microalga. The implication is that Pb II and TiO_2 NPs in a mixture inhibit the release of GPx in the cells thereby reducing the protection of the cells against ROS. This is in disagreement with the result of FAN et al. (2016) who reported reduced GPx activity in *Scenedesmus obliquus* exposed to nano-Al_2O_3 and copper. However, there was increased GR response in the presence of Pb/TiO_2NPs indicating an increase in protection against oxidative stress similar to this study.

The increased MDA in all treatments compared to the control was an indication of lipid membrane damage and cell lysis. Our results is consistent with XIONG et al. (2013) who reported increased MDA level in *Chlorella vulgaris* exposed to a xenobiotic metals.

Conclusion

Based on the specific properties of TiO_2 NPs, it is more likely it adsorbs other non-essential metals in water. The study suggests that co-exposure of TiO_2 NPs and Pb(II) decreased the bioavailability of Pb(II). This study demonstrated an adsorptive interaction between TiO_2 NPs and Pb(II). Data from this study revealed that combined exposure of the two chemicals decreased the biomass of *Chloroidium ellipsoideum* and increased antioxidant activities with dosage. We also observed that the mode action of Pb(II) and TiO_2 NPs has an antagonistic effect on biomass and antioxidant activities. Further studies are required to understand the mechanical pathway of the interactions of this binary mixture.

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EFFECT OF THE EXTERNAL ELECTRIC FIELD ON THE STRUCTURE AND REACTIVITY OF DEOXYRIBONUCLEIC ACIDS

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Key words: adenine, cytosine, DNA, guanine, phosphorylated deoxyribose, thymine.

Abstract

The study of the effects of static external electric fields (EEF) on the structure of several biologically important compounds has been now extended to the structure of deoxyribonucleic acids and interactions via intermolecular hydrogen bonds in the thymine-adenine and cytosine-guanine pairs. The present study involves computations of changes in energy, and dipole moments, charge density and bond lengths of fragments of deoxyribonucleic acid in response to applied external electric fields of 0.00, 5.14, 25.70 and 51.40 $\cdot$ 10^6 MV cm^{-1}. The computations were performed with the help of a commercial package (HyperChem 8.0) software together with the PM3 method for optimization of the conformation of the molecules. The raise in the EEF strength to 5.14 $\cdot$ 10^6 MV cm^{-1} has a subtle effect on the molecular energy of the systems. On elevating the strength up to 25.70 $\cdot$ 10^6 MV cm^{-1} that decrease in molecular energy was more significant. EEF has a tremendous effect on their reorientation in the Cartesian system, geometry of deoxyribonucleic acid and the ability of particular bases within it to form intermolecular hydrogen bonds. Observed changes evoked by the EEF were specific for particular molecules. They resulted mainly from the polarization of the bonds and from steric deformations of the molecules. Based on the energy criterion, regardless of the EEF strength applied, the ACGT (adeniane-cytosine-guanine-thymine) fragment with *T1, C2, G2* and *A1* tautomers is more stable than that fragment bearing *T1, C1, G1* and *A1* tautomers. The EEF of the strength up to

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$51.40 \cdot 10^6$ MV cm^{-1} breaks neither *T-A* nor *C-G* intermolecular bonds but only influence their lengths. EEF, independent of its strength, only slightly influenced the charge density of the phosphoryl group of phosphorylated deoxyribose. A possible splitting of the bonds in that group was only slightly facilitated.

Introduction

All organisms are permanently exposed to static electric fields (SEF) in nature. Natural SEF is the highest at ground level (with electric field intensities of usually 100–150 V m^{-1}) and decreases with an increase in the distance from the ground level. Its strength depends upon temperature, humidity and the presence of ionized molecules (BERING, FEW and BENBROOKE 1998). In the environment transformed by humans, additional, artificial forms of SEF are quite common. Typically, in houses its strength does not exceed 500 V m^{-1}. However, around old types of TV sets and other high-voltage equipment it can raise up to 1 to 20 kV m^{-1}. Typical technical devices generating SEF are direct current (DC) transmission lines, cathode ray tube displays, electric trams and railways. High-voltage direct current (HVDC) transmission lines produce SEF up to the 35 kV m^{-1} (±600 kV HVDC transmission line) strength, DC motors in railway systems generate up to 0.3 kV m^{-1} inside the train, and between 10–20 kV m^{-1} at a distance of 30 cm from cathode ray tube displays. Surprisingly the strength up to 500 kV m^{-1} can be measured at the human body from static charge on clothing. SEF is well absorbed by water in the surface layers of organisms. Usually skin is efficient barrier.

Artificial SEF and electromagnetic fields are called electrosmog (BRAUNER 1996). In humans it generates problems with concentration, memory, sleeping, bad mood and fatigue. There are also links between electrosmog, Alzheimer disease and leukemia (MARSHALL and RUMANN HEIL 2017). Little is known on the influence of SEF on genetic material. Chromosomal abnormalities noted in tumor cells after a 14-day static SEF exposure (8–16 kV m^{-1}) in living mice are in contrast to common believe that SEF does not penetrate living systems and, hence, is not harmful (MARINO et al. 1974, MITCHELL et al. 1978, ARRUDA-NETO 2015, PETRI et al. 2017). Thus, a coincidence between SEF and skin benign lesions, cancer and melanoma should be taken under consideration. For this sake, artificially generated SEF stimulates a concern about its influence upon human organisms and generally on flora and fauna (NATIONAL RESEARCH COUNCIL (US),1993, VAN RONGEN et al. 2007, MARUVADA 2012,VIAN et al. 2016, PETRI et al. 2017).

In order to recognize the effect of the static external electric field (EEF) of varying strength upon the structure and reactivity of several biologically essential molecules, numerical simulations were performed. Such simulations were formerly performed for common simple molecules as water, nitrogen, carbon dioxide and ammonia (MAZURKIEWICZ and TOMASIK 2010), and biologically important molecules, that is, monosaccharides (MAZURKIEWICZ and TOMASIK 2012), alkanols (MAZURKIEWICZ and TOMASIK 2012a), porphin and metalloporhyrins (MAZURKIEWICZ and TOMASIK 2013), proteogenic amino acids (MAZURKIEWICZ and TOMASIK 2013a), selected di- (MAZURKIEWICZ and TOMASIK 2014) and tri-peptides (MAZURKIEWICZ et al. 2015), selected lipids, that is fatty acids and their glycerides (MAZURKIEWICZ et al. 2016) and complex lipids (MAZURKIEWICZ et al. 2017) and recently (MAZURKIEWICZ et al. 2018) to selected pyrimidine and purine bases. That research was stimulated by the role of electricity in the human life.

DNA is a highly charged molecule. Sudden defects of DNA like strand breaks generate SEF of quadrupole nature, and it lasting till the structure will be repaired. SEF can produce also nanochanges in DNA and/or RNA structure (JACQUEMIN et al. 2014). Recently performed computations (ARABI and MATTA 2018) showed that fields $\geq +3.60 \cdot 10^9$ V m^{-1} facilitate the mutation in the GC base pair and reduce the rectification of point mutations.

This paper presents numerical simulations performed for fragments of deoxyribonucleic acid and DNA in EEF of varying strength. In all our former studies computations were performed for EEF of 5.14, 25.70 and 51.40 MV cm^{-1}. In the latter case, in the dry air, the minimum spark gap reach over 50 m. Such drastic conditions can be followed near the lightning during a storm.

The computations were performed employing chiefly the PM3 method. However, a fragment of the presented data was obtained involving the DFT approach in order to demonstrate that both methods offer qualitatively the same tendencies in the influence of the EEF strength upon studied structures and their properties.

Computations

PM3 Computations

The HyperChem 8.0 software (HYPERCHEMRELEASE 8.0.7) was used together with the PM3 method (STEWART 1998) for optimization of the conformations of the molecules under study. Optimization was performed for molecules out of the field as well as in the presence of the field. Charge distributions, potentials and dipole moments for the molecules placed in EEF of 5.14, 25.70 and 51.40 MV cm^{-1} were calculated. The molecules were situated along the x-axis. The y- and z-axes were perpendicular in plane and perpendicular to plane containing this structure, respectively. Hence the simulation was performed for molecules oriented in space relative to the applied EEFs. EEF acted along the x-axis.

DFT Computations

The geometry optimizations were performed for the ground state using density-functional theory (DREIZLER and GROSS 1990) DFT method with Becke's three-parameter hybrid exchange functional (BECKE 1988, 1993) with Lee-Yang-Parr gradient-corrected correlation functional (LEE et al. 1988) B3LYP and basis set 6-31G. No constraints to bonds/ angles/dihedral angles were applied in the calculations. DGauss engine in the Cache (GAUSS 1985) and GAUSSIAN 98 (FRISCH et al. 1988) was employed.

Results and Discussion

As components of DNA and RNA, pyrimidine and purine bases play a key biological role. Either ribose in DNA or deoxyribose in RNA form a chain by 3 → 6' esterification with phosphoric acid. Resulting chain with either pyrimidine or purine bases subsequently bound to the 1-position of the sugar units (Figure 1) turns into a helix.

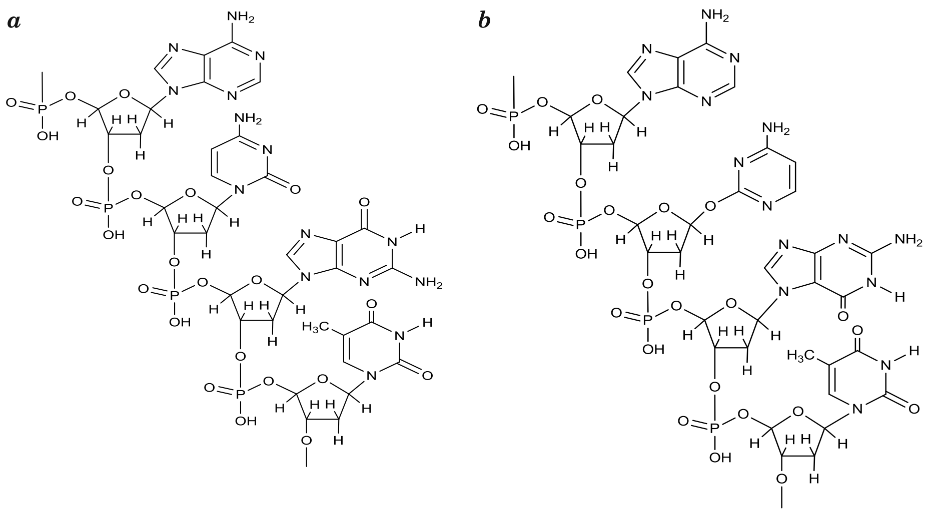

Fig. 1. A simplified fragment of the DNA chain (ACGT) with commonly accepted (a) and with those resulting from computations (b) dominating tautomers of cytosine and guanine

In the simplified structure of the *ACGT* fragment (Figure 1) commonly recognized dominating tautomers of cytosine, guanine and adenine are taken into account (Figure 1*a*). However, our recent numerical computations (MAZURKIEWICZ et al. 2018) performed for those bases revealed that when out of field, for cytosine (*a*) and guanine (*b*) dominate tautomers presented in Figure 2 . In EEF of the 5.14 MV cm^{-1} strength in cytosine also dominates tautomer (*a*) and in EEF of the 51.40 MV cm^{-1} in adenine the tautomer (*c*) dominates.

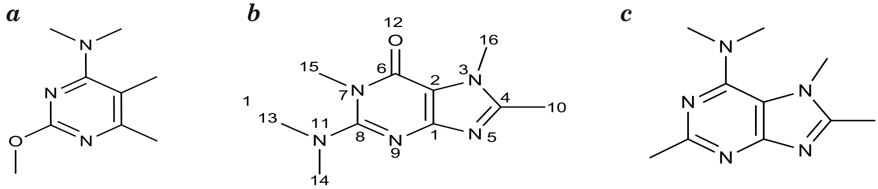

Fig. 2. Dominating tautomers of cytosine (*a*), guanine (*b*) and adenine (*c*)

These computations for the system bearing the *C1* and *G1* tautomers showed that its energy decreased in the EEF with an increase in its strength (Table 1). Simultaneously its total dipole moment considerably increased.

Table 1

Energy of the *ACGT* fragment of DNA out of EEF and in the EEF of increasing strength and changes in its dipole moment under such circumstances*

EEF strength [MV cm^{-1}]	Energy (E) [kcal mole^{-1}]	$E_I - E_0$	Dipole moment [D]			
			total	*x*-axis	*y*-axis	*z*-axis
0.00	-354361	0	18.00134	-17.25998	0.84573	-5.04242
	-357598**	0	7.49662	3.01923	5.87363	2.35492
5.14	-354369	-8	19.33981	-18.33981	-1.03399	-5.12429
	-357597**	-1	6.88824	2.39524	5.89156	2.64578
25.70	-354392	-31	28.38129	-27.92123	-1.99175	-4.68350
	-357653**	-55	19.07635	-15.92908	8.02015	6.77117
51.40	-354445	-84	47.34115	-47.93203	0.93418	0.04134
	-357680**	-82	28.69667	-26.28798	7.93017	8.33989

* If not denoted the results are for the *ACGT* fragment with *A1, C1, G1* and *T1* tautomers

** Data for the ACGT fragment carrying *A1,C2, G2* and *T1* tautomers

Simultaneously, the *C2* and *G2* tautomers incorporated into that ACGT fragment stabilized the system in terms of energy.

Dipole moment of the system bearing the *C1* and *G1* tautomers also increased with the EEF strength and regardless its value the orientation of the molecule along x-axis predominated, particularly on exposure to the EEF of 51.40 MV cm^{-1}. Replacing the *C1* and *G1* tautomers in the system with *C2* and *G2* tautomers, respectively, resulted a considerable decrease in the total dipole moment. In contrast to the former system, that modified prefers situating mainly along the y-axis with the preference for changing orientation in the space along the *x*-axis as the EEF strength increases.

Separate computations performed for a fragment composed of two phosphorylated deoxyriboses (Figure 3) showed how EEF depending on its strength influenced the phosphoric acid moiety linking two deoxyribose units. Table 2 reveals that EEF independently of its strength only slightly influenced the charge density of the phosphoryl group. A possible splitting of the bonds in that group under the influence of EEF was only slightly facilitated.

However, the considerable polarization of the O^3-H^4 bond should be emphasized.

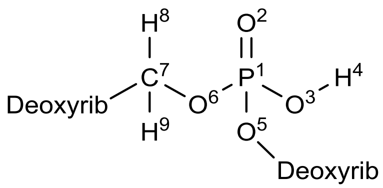

Fig. 3. Two phosphorylated deoxyriboses

Table 2

Charge density distribution in phosphorylated deoxyribose unit out of field and EEF of varying strength*

EEF strength [MV cm^{-1}]	Charge density at atoms*								
	1	2	3	4	5	6	7	8	9
0.00	2.187	-0.847	-0.630	0.245	-0.630	-0.630	0.115	0.018	0.045
5.14	2.187	-0.851	-0.628	0.246	-0.649	-0.637	0.114	0.018	0.042
25.70	2.186	-0.866	-0.627	0.251	-0.645	-0.633	0.111	0.024	0.030
51.40	2.184	-0.889	-0.628	0.263	-0.631	-0.631	0.112	0.014	0.028

* See Figure 4 for notation of particular atoms

In nature two separated helices of either DNA or RNA form a double helix stabilized by intermolecular hydrogen bonds between their *C* and *G*, as well as *T* and *A* moieties. In double helix, *T* is replaced by uracil, *U*. Computations were performed for model *C*1-*G*1 and *T*1-*A*1 as well as *C*2-*G*2 and *T*1-*A*2 pairs (Figure 4) in order to check how EEF could influence their hydrogen bonding. The results are given in Tables 3–5.

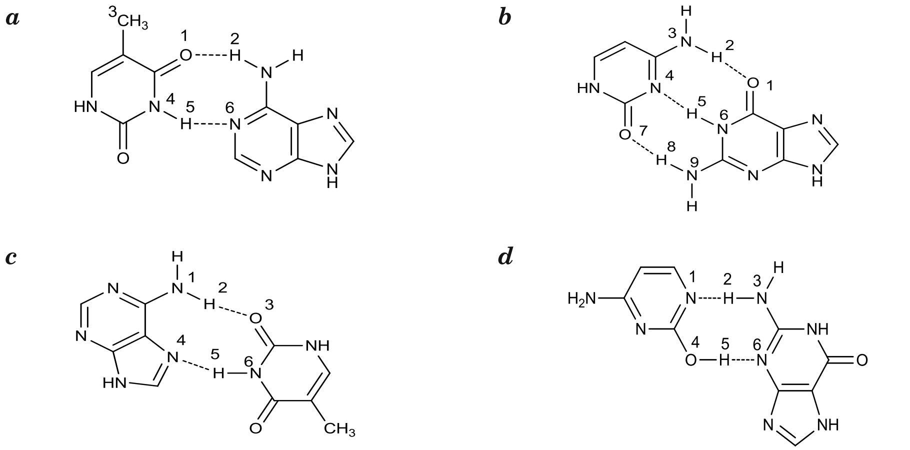

Fig. 4. Intermolecular hydrogen bonds in *T*1-*A*1 (*a*) and *C*1-*G*1 (*b*), *T*1-*A*2 (*c*) and *C*2-*G*2 (*d*) pairs

Table 3

Hydrogen bond lengths in the *T-A* and *C-G* pairs*

EEF [MV cm^{-1}]	Bonds [Å]								
	1-2	2-3	1-2 + 2-3	4-5	5-6	4-5 + 5-6	7-8	8-9	7-8 + 8-9
				T-A					
0.00	1.8209	1.0079	2.8288	1.0356	1.7806	2.8162	–	–	–
5.14	1.8151	1.0061	2.8212	1.0341	1.7871	2.8212	–	–	–
25.70	1.8136	1.0103	2.8239	1.0302	1.8146	2.8548	–	–	–
51.40 –	1.8473 *1.0082*	1.0100 *1.8064*	2.8573 *2.8146*	1.0324 *0.9937*	1.8351 *2.7478*	2.8675 *3.7415*	–	–	–
				C-G					
0.00	1.8643 **1.7342** *1.8090* ***1.8025***	0.9692 **1.0051** *1.0212* ***0.9965***	2.8335 **2.7393** *2.8302* ***2.7990***	1.0697 **1.0562** *0.9766* ***0.9995***	1.6347 **1.5215** *1.7836* ***1.6985***	2.7044 **2.5777** *2.7602* ***2.6980***	1.0462 **0.9922** – –	1.5551 **1.6028** – –	2.6093 **2.5950** – –
5.14	1.8597 *1.8052*	0.9697 *1.0211*	2.8294 *2.8263*	1.0675 *0.9766*	1.6404 *1.7860*	2.7079 *2.9626*	1.0375	1.5755	2.6130
25.70	1.8443 *1.7920*	0.9727 *1.0212*	2.8170 *2.8132*	1.0580 *0.9765*	1.6675 *1.7938*	2.7255 *2.7705*	1.0166	1.6323	2.6489
51.40	1.8254 *1.7196*	0.9773 *1.0359*	2.8027 *2.7555*	1.0476 *0.9695*	1.7033 *1.8515*	2.7509 *2.8210*	1.0012 –	1.6866 –	2.6378 –

* Upper values are for the *C*1-*G*1 and *T*1-*A*1 systems whereas the lower values in italics are for *C*2-*G*2 and *T*1-*A*2 systems, respectively, calculated involving PM3 method. Figures in bold are calculated involving the DFT approach

Table 4

Charge density distribution on the atoms directly involved in the intermolecular bonds formation in the *T*1-*A*1 and *T*1-*A*2 systems

EEF [MV cm^{-1}]	Atoms*					
	1	2	3	4	5	6
0.00	-0.406	0.119	0.119	-0.073	0.197	-0.263
5.14	-0.408	0.123	0.128	-0.073	0.194	-0.264
25.70	-0.415	0.127	0.108	-0.078	0.182	-0.256
51.40**	-0.414 *-0.144*	0.102 *0.127*	0.052 *-0.510*	-0.094 *0.345*	0.183 0.098	-0.274 *0.079*

* See Figure 5*a* for the atoms notation for the *T*1-*A*1 system and Figure 5*c* for the *T*1-*A*2 system
** Lower cases in italics relate to the charge density distribution in the *T*1-*A*2 system

Table 5

Charge density distribution on the atoms directly involved in the intermolecular bonds formation in the *C*1-*G*1 and *C*2-*G*2 systems*

EEF [MV cm^{-1}]	Atoms**								
	1	2	3	4	5	6	7	8	9
0.00	-0.126 *-0.280*	0.239 *0.143*	-0.241 *0.017*	0.040 *-0.223*	0.237 *0.272*	-0.357 *-0.172*	-0.311	0.322	-0.352
5.14	-0.130 *-0.279*	0.241 *0.145*	-0.243 *0.025*	0.044 *-0.227*	0.235 *0.272*	-0.357 *-0.180*	-0.304	0.322	-0.350
25.70	-0.150 *-0.276*	0.251 *0.152*	-0.250 *0.057*	0.055 *-0.243*	0.227 *0.071*	-0.356 *-0.213*	-0.286	0.319	-0.333
51.40**	-0.181 *-0.318*	0.265 *0.191*	-0.260 *0.118*	0.062 *-0.248*	0.215 *0.247*	-0.340 *-0.226*	-0.267	0.311	-0.296

* See Figure 5*b* for the atoms notation in the *C*1-*G*1 system and Figure 5*c* in the *C*2-*G*2 system
** Lower cases in italics relate to the charge density distribution in the *C*2-*G*2 system

*T*1 with A1 form the pair involving two hydrogen bonds (Figure 4*a*). EEF of 5.14 MV cm^{-1} decreased the length of the C = O….H-NH (1-2) bond and, simultaneously, increased the length of the N-H…N(aza) (5-6) bond. These changes were accompanied by shortening the length of the both H-N valence (2-3 and 4-5) bonds to a such extent that total distance between two bases was reduced. The original planar structure of the pair of those bases was retained. The application of the EEF of 25.70 MV cm^{-1} still reduced the length of the 1-2 bond but increased the length of the 2-3 bond. The bonds 4-5 and 5-6 shortened and extended, respectively. Insight in the three dimensional structure of that pair revealed that it was bent to form a structure of a shallow saddle (Figure 5a). At EEF of 51.40 MV cm^{-1} only the 2-3 bond slightly shortened and the other bonds considerably expanded as the deformation of the saddle structure progressed (Figure 5*b*).

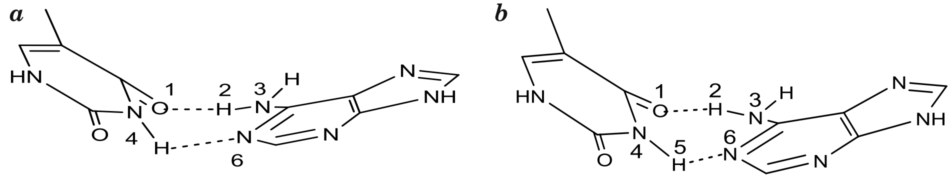

Fig. 5. The saddle structure of the *T*1-*A*1 pair in the EEF of the 25.70 MV cm^{-1} (*a*) and 51.40 MV cm^{-1} (*b*) strength

As shown in our recent paper (MAZURKIEWICZ et al. 2018), in the EEF field of the 51.40 MV cm^{-1} strength the *A*1 tautomer lost its dominating role in favor of the *A*2 tautomer. Computations performed for the *T*1-*A*2 pair (Table 3) revealed a considerable decrease in the 1-2 and 4-5 bond length and, simultaneously, elongation of the 2-3 and 5-6 bonds to a such extent that the sum of the lengths of the 1-2 and 2-3 bonds was to that computed for the *T*1-*A*1 pair and the sum of the 4–5 and 5-6 bond lengths was significantly higher. In contrast to the behavior of *T*1-*A*1 pair in the EEF of that strength, the *T*1-*A*2 pair although, reoriented in the Cartesian system, remained planar.

The formation of the *C*1-*G*1 pair involved three hydrogen bonds (Figure 4*b*). The EEF strength increase was followed by elongation of the = N-H….O = C (2-3) and shortening of both the N(aza)… H-N (4-5) and C = O….H-NH (7-8) hydrogen bonds. Simultaneously, the HN-H (1-2), N(aza)-H (5-6) and HN-H (8-9) valence bonds shortened, expanded and expanded, respectively. In that manner the EEF caused shortening of the 1–3 distance and elongation of the 4–6 and 7–9 distances. Independently of the EEF strength the *C*1-*G*1 pair remained planar.

The replacing of the *C*1 and *G*1 tautomers in the *ACGT* system with the *C*2 and *G*2 tautomers resulted, first of all, in reduction of the intermolecular hydrogen bonds from 3 to two (Figure 5). Compared to the ACGT fragment with the *C*1 and *G*1 tautomers there was a shortening of the 1-2 and 4-5 bonds and elongation of the 2-3 and 5-6 bonds, respectively. The sum of the 1-2 + 2-3 bonds was shorter, whereas the sum of the 4-5 + 5-6 bonds was longer.

Insight in Table 4 revealed that in the *T*1-*A*1 pair the EEF increasing its strength increased slightly the negative charge density on the carbonyl oxygen atom of thymine as well as on the aza atom of the pyrimidine part of adenine. The charge density on both hydrogen atoms increased with the strength of EEF but these changes varied on a chimeric manner because of the deformation of the structure from planar towards a saddle-like (Figure 5) and accompanying changes in the bond lengths (Table 3). Such deformation caused a twist of the exocyclic amino group of the adenine moiety partly cancelling the resonance interaction with the π-orbital of adenine. It explained a relatively large decrease in the positive charge density on the nitrogen atom of that group. In the *T*1-*A*2 system in the EEF of the 51.40 MV cm^{-1} strength, the negative charge density shifted from the aza atom 6 to the exocyclic amino group nitrogen atom 3.

Inspection of data in Table 5 similarly revealed that an increase in the EEF strength increased the ability of the carbonyl oxygen atoms of *C*1 and *G*1 favoring the hydrogen bond formation irregularly influenced the positive charge density of the hydrogen atoms participating in the formation of those hydrogen bonds.

In the *C*1-*G*1 system, the negative charge density on the carbonyl oxygen atom (O7) in the *C*1 moiety decreased with the EEF strength whereas, simultaneously, very small positive charge density on the *C*1 aza-atom N4 slightly increased. The nitrogen atom of the exocyclic amino group of *C*1 also carried considerable negative charge density which increased with the EEF strength. The latter effect was responsible for an increase in the positive charge density of the hydrogen atom participating in the hydrogen bonding to the G1 carbonyl group oxygen atom (O3) of *G*1. The aza-atom, N6, and the nitrogen atom of the exocyclic amino group (N9) of *G*1 carried considerable negative charge density, both decreasing as the EEF strength increased. Simultaneously, decreased the positive charge density on the hydrogen bonds forming hydrogen atoms, H5 and H8.

In the *C*2-*G*2 system the negative charge density on the N1 atom was much higher and simultaneously, the negative charge density on the N6 atom turned lower than that in the *C*1-*G*1 system. The charge density on the O3 atom turned from negative in the *C*1-*G*1 system into positive and the positive charge density on the N4 atom in the latter system turned into considerably negative and increasing with the EEF strength.

Conclusions

Observed changes evoked by the EEF are specific for particular molecules. They results mainly from the polarization of the bonds and from steric deformations of the molecules. Based on the energy criterion regardless the EEF strength applied, the ACGT fragment with *T*1, *C*2, *G*2 and *A*1 tautomers is more stable than that fragment bearing *T*1, *C*1, *G*1 and *A*1 tautomers. That fragment with the *T*1, *C*2, *G*2 and *A*1 bases is less polar than that commonly considered correct fragment with *T*1, *C*1, *G*1 and *A*1 tautomers. The EEF of the strength up to 51.40 MV cm^{-1} breaks neither T-A nor C-G intermolecular bonds but only influences their lengths.

Solely, in case of *T*1-*A*1 pair the 25.70 and 51.40 MV cm^{-1} EEF deteriorates the planarity of the system. EEF independently of its strength only slightly influences the charge density of the phosphoryl group of phosphorylated deoxyribose. A possible splitting of the bonds in that group is only slightly facilitated.

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EVALUATION OF THE ROSE HIPS OF *ROSA CANINA* L. AND *ROSA RUGOSA* THUNB. AS A VALUABLE SOURCE OF BIOLOGICAL ACTIVE COMPOUNDS AND ANTIOXIDANTS ON THE BALTIC SEA COAST

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Abstract

The work involved the study of the contents of ascorbic acid, carotenoids, total flavonoids, total tannins, total phenolics, and antioxidant properties of rose hips of two species: *Rosa canina* L. and *Rosa rugosa* Thunb. collected from wild bushes growing on the Curonian Spit, Sambia peninsula, and the Vistula Spit (Kaliningrad region, Russia). Species-specific accumulation of ascorbic acid, carotenoids, flavonoids, phenolics, and total antioxidants was shown. The content of the above components was significantly higher in hips of *Rosa rugosa* than in rose hips of *Rosa canina*. With the exception of the total content total water-soluble antioxidants and flavonoids, the content of biologically active compounds was higher in fully ripe fruits compared to unripe and half-ripe fruits. The accumulation of studied phytonutrients depended also on growth locations. In this study was shown high nutritional value of rose hips, especially of the species *Rosa rugosa* Thunb.

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Introduction

Finding new natural sources of antioxidants remains an important issue, as this will not only enable to enhance the food quality but also to generally improve people's life, wellbeing, and health. The impaired natural balance of the free radical oxidation rate and the antioxidant protection activity of human body that may be caused by the adverse conditions (environmental pollution, chronic emotional stress, high content of fast digesting carbohydrates and fats in the diet with simultaneously lowered amount of bio-antioxidants) is the important factor in pathogenesis of many diseases, including cardiovascular disorders, cancer, neurodegenerative and endocrine diseases (HALLIWELL 2012, FIEDOR and BURDA 2014, CIRILLO et al. 2016).

In the endeavours to find antioxidant producing plants, of particular interest are the fruits of rose plants (*Rosa* sp.), which have been long used as raw material for medications, vitamins, and food. Rose hips have been discovered to be rich in polyphenols (mainly flavonoids including proanthocyanidins and catechins), triterpene acids, essential fatty acids, galactolipid, folate, vitamin A, C and E, mineral (Ca, Mg, K, S, Si, Se, Mn and Fe), among other bioactive components (PATEL 2017). They can be used not only for direct consumption but also for the extraction of active ingredients that can be used as food additives (JIMÉNEZ et al. 2017). Rose hips are usually harvested in late summer, early autumn or in autumn. The content of biologically active compounds is proved to depend on a number of factors, i.e. species specificity (KAZAK et al. 2009, CZYŻOWSKA et al. 2015), ripening stage (NOJAVAN et al. 2008, ADAMCZAK et al. 2012, ELMASTAŞ et al. 2017), environmental growing conditions (CHUPAKHINA et al. 2014), etc.

In phytomedicine, the most common species is *Rosa canina* L. Medicinal properties are found in flowers, hips, leaves and roots of the plant. Wild rose is used for making decoctions, juice, and jam. The plant contains vitamin C known for its immunostimulating activity, as well as A, K, E, P, and many valuable substances (BARROS et al. 2011, ŽIVKOVIĆ et al. 2015). It is also known for its ability to lower blood pressure and improve human body's resistance to colds and infections (İLBAY et al. 2013, FAN et al. 2014).

However, there has been recently a recurring discussion in various sources about curative and nutritive properties of *Rosa rugosa* Thunb. (ALTINER and KILIÇGÜN 2008, OLECH et al. 2012, DUDRA et al. 2016). In the areas on the Baltic Sea coast it is an invasive species (BRUUN 2005). Massive introduction of *Rosa rugosa* on the Baltic coast was mainly performed as part of the shore protection. Planting of these shrubs in the Curonian Spit and Vistula Spit in the Kaliningrad region, for example, helped in the stabilization of sand dunes. Yet, in many European countries there have been recently a number of debates over the insistent need to control *Rosa rugosa*, being considered an active invasive species. Researchers in the Baltic countries are concerned that the plant is rapidly spreading and, establishing a dense canopy, changes natural (habitual) landscape (due to the change in phytocoenosis) (ISERMANN 2008, PROVOOST et al. 2011, DOODY 2012). So in Northwest Europe, *Rosa rugosa* forms dominant, large, and dense scrub that excludes, at a local scale, native species (ZHANG et al. 2018). And yet, before any final decision can be taken, numerous valuable qualities of *Rosa rugosa* should be thoroughly weighed, such as its curative value, vitamin content and nutritional value.

With this in view, the aim of this research was to study antioxidant properties of rose hips of two species (*Rosa canina* L. and *Rosa rugosa* Thunb.) collected from different locations at the Baltic Sea coast (in Kaliningrad region) in depending on ripening stage.

Materials and Methods

Chemicals

Trolox, quercetin, gallic acid, 2,6-dichloroindophenol, metaphosphoric acid were purchased from Acros Organics (New Jersey, USA). L-ascorbic acid, Folin-Ciocalteu reagent, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,4,6-tripyridyls-triazine (TPTZ) were purchased from Sigma (St. Louis, MO, USA). All other reagents and solvents were analytical grade from Vecton (Russia).

Plant material

The rose hips (pseudofruits) of *Rosa canina* L. and *Rosa rugosa* Thunb. were collected from beginning of August to the end of September 2016 from wild bushes growing in three different locations on the Baltic Sea coast in Kaliningrad region (Russia). The places of collection are represented on the map (Figure 1). The rose hips were collected at three ripening stages: 1) unripe (green and hard), according BBCH scale stage 79 709 Fruits have reached 90% of final size, 2) half-ripe (orange and hard) according BBCH scale stage 85 805 Progression of cultivar-/species-specific fruit colouring: increasing intensity of colour, and 3) fully ripe (red and soft), according BBCH scale stage 88 808 Full ripeness: cultivar-/species-specific fruit colouring and seed ripeness (Meier et. 2009). The fruits were collected from six bushes in each location. From each bush were taken five rose hips that were combined to one sample.

Fig. 1. The map showing three places in Kaliningrad region (Russia) where rose hips (pseudofruits) of *Rosa canina* L. and *Rosa rugosa* Thunb were collected. Point *A*: Location 1 – Curonian Spit; Point *B*: Location 2 – Sambia peninsula (the fruits were collected on the north part of peninsula, close to village Roshchino); Point *C*: Location 3 – Vistula Spit

Rose hips without calyxes were washed several times with water, and the seeds of the pseudofruits were cleaned. Approximately 20–50 g of fruits samples from each bush was lyophilized (FreeZone Triad, Labconco, USA) and stored at –20°C until analysis (Liebherr, Germany). The vitamin C content was measured in fresh fruits directly on the day of collection. All other analyzes was performed in lyophilized samples.

Ascorbic acid

Ascorbic acid was determined as described by Barros et al. (2010). Plant extract preparation: 0.15–0.20 g fresh plant material was homogenized with 10 mL of 1% metaphosphoric acid and centrifuged at 4500 rpm for 15 min at 4°C. The supernatant (1 mL) was mixed with 2,6-dichloroindophenol (9 mL) and the absorbance was measured within 30 min at 515 nm against a blank. Content of ascorbic acid was calculated on the basis of the calibration curve of L-ascorbic acid, and the results were expressed as mg of ascorbic acid per g of dry weight.

Carotenoids

Accurately weighted 0.5 g lyophilized plant sample was taken, and homogenized in tissue homogenizer (Ultra-Turrax Tube Drive, IKA, Germany) with 10 mL of 80% acetone. Homoginized sample mixture was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant were separated and 0.5 mL of it is mixed with 4.5 mL of the solvent. The optical density of the above mixture was determined at 470 nm, 646.8 nm and 663.2 nm (UV-3600, Shimadzu, Japan). The concentration of carotenoids was calculated according to (SUMANTA et al. 2014). Finally the content of carotenoids in plant was converted to mg per gram dry weight.

Total flavonoids content (TFC)

The total flavonoids content was measured by the $AlCl_3$ colorimetric assay according described by SEVKET et al. (2016). An aliquote (0.1 mL) of methanolic plant extracts was added to 10 mL volumetric tube containing 4 mL dH_2O. Then, 0.3 mL 5% $NaNO_2$ solution was added. After 5 minutes 0.3 mL 10% $AlCl_3$ solution was poured into the flask and maintained for another 6 minutes, after which 2 mL 1 M NaOH solution was added. The total volume was completed up to 10 mL with dH_2O. The solution was mixed and the absorption was measured at 510 nm (UV-3600, Shimadzu, Japan). The total flavonoids content was calculated using a calibration curve, and then expressed as mg quercetin equivalent (QE) per gram dry weight.

Total tannins content (TTC)

The total tannins content was determined by the Prussian Blue method with some modifications (GUPTA and VERMA 2011). Plant extract preparation: 0.2–0.5 g lyophilized plant material was homogenized with 10 mL of extraction solvent (1% HCl ethanol solution). The extracts were centrifuged for 30 min at 4500 rpm. Supernatants were collected and stored at 5–8°C in dark until analysed. 250 µl of extracts was taken. It was diluted within 25 mL of distilled water and added 3 mL of 0.5 M $FeCl_3$ in 0.1 N HCl and 3 mL of 0.008 M $K_3Fe(CN)_6$. Colour develops immediately after 10–15 min. The optical density of the above solution was determined at 720 nm (UV-3600, Shimadzu, Japan). The calibration curve was made by preparing gallic acid solutions at different concentrations in ethanol. The total tannins contents are expressed in mg of as gallic acid equivalent (GAE) per gram dry weight.

Total phenolic compounds content (TPC)

Phenolic compounds were extracted from lyophilized fruits with 96% ethanol solution and determined by Folin-Ciocalteu method (Padhi et al. 2017). Briefly, 100 µL of gallic acid standard or plant extract was mixed with 300 µL 0.2 M Folin-Ciocalteu reagent in a tube, and incubated for 10 min at room temperature in darkness. Next, 6 mL of 6.75% sodium carbonate (Na_2CO_3) solution was added to each tube, and the tubes were incubated for 30 min at room temperature in darkness. The optical density of the above solution was determined at 765 nm (UV-3600, Shimadzu, Japan). TPC was expressed as mg gallic acid equivalent per gram dry weight.

Total water-soluble antioxidants content (TWAC)

The total water-soluble antioxidants content have been estimated by an amperometric method using a TsvetYauza-01-AA (NPO Khimavtomatika Inc., Moscow, Russia) according to Yashin (Yashin 2008). Plant extract preparation: 0.2–0.5 g of lyophilized plant material was homogenized with 50 mL of eluent (solution of phosphoric acid with the molar concentration of 2.2 mM). The mixture was then filtered and used for analyse in day of preparation. The calibration curve was made by preparing quercetin solutions at different concentrations. The total antioxidant contents are expressed in mg of as quercetin equivalent (QE) per gram dry weight.

Total antioxidant capacity (TAC)

The total antioxidant capacity was measured using DPPH (1,1-diphenyl-2-picrylhydrazyl) radical, $ABTS^{\cdot+}$ (2,2'-azino-bis(3-ethylbenzothiazoline-6- sulfonic acid) radical and FRAP (ferric reducing antioxidant power) assays. Plant extract preparation: 0.1–0.2 g lyophilized plant material was homogenized with 10 mL of 96% ethanol solution.

Each extract of rose hip fruits was mixed with 2.85 mL freshly prepared 0.1 mM solution of DPPH in ethanol. The sample was incubated for 30 min at room temperature in darkness. The reduction of absorbance at 515 nm (UV-3600, Shimadzu, Japan) was measured spectrophotometrically (Tõnutare 2015).

ABTS and FRAP assays was performed as described by Taneva et al. (2016). ABTS radical was generated by mixing aliquot parts of water solution of 7.0 mM (ABTS) and 2.45 mM potassium persulfate. For the assay, 2.85 mL of this $ABTS^{\cdot+}$ solution was mixed with 0.15 mL of obtained extracts. After 15 min at 37°C in darkness the absorbance was measured at 734 nm (UV-3600, Shimadzu, Japan) against ethanol.

The FRAP reagent was freshly prepared by mixing 10 parts 0.3 M acetate buffer (pH 3.6), 1 part 10 mM 2,4,6- tripyridyls-triazine (TPTZ) in 40 mM HCl and 1 part 20 mM $FeCl_3 \cdot 6H_2O$ in dH_2O. The reaction was started by mixing 3.0 mL FRAP reagent with 0.1 mL of investigated extract. The reaction time was 10 min at 37°C in darkness and the absorbance was measured at 593 nm (UV-3600, Shimadzu, Japan) against blank prepared with ethanol.

All results from the determination of antioxidant capacity were expressed as µmol Trolox equivalents (µmol TE) per gram dry weight.

Statistical analysis

All analyses were performed in five repetitions. The results of analyses were reported as mean ± standard deviation (SD). Three-way analysis (ANOVA) was performed using the SigmaPlot 12.3 (Systat Software GmbH, Erkrath, Germany). Before ANOVA data were checked for normality and the homogeneity of variance. Statistical comparisons were performed using the Tukey's multiple comparison test. Differences were considered significant at $p \leq 0.05$. A correlation analysis based on Pearson's chi-squared test was conducted. Similarity in the content of biological active compounds and antioxidants due to species, ripening stages and growth locations was evaluated by hierarchical cluster analysis by Ward's method in Stata v.13, using the Euclidean distance as a similarity measure. The reliability of clusters was evaluated by bootstrapping with 1000 replicates in PAST v. 3.17.

Results

Ascorbic acid

This study proved species-specific ascorbic acid accumulation. Vitamin C content was evidently higher in *Rosa rugosa* fruits ($p \leq 0.05$), which contained Vitamin C at over 40 mg g^{-1} dry weight (Figure 2 , Table appx. 1). The content of ascorbic acid in rose hips depended also on the stage of their ripening. The maximum level was determined in the fruits collected at the stage of full ripeness. At the same time, the unripe and half-ripe fruits of *Rosa canina* were characterized by almost the same level of vitamin C, while its content in the unripe fruits of *Rosa rugosa* was significantly lower compared to fully ripe and half-ripe fruits (Figure 2). The results of the

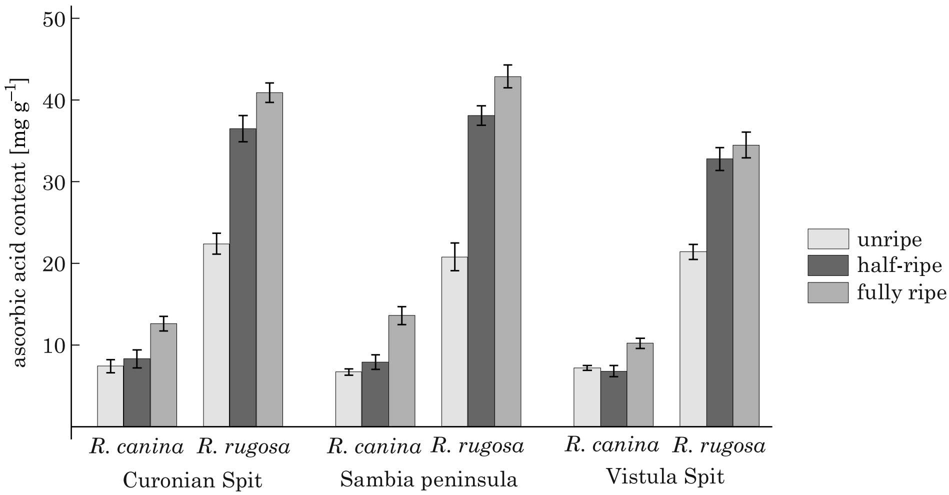

Fig. 2. Ascorbic acid content in fruits of *Rosa canina* and *Rosa rugosa* collected at different ripening stages and from three growth locations

evaluation via 3-factorial ANOVA (Table appx. 1) showed that growth location has a significant effect on the ascorbic acid content in rose hips. A lower content of ascorbic acid was found in the fruits collected on the Vistula Spit. Significant differences between the fruits collected in two other places were not identified.

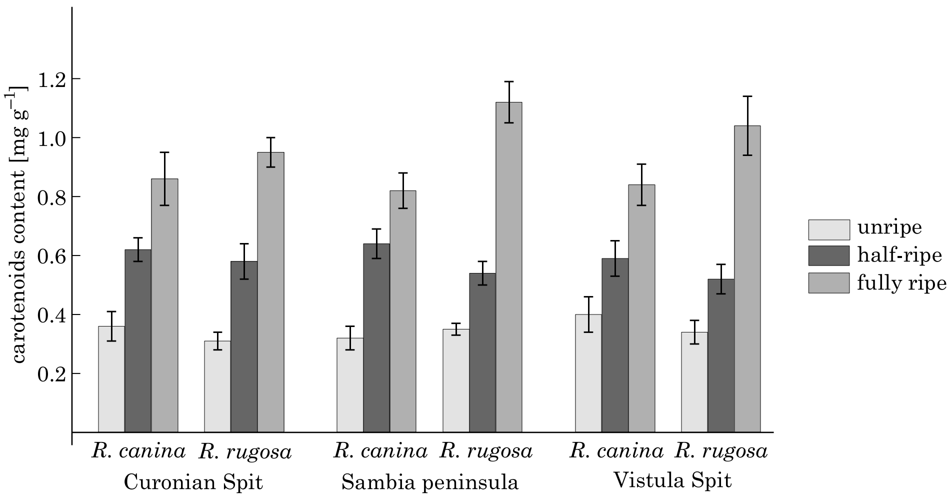

Fig. 3. Carotenoids content in fruits of *Rosa canina* and *Rosa rugosa* collected at different ripening stages and from three growth locations

Carotenoids

As shown in Figure 3 , the fruits of *Rosa canina* and *Rosa rugosa* had nearly the same content of carotenoids (0.61–0.64 mg g^{-1}). However, a statistically significantly higher content of carotenoids was established in the fruits of *Rosa rugosa* (Table appx. 1). The stage of ripeness was the decisive factor that determined the level of carotenoids in rose hips of both species. The level of carotenoids in fruits increased up to three times during their ripening. The growth location had no significant effect on the content of carotenoids.

Phenolic compounds

The total flavonoids content in fruits of *Rosa rugosa* were 1.5–1.7 times higher than in the fruit of *Rosa canina* (Figure 4). The ripening stage had significant influence on flavonoids content in rose hips, but the correlation between the levels of flavonoids and mature of fruits was not established. The maximal content was in half-ripe fruits, and the unripe and fully ripe fruits did not differ significant. The content of flavonoids in rose hips collected from various locations was different. The higher level was in fruits from Curonian Spit.

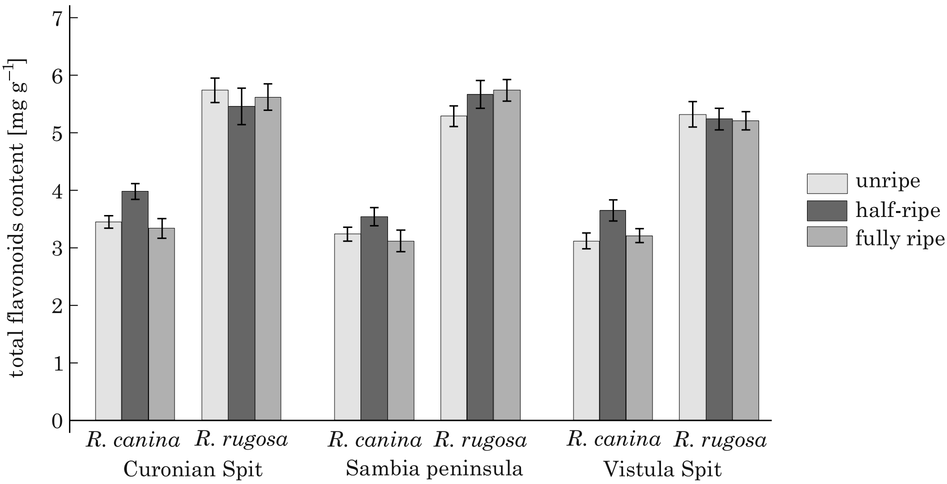

Fig. 4. Total flavonoids content in fruits of *Rosa canina* and *Rosa rugosa* collected at different ripening stages and from three growth locations

The total tannins content in the fruit of *Rosa canina* was significantly higher than in the fruit of *Rosa rugosa* (Figure 5). On average, the difference was about 37% (3.23 and 2.03 mg g^{-1} for *Rosa canina* and *Rosa rugosa* respectively, Table appx. 1). In the process of ripening, total tannins content increased and reached a maximum in ripe fruits. According to the results of 3-factorial ANOVA the location has also significant effect on tannins accumulation in rose hips (Table appx. 1). The higher levels were in fruits from Curonian Spit and Sambia peninsula.

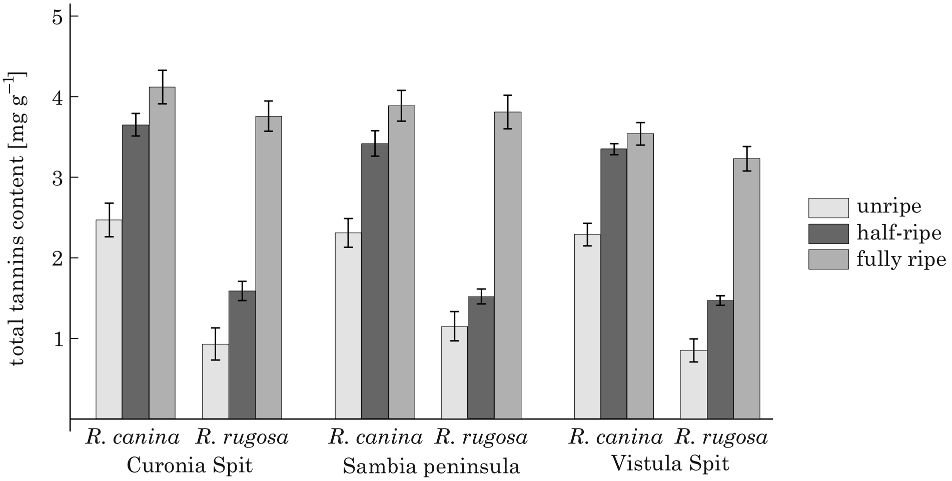

Fig. 5. Total tannins content in fruits of *Rosa canina* and *Rosa rugosa* collected at different ripening stages and from three growth locations

The fruits of *Rosa rugosa* characterized also by higher total phenolic compounds content, especially ripe fruits. For half-ripe and unripe fruits the difference between species was not so great (Figure 6). However, for both species the total phenolics content in rose hips increased during their

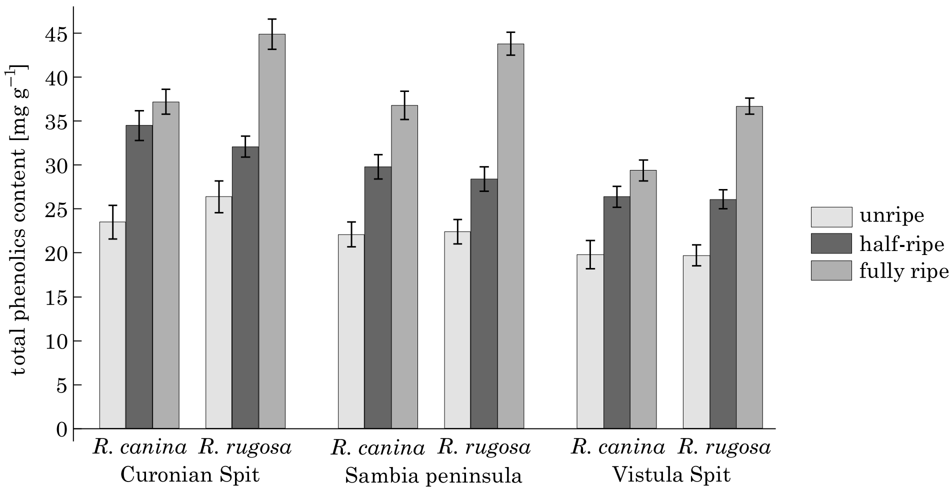

Fig. 6. Total phenolics content in fruits of *Rosa canina* and *Rosa rugosa* collected at different ripening stages and from three growth locations

ripening. The polyphenols content was different in fruits collected from various growth locations. The highest level was in rose hips from Curonian Spit, medium – from Sambia peninsula, and lowest – from Vistula Spit (Table appx. 1).

Total water-soluble antioxidants content and antioxidant capacity

Total water-soluble antioxidants were also varying significantly between the species (Table 1, Table appx. 2). This value was significantly higher (up to 2 times) for the fruit of *Rosa rugosa* ($p \leq 0.05$). The hips of this species may probably have higher total water-soluble antioxidants due to the higher content of ascorbic acid (r = 0.898, $p \leq 0.05$) and flavonoids (r = 0.955, $p \leq 0.05$,

Table 1

Antioxidant properties of rose hips collected from different location on the Baltic Sea coast (2016 year)

Species	Maturity	Total water-soluble antioxidants content [mg QE g^{-1}]	Total antioxidant capacity [μmol TE g^{-1}]		
			DPPH	ABTS	FRAP
Curonian Spit					
Rosa canina L.	unripe	23.4±1.7	114.1±3.2	298.6±3.6	512.1±5.8
	half ripe	21.3±2.1	126.3±4.6	329.2±6.4	531.1±8.8
	fully ripe	22.0±1.2	127.6±5.4	369.4±4.2	574.1±4.8
Rosa rugosa Thunb.	unripe	41.9±2.0	311.4±9.3	471.6±12.2	621.4±7.2
	half ripe	42.6±2.4	325.4±10.8	568.4±9.7	674.5±11.8
	fully ripe	45.5±3.3	356.3±11.7	721.7±13.6	689.1±9.2
Sambia peninsula					
Rosa canina L.	unripe	25.6±1.4	124.1±4.1	275.4±4.2	498.7±6.4
	half ripe	19.8±1.9	128.4±5.2	314.4±9.6	568.1±10.7
	fully ripe	21.4±1.4	132.2±3.1	344.1±3.6	589.7±5.4
Rosa rugosa Thunb.	unripe	45.8±2.2	325.6±8.4	487.9±10.8	610.3±8.4
	half ripe	44.5±1.8	331.2±9.7	574.1±10.1	665.2±10.7
	fully ripe	47.8±2.1	342.9±4.9	703.7±10.6	674.4±6.7
Vistula Spit					
Rosa canina L.	unripe	19.7±1.1	108.2±2.9	304.6±3.8	468.9±5.4
	half ripe	17.9±1.5	119.7±4.1	297.4±5.6	512.3±7.3
	fully ripe	19.8±0.7	116.3±4.3	312.7±2.8	541.4±3.8
Rosa rugosa Thunb.	unripe	38.7±1.7	298.7±7.4	467.2±12.6	594.2±6.4
	half ripe	36.4±1.6	319.4±8.6	542.3±6.8	632.4±9.5
	fully ripe	42.1±2.4	312.4±8.6	679.8±11.8	634.6±7.2

Table 2

Correlation matrix with the Pearson coefficient values for the bioactive compounds and antioxidants in rose hips

	AsA	Car	TTC	TFC	TPC	TWAOX	DPPH	ABTS	FRAP
AsA	1,000								
Car	0,414	1,000							
TTC	-0,188	0,775*	1,000						
TFC	0,881*	0,078	-0,484*	1,000					
TPC	0,499*	0,899*	0,706*	0,223	1,000				
TWAOX	0,898*	0,116	-0,458	0,955*	0,242	1,000			
DPPH	0,923*	0,135	-0,463	0,977*	0,248	0,978*	1,000		
ABTS	0,973*	0,475*	-0,114	0,877*	0,547*	0,902*	0,918*	1,000	
FRAP	0,936*	0,421	-0,148	0,856*	0,563*	0,859*	0,894*	0,908*	1,000

*Correlation is significant at the 0.05 level (2-tailed), $p < 0.05$; AsA – ascorbic acid, Car – carotenoids, TTC – total tannins content, TFC – total flavonoids content, TPC – total phenolics content, TWAOX – total water-soluble antioxidants content, DPPH, ABTS, FRAP – antioxidant capacity measured by DPPH, ABTS and FRAP assays respectively

Table 2). The estimation of total antioxidant capacity using DPPH, ABTS, and FRAP assays also showed a higher antioxidant value of rose hips of *Rosa rugosa*. At the same time, the results obtained with various assays dispersed. Thus, by using DPPH assay the total antioxidant capacity of rose hips *Rosa rugosa* was almost 3 times higher, by using ABTS assay 2 times higher, and by using FRAP assay only 1.2 times higher (Table 1, Table appx. 2). The higher value of total water-soluble antioxidants content was determined in unripe and fully ripe fruits. Generally, the antioxidant capacity of rose hips increased in process of ripening. The higher values of total antioxidant capacity were identified in ripe fruits compared with unripe and half-ripe, excluding antioxidant capacity defined by using DPPH assay. In this case there was no significant difference between half-ripe and fully ripe rose hips (Table appx. 2). As well as total water-soluble antioxidants content and antioxidant capacity were lower in rose hips collected from Vistula Spit and higher in fruits from Curonian Spit.

Discussion

In the present study, species-specific accumulation of ascorbic acid was investigated. It was shown that the fruits of *Rosa rugosa* had significant more Vitamin C as the fruits of *Rosa canina*. Species-specific accumulation of ascorbic acid was also demonstrated in the study of KAZAK et al. (2009). The authors proved that Vitamin C content in *Rosa canina* L. was

1.3–4.0 times higher than in *Rosa damascene* Mill. depending on the studied part of the plant (fruit, fruit flesh or seed). Research by CZYŻOWSKA et al. (2015) also proved that raw materials from *Rosa rugosa* hips had higher content of ascorbic acid in comparison with *Rosa canina* plants.

Rose hips are a good source of carotenoids and can be used as a raw material in production of health-promoting products. Typical carotenoids in rose hips are mainly lycopene, lutein, and *β*-carotene (BARROS et al. 2011). Some authors qualified high amount of rubixanthin in rose hips (AL-YAFEAI et al. 2018b). But in study by AL-YAFEAI et al. (2018b) the content and variety of carotenoids were strong depended on rose species. In our study was also demonstrated that fruits of *Rosa rugosa* had more carotenoids compared to *Rosa canina*.

The value of rose hips as being the source of biologically active compounds is also in that the stability of ascorbic acid in the fruit is sustained because the fruit contains phenolic compounds (anthocyanins, tannins, leucoanthocyanins and flavonols) with different chemical structure but yet similar biological action (CARR and VISSERS 2013). In work (STĂNILĂ et al. 2015) big variety of phenolic compounds in the ripe fruit of *Rosa canina* var. Lutetiana & flexibilis was shown. The authors identified up to 19 individual compounds out of the groups of anthocyanins, flavonols, and their glycosides, and phenolic acids. In the present study was shown that the total content of flavonoids and polyphenols was higher in fruits of *Rosa rugosa*, but the total content of tannins was opposite higher in *Rosa canina*. In work (KOCZKA et al. 2018) the concentration of TPC was obtained in a decreasing order of *R. spinosissima* > *R. canina* > *R. rugosa* > *R. gallica*.

Nowadays, there are many very different methods for determining antioxidant activity in plant extracts. The amperometric detection offers a number of advantages. These include a low detection limit, high selectivity (determined are only those compounds whose molecules can be oxidized, while other compounds are not determined, even if in high concentrations), a small volume of the electrochemical cell (0.1–5 µL), and easy servicing (YASHIN 2008). However, as shown in the study of CZYŻOWSKA et al. (2015), when comparing antioxidant activity of wines from R. *canina* and R. *rugosa*, the measuring method should be taken into account. Thus, when using DMPD method, the wine from R. *rugosa* showed higher antioxidant activity, whereas with ABTS method it was higher in the wine from R. *canina*, and there were no significant differences when DPPH method was used. Measuring antioxidant activity in the leaves of different species with DPPH method also revealed no difference between *Rosa rugosa* and *Rosa canina* (NOWAK and GAWLIK-DZIKI 2007).

During the ripening the amount of some compounds decreases, while the amount of others – increases. Although the majority of authors still noted that the level of biologically active compounds increases with the ripening of rose hips. Thus, in the study of NOJAVAN et al. (2008), Vitamin C content in the rose hips of *Rosa canina* was found to be increasing with ripening. And the study of ADAMCZAK et al. (2012) proved that the fully ripe fruit had the maximum content of anthocyanins and flavonols. According to the work by AL-YAFEAI et al. (2018) increasing in total carotenoids content due to the accumulations of (all-E)-β-carotene, (all-E)-rubixanthin and (all-E)-lycopene. The synthesis of these secondary metabolites in plant changes according to changes in enzyme activity. Enzyme activities are influenced by many factors including stage of growth and development of the plant. Depending on the enzyme activities can also change the content of biological active compounds in the pseudofruits of *Rosa* species (ELMASTAŞ et al. 2017).

Secondary metabolites, including many biologically active components and antioxidants, are sensitive components of plants, whose levels in a given plant is directly related to the state of environment (pollution of the soil and/or air with xenobiotics) and ecological conditions that determine the growth and development of plants (temperature, light, humidity). This topic is especially relevant for coastal areas, due to climatic features and specific vegetation period of plants. The climate of Kaliningrad region is temperate, marine transitional to continental. The summer is relatively cold and the July mean temperature ranges from +17 to +18°C. The winter is mild. The January mean temperature is -2–-4°C. The average annual precipitation is 750 mm, ranging from 400 to 1100 mm, depending on the prevalence of either continental or marine air masses (FEDOROV et al. 2016). In our study was shown that growth location has significant influence on accumulation of all biological active compounds, with the exception of carotenoids. The main trend was a decrease in the level of biologically active compounds and antioxidants in rose hips, in the direction from the Curonian Spit to the Vistula Spit.

Based on the content of ascorbic acid, carotenoids, flavonoids, tannins, polyphenols, water-soluble antioxidants, and antioxidant capacity of rose hips the hierarchical cluster analysis was performed (Figure 7). The results of this analysis showed that the main factor determining the differences in accumulation of biologically active compounds is the specie of rose followed by ripening stage and growth location.

A lot of studies have reported high biological active compounds content and antioxidant capacities of natural plant sources. As correctly pointed

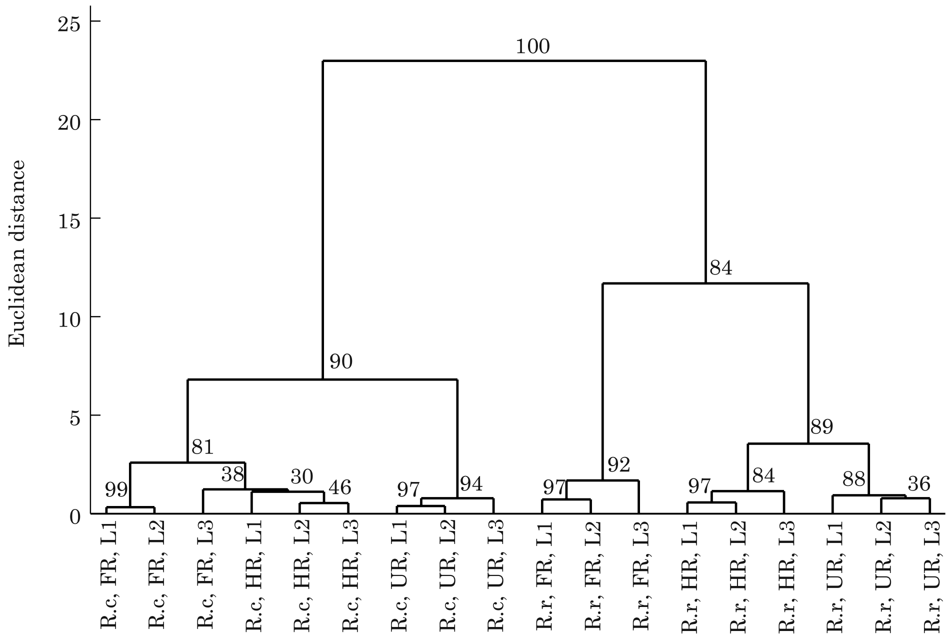

Fig. 7. Dendrogram for the classification of *Rosa* L. fruits collected at different ripening stages and from different locations by Ward's method with respect to the content of studied biological active compounds and antioxidant capacity. Figures at the nodes indicate bootstrap support values after 1000 replicates. R.c. – *Rosa canina*, R.r. – *Rosa rugosa*, FR – fully ripe, HR – half-ripe, UR – unripe, L1 – Curonian Spit, L2 – Sambia peninsula, L3 – Vistula Spit

out in the article of Dr. PETAL (2017) "While consumers are willing to pay exorbitant price for dietary supplements, a natural source of multi-nutrients going to waste is paradoxical". Rose hips are valuable plant resource, containing various biologically active compounds. In our study was established the content of some phytonutrients depending on species and ripening stage of rose hips. *Rosa rugosa* showed higher nutritional value than *Rosa canina* according to the content of vitamin C, the total amount of flavonoids and phenolics, water-soluble antioxidants and antioxidant activity of the rose hips. At the same time, the level of biologically active compounds, especially of ascorbic acid, carotenoids, tannins, and total phenolic compounds, in the fruits of both species increased during the ripening and reached the maximum in fully ripe rose hips. It was also exposed that the content of biological active compounds strongly depends on the place of plant growth and this factor should be taken into account by the collection of rose hips for example for the production of health-promoting products. Thus, in this study was shown high nutritional value of rose hips, especially of the species *Rosa rugosa* Thunb. The results should be taken into account when deciding on the possible limitation of the spread of this species on the Baltic Sea coast, as well by the including the rose hips in the daily diet or designing on their basis functional foods.

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Appendix

Table appx. 1

Results of 3-factorial ANOVA for bioactive compounds

		AsA	Car	TFC	TTC	TPC
Main effects						
Species (Sp)	*R. canina*	8.97*b*	0.61*b*	3.41*b*	3.23*a*	28.8*b*
	R. rugosa	32.3*a*	0.64*a*	5.48*a*	2.03*b*	31.7*a*
Ripening stage (RS)	unripe	14.3*c*	0.35*c*	4.37*b*	1.67*c*	22.3*c*
	half ripe	21.7*b*	0.52*b*	4.60*a*	2.50*b*	29.6*b*
	fully ripe	25.8*a*	0.94*a*	4.38*b*	3.73*a*	38.1*a*
Location (L)	Curonian Spit	21.4*a*	0.61*a*	4.61*a*	2.75*a*	33.1*a*
	Sambia peninsula	21.7*a*	0.63*a*	4.44*b*	2.68*a*	30.6*b*
	Vistula Spit	18.8*b*	0.62*a*	4.23*b*	2.43*b*	26.4*c*
Significance	Sp	*	*	*	*	*
	RS	*	*	*	*	*
	L	*	ns	*	*	*
	Sp*RS	*	*	*	*	*
	Sp*L	*	*	ns	ns	ns
	RS*L	*	*	*	*	*
	Sp*RS*L	ns	*	*	ns	ns

Data was evaluated via three-way ANOVA, factors: species, ripening stage and location, $a = 0.05$, followed by Tukey HSD test (mean, $n = 6$). Identical letters indicate that values do not differ significantly. Asterisks indicate significantly influential factors. AsA – ascorbic acid, Car – carotenoids, TTC – total tannins content, TFC – total flavonoids content, TPC – total phenolics content

Table appx. 2

Results of 3-factorial ANOVA for antioxidants

		AOX	DPPH	ABTS	FRAP
Main effects					
Species (Sp)	*R. canina*	21.2*b*	121.9*b*	316.2*b*	532.9*b*
	R. rugosa	42.8*a*	324.8*a*	579.6*a*	644.0*a*
Ripening stage (RS)	unripe	32.5*a*	231.3*b*	384.2*c*	550.9*b*
	half ripe	30.4*b*	225.1*ab*	437.6*b*	597.3*a*
	fully ripe	33.1*a*	231.7*a*	521.9*a*	617.2*a*
Location (L)	Curonian Spit	32.8*a*	226.9*a*	459.8*a*	600.4*a*
	Sambia peninsula	34.2*a*	230.7*a*	449.9*ab*	601.1*a*
	Vistula Spit	29.1*b*	212.5*b*	434.0*b*	563.9*b*
Significance	Sp	*	*	*	*
	RS	*	*	*	*
	L	*	*	*	*
	Sp*RS	*	*	*	*
	Sp*L	*	*	*	*
	RS*L	ns	*	*	*
	Sp*RS*L	ns	*	*	*

Data was evaluated via three-way ANOVA, factors: species, ripening stage and location, $a = 0.05$, followed by Tukey HSD test (mean, $n = 6$). Identical letters indicate that values do not differ significantly. Asterisks indicate significantly influential factors. AOX – total water-soluble antioxidants content, DPPH, ABTS, FRAP – antioxidant capacity measured by DPPH, ABTS and FRAP assays respectively

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RAINWATER HARVESTING SYSTEM IN A MULTI-FAMILY BUILDING LOCATED IN POLAND

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Key words: rainwater utilization, rainwater harvesting, water saving.

Abstract

Rainwater harvesting, storage, and utilization systems are widely used in many countries in order to reduce tap water usage. In Poland rainwater is utilized in very limited manner despite the fact that potable water resources per capita are one of the lowest in Europe. There are many different ways of using rainwater within a household, public or any other building. It might be used for watering gardens, flushing toilets and due to its softness for cars and windows washing, and in washing machines. In this paper authors described rainwater utilization system for flushing toilets in a multi-family building located in Poland. In this type of building rainwater commonly does not fully cover the demand and during dryer periods needs to be supported by tap water, nevertheless, it generates noticeable savings on potable water. This paper presents economic and environmental aspects of using rainwater harvesting system in a multi-family building under conditions of Polish climate. Calculations of cost, types of systems and designing methods, including tank sizing are presented and discussed.

Introduction

Rainwater utilization is an inherent problem, from the moment when first buildings were constructed. However, the aim of rainwater system typically was to drain the water as far as possible from the place of living. Different situation was in the countries with a very dry climate, where water is priceless and was always collected to the very last drop. Until the nineties of the 20th century it was considered that storm water problem solution in the cities was to quickly collect and remove it to a receiver, but

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progressive urbanization and replacing natural land cover with tight surfaces, e.g. concrete, led to approach change (WOJCIECHOWSKA et al. 2015). Nowadays people try to lower the impact on the environment by using renewables, but water unfortunately does not qualify as one of them. Water is a valuable and exhaustible resource, the demand on it is constantly growing and it is anticipated that this tendency will obtain (DÖLL et al. 2009).

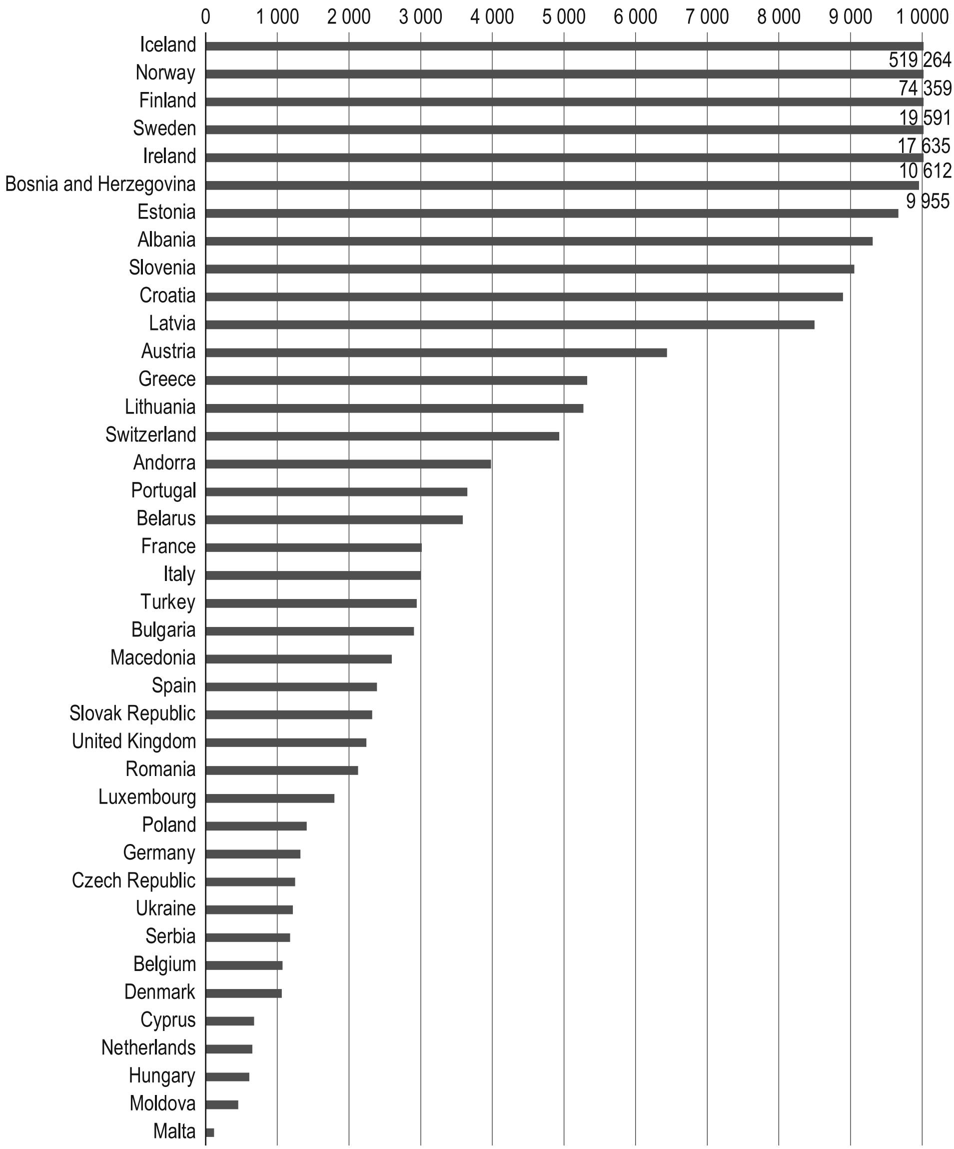

Fig. 1. Freshwater resources per inhabitant – long-term average [cubic meters] (FAO 2014)

Water resources are decreasing due to human negative impact on the environment (DUDKIEWICZ and LASKA 2019). These factors create necessity of more reasonable water usage and searching for alternative sources like rainwater. In addition, there is an economical factor – people always prefer to pay less for the bills if there exists such a possibility. Poland is a country with low level of water resources (STEC and SŁYŚ 2017) – Figure 1 comparing to other European Union countries (FAO 2014), it takes further than 20th place (GUTRY-KORYCKA et al. 2014) which means that people should pay more attention to water usage. Utilizing rainwater also has another aspect, especially in big cities due to high surface sealing through which water cannot permeate, rainwater can cause floods especially so-called "flash floods". Urbanized area, which can be assumed as a tight surface, takes now ca. 5,4% of total surface in Poland (Figure 2)

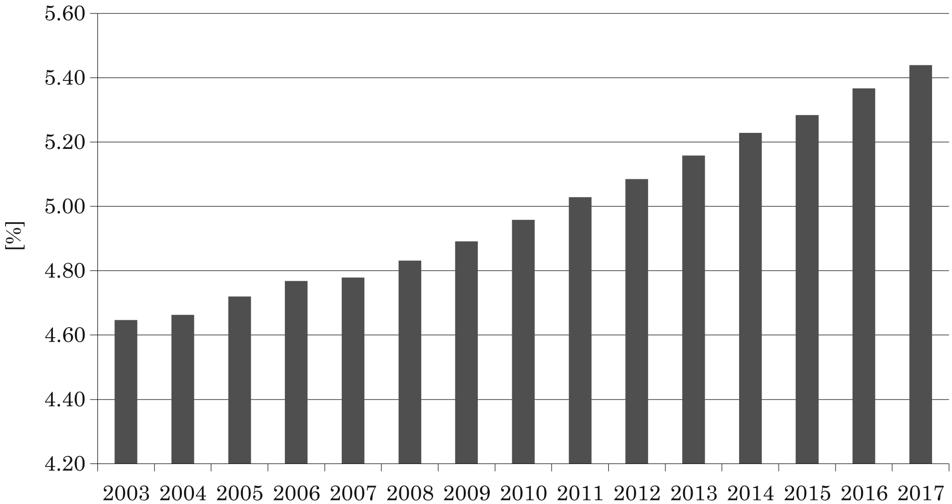

Fig. 2. Percentage quota of urbanized and built-up areas in Poland (GUS 2018)

and is constantly growing (GUS 2018). On the contrary this water, due to its softness, is a perfect cleaning agent for washing many different things, i.e. car washing, windows, and, which is the most useful in a household, laundry. It requires some minor treatment, like filtration and temporary disinfection. After filtering it can be used as well for flushing toilets, which in average covers 30% of total household water usage (Figure 3) and even 43% in public buildings (LUDWIŃSKA and PADUCHOWSKA 2017). Rainwater might be purified to the drinking water level, which can be useful in certain cases, but this process is very expensive and simply unprofitable from the economical point of view. People should therefore significantly change approach to dealing with rainwater and start treating it as a precious

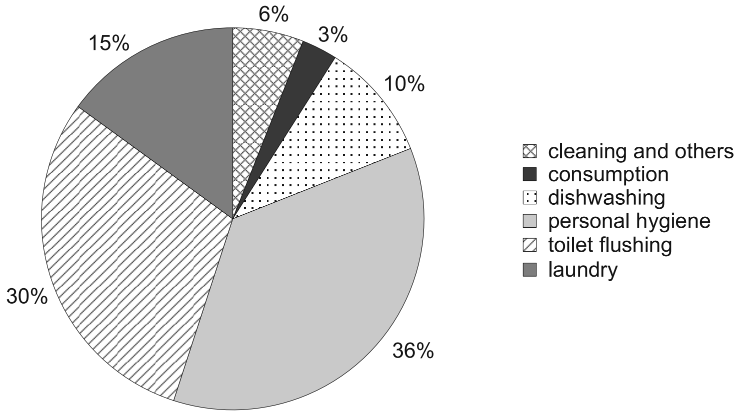

Fig. 3. Household typical water usage
Source: own study on the basis of LUDWIŃSKA and PADUCHOWSKA 2017

resource instead of a wastewater or sewage. In Poland there are regulations determining collecting and removing storm water to the sewage system or harvesting it and utilizing within the area around the building (Rozporządzenie Ministra Infrastruktury... DZ.U.2015.1422, 2018). Rainwater resources depend mainly on meteorological conditions, which may vary significantly in different regions and annual weather cycles. In Poland it varies from c.a. 500 to 1400 mm/year, in Olsztyn: 600–700 mm/year (IMGW 2018). It should also be mentioned that utilizing rainwater for household purpose lowers sewage volume introduced to wastewater collecting system thereby it decreases the whole sewerage system and treatment plants capacity. The objective of this paper is to present rainwater usage possibilities and their benefits based on a multi-family building example located in Poland.

Rainwater harvesting and usage systems

Rainwater can be collected from the rooftop and from other surfaces surrounding a building. Quality of water depends mainly on location, whether it is a polluted industrial region or place surrounded by forest and lakes, or by other environmental factors. In particular the type of collecting surface is crucial. Pollution that might occur in harvested water are: bird feces, leftovers of food carried by birds, organic matter e.g. leaves, street dirt and sand, chemical contaminant from industry, and other pollutions like oil or petrol. Rainwater harvesting systems are typically equipped with a tank, where water is collected after filtration. This process removes organic matter, partly dust and sand. In case of collecting water from pavements or streets grease separators and settlers must be utilized to remove oil and other floating particles before introducing water into collecting tank. From the tank water is next pumped to smaller tanks nearby the sanitary equipment or directly to the devices. Collecting tanks might be situated at the higher floors to enable gravity flow and omitting pump application. It is strongly advised to use disinfection, e.g. UV-rays to prevent *Legionella* in systems utilizing rainwater for other purposes than watering gardens and flushing toilets.

The simplest form of rainwater harvesting system is a tank, in which water is collected from the rooftop. Then it is used for garden watering or car cleaning without any treatment, besides a simple filter capturing leaves. More complicated systems, which deliver water to the building as well, can be divided according to the way of distributing it to the sanitary devices (LEGGETT 2001). Systems with indirect pumping (Figure 4) are based on two tanks, the smaller one is installed on the highest floor, above all the sanitary devices. Water is pumped from the main tank to the smaller one and subsequently it gravitationally flows to sanitary devices.

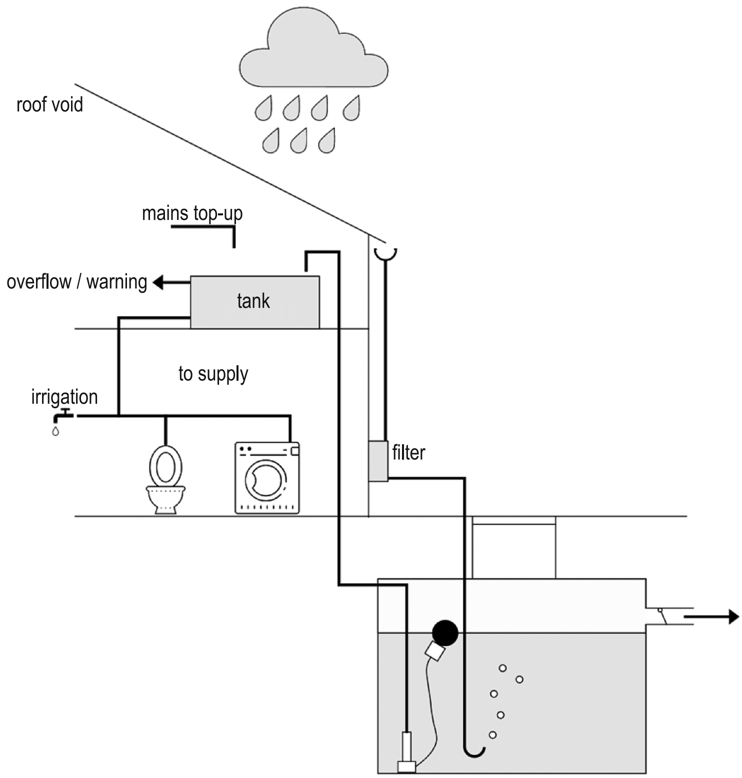

Fig. 4. Scheme of the indirect pumping system (LEGGETT 2001)

These tanks are additionally supplied with tap water in case of low rainfall during dryer periods. It is advised to mount them at least 1 meter above the sanitary devices level. Excess water should be discharged to sewage or drainage system. The advantage of this system type is lower pump expenditure due to less on/off turning. Notwithstanding the cost is higher comparing to the single-tank systems due to additional tank and, in certain cases, construction reinforcement necessity due to the tank weight. Alternative rainwater harvesting system type is direct pumping system (Figure 5), where water is collected in one tank and directly from it pumped to the sanitary devices. This system requires tap water supply in case of low rainfall and an overflow valve connected to sewage system.

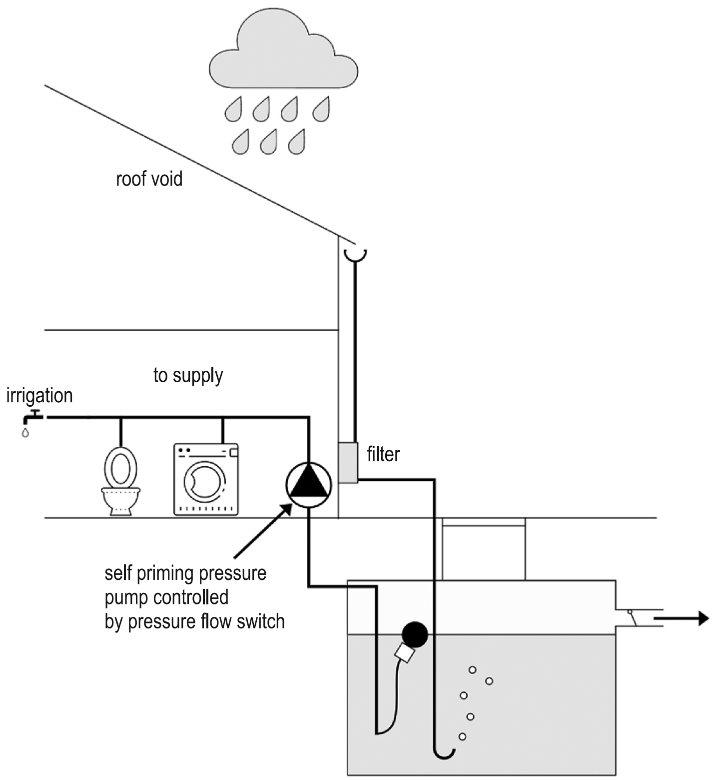

Fig. 5. Scheme of the direct pumping system (LEGGETT 2001)

One disadvantage of this system type is higher pump failure probability as a result of more frequent on and off turning. Secondary tank is not necessary. The third system is a gravity system (Figure 6) where the tank is placed on the highest floor or just under the rooftop (at least 1 m above sanitary devices) and the water flows gravitationally to sanitary devices.

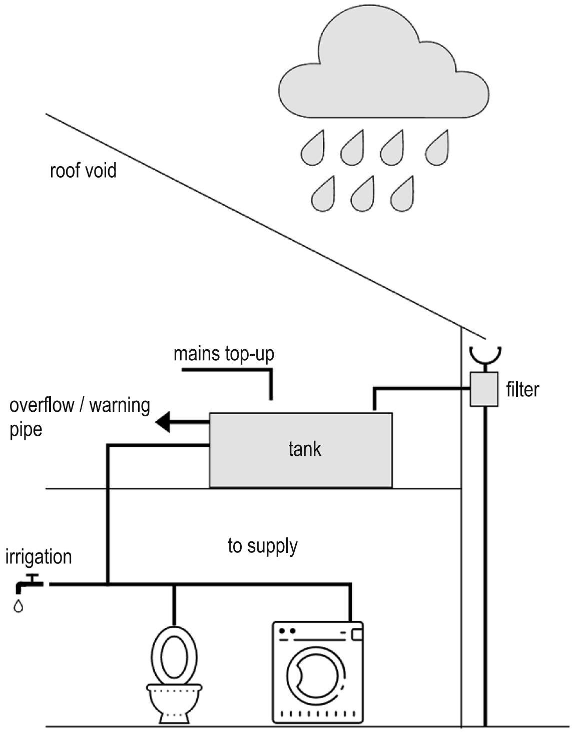

Fig. 6. Scheme of the gravity system (LEGGETT 2001)

The tank cannot be situated too high in terms of letting gravitational inflow of the water from the rooftop. It also requires adequate construction reinforcement on behalf of the very high tank weight, e.g. 10 tons. This system does not require any pump which results in reliability increase and cost effectiveness. Tap water and overflow valve should be installed as in the previous system types.

Design of rainwater utilization systems

One of the most important factors deciding on implementing installation with rainwater use is medium yearly rainfall in given region and the volume of water possible to collect from the rooftop or another collecting area. Design process of rainwater utilization systems is shown on Figure 7. Rainwater collecting surface is a total surface from which water can be collected, specified in m^2. Predominantly for this purpose a building roof is used, however, as it was already mentioned, water can be collected from pavements, streets and parking places. In case of collecting rainwater

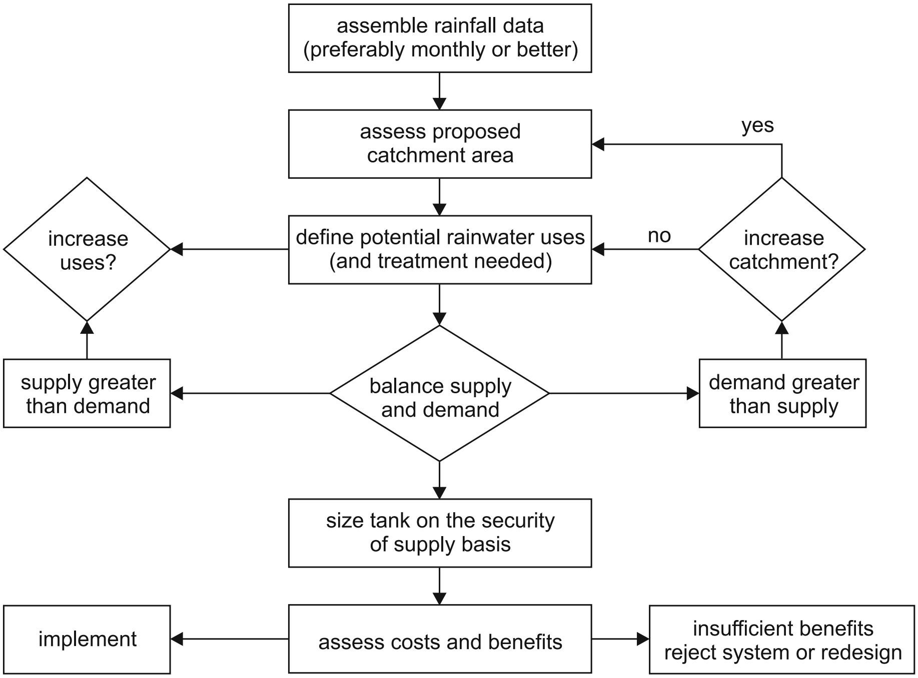

Fig. 7. Rainwater utilization system decision and design process (LEGGETT 2001)

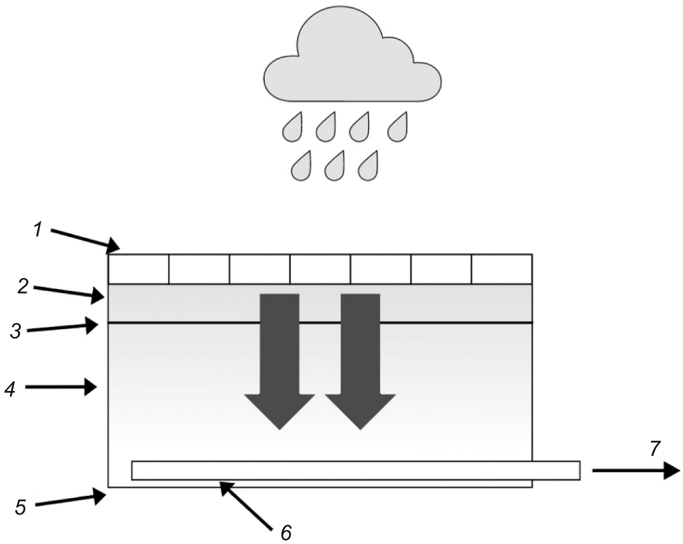

Fig. 8. Permeable pavement (LEGGETT 2001): *1* – permeable surface; *2* – bedding layer; *3* – permeable geotextile; *4* – permeable sub-base with storage voids; *5* – for attenuation: robust welded geo-membrane; *6* – collection pipe network; *7* – rainwater to further treatment, storage or directly to re-use

from other surfaces than rooftop much higher filtration and disinfection is required on account of the dust, sand, oil, petrol and other pollution that can be found on streets and pavements. An exception here can be so called *permeable pavements* (Figure 8) thanks to filtrating dust through utilizing proper layers absorbing pollution and causing proper conditions for biological oil degradation (SCHOLZ and GRABOWIECKI 2007). While designing rainwater harvesting system collecting area is taken as a flat surface which means that roof or pavement slope is not taken into account. It is normally necessary to divide area into smaller parts on behalf of different rooftop or pavement materials. The predominant wind directions should be taken into account as well as surrounding buildings and trees that can lower water volume reaching the planned area. Total volume of water does not run into the tank because of evaporation, absorption by materials, or its loss caused by bounce from the roof and falling on the lawn. Flat rooftops, covered with crushed stone, maintain huge amount of water, which slowly evaporates, while from the sloping roof made from tile or plate water flows down almost immediately. Roofs covered with straw or so-called *green roofs* covered with grass absorbs huge amount of water which slowly evaporates and only some small volume is drained to a tank or sewerage system. Run-off coefficients for different surface types are presented in Table 1.

Table 1

Rainwater run-off coefficient (ŚWIGOŃ 2008)

Surface type	ψ
Roofs covered with plate or slate	0.90–0.95
Asphalt and conrete roads	0.85–0.90
Roofs covered with tile	0.80–0.90
Paving surfaces	0.75–0.85
Flat roofs and wooden elements	0.50–0.70
Grass-concrete parkings (lawn grilles, loose row and stone cubes, broken stone surfaces)	0.25–0.60
Gravelroads	0.15–0.30
Cultivated lawns	0.10–0.20
Parks, gardens, fields, greens	0.05–0.10

Atmospheric conditions and surrounding area (possible industries and factories) have a big impact on rainwater quality before it reaches collecting surface. Chemicals such as sulphur dioxide, nitrogen oxides or carbon dioxide are absorbed by raindrops making rain sour. Next pollution stage occurs at the moment of water flow on the roof, street or pavement. Roofs are quite often covered with plate or other materials having some metal parts, e.g. steel, brass, copper etc. Therefore, water can contain higher metals concentration. In certain case it can result in lower bacteria development so in this context it can be taken as an advantage. Brittle materials on the roof lead to overgrowing with moss due to longer water keeping on the surface. This can cause increased bacteria development. Roofs made of steel or glass, as well as oblique roofs, especially these with high inclination angle, cause faster flow of the water, which cleans the surface and stops moss growth or bacteria development. Concrete roofs might to some extend improve the acid pH reaction, and "green roofs" result in lower water clarity, change of its colour to brown, which requires additional filtration. Roofs and gutters should be mechanically cleaned periodically in terms of eliminating as much pollution as possible. The best material for the pipework is polyethylene (PE). Pollution such as street dust, food leftovers, bird feces can cause occurrence of pathogens. Conditions caused by this pollution can be suitable for bacteria development such as *Legionella* or *Pseudomonas aeruginosa* (CHIDAMBA and KORSTEN 2015). The number of bacteria in the rainwater depends also on storage conditions. If the collecting tank is exposed to the daylight, an algae growth can occur which is perfect nourishment for bacteria and its further development. It is therefore recommended to situate tanks in shady places, preferably underground. The tank should not be oversized to prevent water stagnation. To obtain biological activity it is important to ensure proper oxygen conditions in the lower parts of the tank. This can be achieved by proper tank construction and way of introducing water. Oxygenation can be caused by introducing water to the lower part of the tank, bearing in mind not to put in motion matter which sedimented already before. Activity of the anaerobic bacteria is caused by too high amount of organic matter and other nutrients and can entail obnoxious smell of sulphuric acid. Tanks should therefore be ventilated.

Rainwater storage

Storage tanks can be made of different materials, such as plastic or concrete and have different shapes. One of the basic issues is the tank volume which has impact on whole system cost and savings that installation may bring. Then it is necessary to properly balance water demand and the available rainwater volume (MROWIEC 2008). The tank size depends on harvesting surface, medium rainfall, water demand, retention time (storage period), cost of the tank and its accessories. Potential rainwater volume collected from the given surface can be calculated as below (LEGGETT 2001):

mean rainwater supply [dm^3/year] = mean annual rainfall [mm] ·
surface area of catchment [m^2] · run-off coefficient · filter coefficient

The result is approximate and there are many elements that can disturb the real collected water volume and potential money savings, first of all unpredictability of weather. There are three typical situations of dimensioning the tank. The first is when given data and calculations indicate that possible rainwater collection would never satisfy the needs. This situation is typical for high multi-family buildings or office buildings with a large number of users and here the tank size depends only on rainwater available to collect. The second situation takes place when possible to collect water significantly exceeds the needs of the building. This is typical for factories or other buildings with low users' density. In that case tank size estimating is based only on the needs and water surplus is poured to sewerage system. The tank should not be oversized since water stagnation favours bacteria development. The third case is when possible to collect rainwater thereabout responds to the needs. The tank should be then dimensioned upon all the available factors, it should not be too small to prevent too often overflows to wastewater collecting system, nor to large, which could be conducive to bacteria growth and needlessly enlarge installation cost. A project conducted in Great Britain called "Buildings That Safe Water" *(*BTSW*)* (LEGGETT 2001) indicated that in most of cases storage tanks were oversized on account of too optimistic assumptions of designers. There are many different methods to calculate tank size used currently by producers and designers. Some of them are greatly complicated, based on different type of data, but there also exist simplified methods based on tables or charts. One of them indicates dimensioning of garden watering (Table 2) and household (Table 3) tanks (PIASNY 2013).

Table 2

Rainwater tanks for garden watering dimensioning (PIASNY 2013)

Garden area for watering [m^2]	Minimal roof area [m^2]	Tank volume [dm^3]
100	20	1 000–1 500
300	40	2 500–3 500
500	70	3 000–5 000
800	90	5 000–7 500
1000	100	7 500–10 000
1500	120	10 000–15 000

Table 3

Rainwater tanks for household dimensioning (Piasny 2013)

Number of residents	Minimal roof area [m^2]	Tank volume [dm^3]
1–2	35	2 500–3 000
3	65	2 500–5 000
4–5	80	5 000–7 500
6–9	100	7 500–10 000

Catalogues of different brands such as *Kingspan* suggest calculating tank size according to an average from yield of rainwater and water demand, assuming 21-day retention period. *Roth* company suggests estimating 1 m^3 of tank for each 25 m^2 of the collecting surface. There is also analytical method with the formulas given:

$$E = NA\eta \qquad V = E \cdot 6\%$$

where:
N – mean annual rainfall [mm]
A – surface area of catchment [m^2]
η – run-off coefficient
E – mean rainwater supply [dm^3]
V – required tank volume [dm^3]

All the accessories, pipes, tanks, filters, pumps etc. require regular service to ensure faultless and long-lasting system operation. In the previously mentioned project BTSW the most common system malfunction was pump fault caused by filter soiling, not having them cleaned on time (Leggett 2001).

Rainwater treatment

To clean rainwater three basic methods are used: filtration, biological purification and disinfection. The purpose of filtration is to remove mechanical contamination before introducing water into the tank and after water storage in it. Biological purification is used in terms of removing nutritional substances for bacteria and preventing bacteria reproduction. Disinfection eliminates microorganisms. Gutter filters can be both internal and external. It is also recommended to use floating filters inside the tank which capture floating pollution from the water. These filters should be also connected directly to the pump with a pipe to ensure suction of the clearest water located just under the water surface (Leggett 2001).

In Germany, based on many years observation of utilizing internal gutter filters and floating filters, the water quality was sufficient for flushing toilets without any further purification. Biological purification is typically used as one of the purification stages in larger installations, where water is collected not only from the roof but also from pavements or streets. This process is carried out after collecting water in the tank. Bacteria that occur in water are used to remove such pollution as oils, organic matter, phosphates, nitrates and ammonia. Phenomenon of biological purification occurs also in previously mentioned permeable pavements. After filtration and eventual biological treatment disinfection might be required in terms of removing bacteria and other living organisms. Most of rainwater harvesting systems, which typically use water for flushing toilets and watering garden do not require disinfection at all, on condition that the primary pollution was not very significant. Nevertheless, if necessary, disinfection can be carried out in a physical manner as UV-rays, but adequate water transparency is required. This type of disinfection is typically used to remove bacteria such as *Legionella* or *E. coli*, which can have negative impact on the users' health. UV lamps consumes some amount of energy, ca. 120–140 kWh per year, which increases the whole system usage cost. This type of disinfection becomes more economical when the water is used more often on account of the fact, that UV-rays are equally effective for every water flow, if proper transparency is provided. Chemical disinfection is not recommended for rainwater harvesting system because of typically small amount of pollution. This process is more valuable when utilizing greywater but this is another topic.

Rainwater harvesting system usage in multi-family building

For the purposes of this article an elaboration of conceptional project has been made for a multi-family 4-storey building localised in Olsztyn. On each floor there are 6 flats, 2 persons for each flat in average. There is no terrain slope around the building. The rainwater is supposed to be used for flushing toilets, which induces savings on tap water. According to the calculations the rainwater does not fully cover the needs, thus replenishment from the water supply is provided. Tap water inflow is regulated automatically through a water level sensor, controller and valve to maintain minimal water volume in the tank during dryer periods. The system is designed as direct pumping system, which means that water supply is held from the main tank directly to the sanitary devices. The pump is enabled upon signal from the pressure sensor mounted on the pipe providing water to the toilets. Tank, made of plastic, is situated outside the building underground, below the frost level. It is equipped with a spillway to drain water excess to the sewerage network during heavy rainfalls. Pipes used are made of polyethylene on account of their resistance to corrosive nature of rainwater. For water purification an internal gutter filter has been selected – WISY, type WFF 150 – Vortex filter DN150. The filter is adopted on the final gutter length to remove such impurities like leaves, roof dirt, bird feces, insects and others.

Water demand has been calculated as below:

$$6 \text{ flats} \cdot 2 \text{ persons} \cdot 4 \text{ storeys} \cdot 4 \text{ times a day toilet flushing} \cdot 6 \text{ dm}^3 \text{ of water for each flush} =$$

$$= 6 \cdot 2 \cdot 4 \cdot 4 \cdot 6 = 1152 \text{ dm}^3\text{/day}$$

$$1152 \cdot 365 \text{ days} = 420\,480 \text{ dm}^3\text{/year}$$

Rainwater possible to use:

Rooftop area:	$41.0 \cdot 12.6 = 516.6$ m^2
Mean rainfall in Olsztyn:	700 mm/year
Run-off coefficient:	0.90 (oblique roof covered with plate)
Filter efficiency:	0.90

$$\text{mean rainwater supply [dm}^3\text{/year]} = \text{mean annual rainfall [mm]} \cdot$$

$$\cdot \text{ surface area of catchment [m}^2] \cdot \text{run-off coefficient} \cdot \text{filter coefficient (LEGGETT 2001)}$$

$$V = 700 \cdot 516.6 \cdot 0.90 \cdot 0.90 = 292\,912 \text{ dm}^3\text{/year}$$

The tank volume calculation is based on *Kingspan* recommendations, with the reservation that retention time taken into account is 10 days, which has been calculated upon a book *Frequency of days with precipitation in Poland* (OLECHNOWICZ-BOBROWSKA 1970), where it was given that periods without any rainfall longer than 10 days occurs very rare. According to the German experience (SAYERS 1999) it is recommended to select tank size in a manner enabling periodical overflows of the water and removing ipso facto floating pollution (suspended solids). Tank size was calculated with the formula given (KINGSPAN 2011):

$$\frac{\text{yield} + \text{demand}}{2} \cdot \frac{10 \text{ days}}{365} = \frac{292\,912 + 420\,480}{2} \cdot \frac{10}{365} = 9772 \text{ dm}^3$$

A rainwater plastic tank chosen is *Rewatec* type *BlueLine II*, 10 000 dm^3 volume. Installation scheme is shown on Figure 9. All the pipework diameters, materials, pumps, valves, filters, etc., for both rainwater and tap water installation, have been calculated according to all currently applicable standards and regulations in Poland. Rainwater is collected from the rooftop through roof gutters Ø 150 mm and directed to the tank through the pipework.

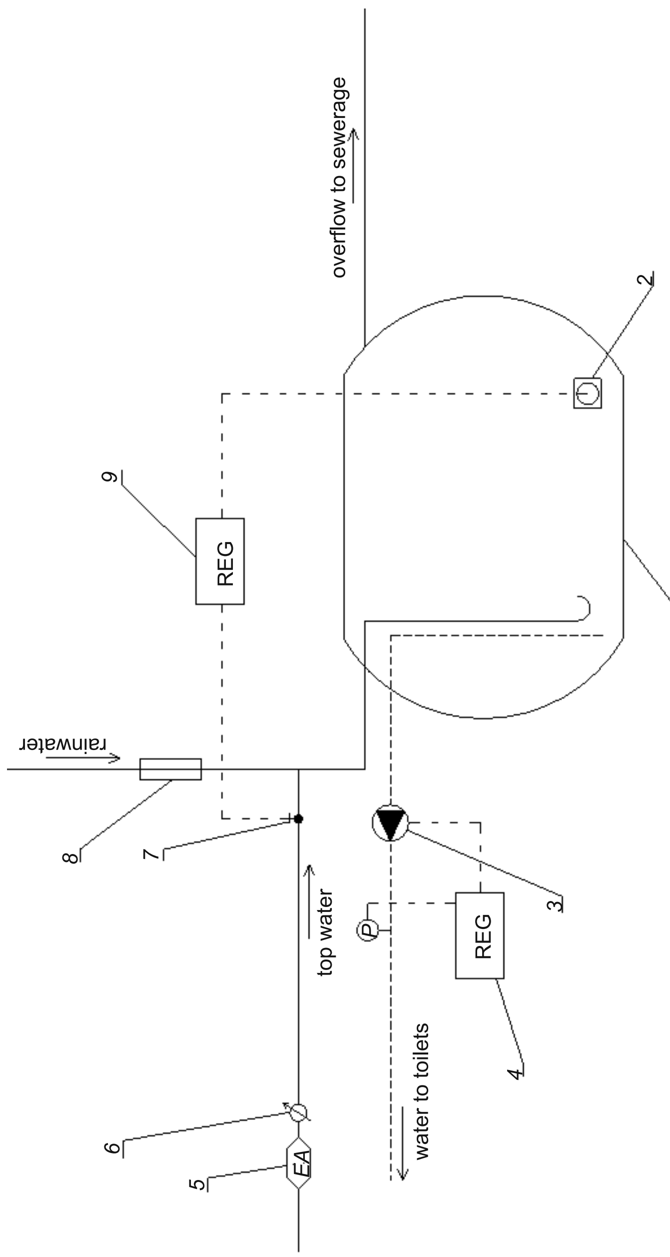

Fig. 9. Rainwater harvesting system example installation scheme: *1* – rainwater collecting tank Blue Line II 1000 dm^3 PE; *2* – water level sensor; *3* – pump activated on basis of pressure sensor; *4* – controller; *5* – anti-contamination filter; *6* – water meter of tap water; *7* – valve with actuator activated on basis of rainwater level sensor; *8* – inner gutter filter; *9* – controller

Rainwater harvesting system economics

The rainwater harvesting system in the multi-family building described above is supposed to bring the savings of 292 912 tap water litres per year, which covers almost 70% of the total demand for flushing toilets in the building.

$$\frac{\text{mean rainwater supply}}{\text{water demand for flushing toilets}} \cdot 100\% = \frac{292\ 912}{420\ 480} \cdot 100\% = 69.7\%$$

Total water demand savings:

$$69.7\% \cdot 30\% \text{ (household toilet flushing)} = 69.7\% \cdot 30\% = 20.1\%$$

Taking into account current water and wastewater prices in Olsztyn (PWIK 2017) the financial savings presented below seem to be optimistic as well. Taxes for storm water have not been introduced in Olsztyn yet, nevertheless, it seems to be probable in the nearest future.

Water price for households: 4.32 PLN/m^3
Wastewater price: 6.44 PLN/m^3

$$\text{water cost savings} = (\text{water price} + \text{wastewater price}) \cdot \frac{\text{water volume savings}}{1000}$$

$$(4.32 + 6.44) \cdot \frac{292\ 912}{1000} = 3152 \text{ PLN/year}$$

The prices vary significantly in different regions of Poland, and in comparison the highest price in Poland is 12.05 PLN for water and 17.25 PLN for wastewater cubic meter in Szklarska Poręba (KSWIK 2018), which almost triples the potential savings. In addition, the environmental aspect should be considered, regarding lower potable water consumption. Whole installation and project costs depend on many factors, mostly on complexity of the installation, designer brand and his prices. Occasionally calculations are held by unqualified house owner and potential user of the installation, which can at glance lower whole cost, but at last expenses may grow due to incorrect long-term system operation. The costs are much lower when new building is constructed since all the machinery, e.g. excavators are present anyway at the construction site thus digging additional hole for the tank is not a problem. When the rainwater utilization system is mounted to an existing building in certain cases it might encounter such difficulties as terrain development with small architecture, which does not allow excavators to easily enter the area. Having the system installed the only maintenance costs seems to be pump work, UV lamps, filter cleaning and some repairs. In the multi-family building described above the system is simplified, without disinfection lamps or biological purification, and with one pump only which lowers whole operating cost.

Conclusions

Rainwater harvesting system in a multi-family building can be successfully used for flushing toilets purpose and bring significant environmental and financial savings, regarding constantly rising water and wastewater prices and upcoming rainwater taxes. In the example building described in this paper it occurred that almost 70% of water demand for flushing toilets can be covered by rainwater, which gives 20% savings on total water demand. One of the most important issues is to properly dimension the rainwater tank bearing in mind periodic overflows. During evaluating of the conceptual system in a multi-family building some problems have been encountered. One of them was the method of accounting for tap water consumption during dry season. This water can be counted by a water meter but it is impossible to divide the consumption into individual flats. The most favourable solution seems to be equal division to each flat. In certain case this might cause dissatisfaction of a group of people. Rainwater harvesting systems should be considered in every new-constructed building as well as in the existing ones especially in Poland where the water resources are quite limited.

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BIOCLIMATIC CONDITIONS IN FOREST COMMUNITIES ALONG THE TOURIST TRAILS OF KAMPINOS NATIONAL PARK

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Key words: bioclimate, forest communities, tourism, Kampinos National Park.

Abstract

Microclimatic conditions in the forest differ significantly from conditions in non-forest areas, and different forest communities have a different impact on the human organism. Not all forest communities are universally beneficial for everyone. Forests in Kampinos National Park (KNP) show a considerable diversity of habitats and communities. The tourist and recreational function of these forests is very important because of their location close to Warsaw. Taking advantage of the therapeutic role of the particular forest communities and their stimulating effect on human vitality may enhance their attractiveness, e.g. through the development of health tourism. The primary study objective was to assess the bioclimatic conditions in the forest communities of KNP, including the determination of their health properties, and to analyse the tourist trails against the background of these conditions.

Most of the tourist trails in KNP go through communities with favourable bioclimatic conditions. Bioclimate beneficial to people occurs along 94% of the length of KNP trails in the spring and 77% in the summer. Unfavourable bioclimatic conditions occur along 3% of KNP trails. The distribution of tourist trails in relation to the bioclimatic conditions of KNP forest communities is very advantageous from the tourist and recreational perspective.

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"No medicine has such an immediate effect
as taking a walk in a beautiful forest"
Qing Li
Shinrin-yoku. The Art and Science of Forest-Bathing.
How Trees Can Help You Find Health and Happiness

Introduction

Since ever, forests have played a very important role in human life, and their recreational and tourist significance continues to grow. The beauty and diversity of forest landscapes allow people to rest and regenerate. Based on the stress-relieving properties of forests, various forms of their therapeutic use have been developed, e.g. the Japanese art of shinrin-yoku (LEE et al. 2012, HANSEN et al. 2017). In Poland, forest therapy is referred to as "sylvotherapy" (ZAWADKA-PIETRZAK and ZAWADKA 2015). The air that we breathe in forests is less contaminated with particulate matter and gas pollutants, and is richer in oxygen and aromatic substances, including bactericidal phytoncides emitted by plants (LI et al. 2009, NOWAK et al. 2014. The changes of the meteorological element values (both on a daily and yearly basis) are more subtle in forests, and noise is replaced by the soothing sounds of nature. The ubiquitous forest green restores one's inner peace and balance (GRAHN and STIGSDOTTER 2003, BERMAN et al. 2008). While the above-mentioned effects improve well-being, the impact of forests on the human organism is much more complex. Staying in the unique forest bioclimate has a positive influence on human health, including the cardiovascular, respiratory and nervous system, and improves the immunity of the body. Various aspects of the influence exerted by the forest environment on the human organism were described by authors such as MAYER and HOPPE (1984), BROWN and CHERKEZOFF (1989), MOSZYŃSKA (2000, 2012), SCHILLER (2001), FORNAL (2004), GRZYWACZ (2011), ZIÓŁEK et al. (2012, 2013), KARDAN et al. (2015), ZAWADKA-PIETRZAK and ZAWADKA 2015, BASTEK and PŁOSZAJ-WITKOWSKA (2016).

The conditions vary across different forest areas and differ from those in non-forest areas (BOGUCKI 1988, KRZYMOWSKA-KOSTROWICKA 1997, FALENCKA-JABŁOŃSKA 2012, DRAGAŃSKA et al. 2016). Since the particular forest communities differ in terms of habitat properties, age of the trees, species composition and density of tree crowns, their impact on the human organism is also different. Various types of communities can even have opposing effects, e.g. a longer stay in a forest can increase or lower blood pressure, or can have a soothing or stimulating effect. Thus, not all forest communities are universally beneficial, and longer stays in some of them are not recommended to everyone. In addition, some kind of nuisance can occur in some communities, e.g. stagnant and stuffy air, or the presence of allergens or bothersome insects. Therefore, it is important to assess the bioclimatic conditions in the particular forest communities and demonstrate their impact on human health and well-being.

A set of environmental factors occurring in forests and affecting people visiting them constitutes the so-called recreational bioclimate. According to TOYNE (1979, see KRZYMOWSKA-KOSTROWICKA 1997), recreational bioclimate encompasses all variable external environmental conditions of the air layer, referred to as the "recreational layer." It covers the zone from 20 cm below the ground to 2 m above the ground, i.e. the zone where human tourist and recreation activity is concentrated. KRZYMOWSKA-KOSTROWICKA (1997) lists the following key bioclimate parameters of the recreational layer in forest communities: sun exposure, humidity, ventilation, oxygen production, ozone production, air ionisation, presence of phytoaerosols (essential oils, including phytoncides) and aeroplankton (plant pollen and particles, fungal spores, bacteria, insects).

National parks are areas where tourist traffic takes place along designated routes passing through plant communities, including forest communities, that have a significant impact on tourists' health and well-being. Taking advantage of the therapeutic role of the particular forest communities and their stimulating effect on human vitality may enhance their attractiveness and contribute to the development of health tourism. The primary study objective was to assess the bioclimatic conditions in the forest communities of KNP, including the determination of their health properties, and to analyse the tourist trails against the background of these conditions. The use of GIS software made it possible to create a map of the bioclimatic conditions occurring in the Park. The bioclimate in KNP forests was investigated taking into account the spring (before the full development of the leaves) and summer season when the most intensive tourist traffic occurs.

Materials and Methods

The survey encompasses the area of Kampinos National Park (KNP) established in 1959. Covering more than 38,500 ha, KNP is one of the biggest national parks in Poland. It comprises the Kampinos Forest located in the ice-marginal valley of the Vistula, in the western part of the Warsaw

Basin. The forests in the Park are a remainder of vast primeval forests that used to grow in the Mazovia region. KNP is a typical forest park. Forests account for nearly three quarters of the Park's area. 66 tree species, including 33 native species, were found to occur in the Park and its buffer zone. The most important role in the tree stand is played by scots pine (*Pinus sylvestris*, 69.5%) – the primary forest-forming species, common alder (*Alnus glutinosa*, 12.5%), pedunculate oak (*Quercus robur*) and sessile oak (*Quercus petraea*) (about 10% in total), silver birch (*Betula pendula*) and downy birch (*Betula pubescens*) (6.5% in total) (*Kampinoski Park...* 2019). Coniferous forests are the predominant woodland communities. Forest areas are adjoined by open land occupied by meadow and other communities. Forested dunes and wetland with peat-bog communities are the most characteristic features of the Park's landscape. Such an environment is conducive to flora and fauna diversity, and the abundance of forest communities provides the living conditions for many species of large mammals. Since 2000, KNP has been a UNESCO World Biosphere Reserve.

Located in close proximity to Warsaw, it is one of two "suburban" national parks in Poland, alongside Wielkopolski National Park. Thanks to its location, KNP is a very popular place of recreation for people living in the Warsaw metropolitan area, hence its tourist and recreational functions are very important. According to data published by GUS (Central Statistical Office), KNP is a national park with the greatest length of tourist trails in Poland: about 360 km of hiking trails and more than 200 km of cycling trails as well as 9 educational trails (*Kampinoski Park...* 2019). The Park is visited by about 1 million tourists per year, which makes it the fifth most popular park in Poland (*Ochrona środowiska...* 2017). Tourist traffic in KNP has been studied by OSIŃSKI 2002, CIESZEWSKA 2008, 2009, DZIOBAN 2013, among other researchers.

Digital data on the Park's plant communities were the basis for the assessment of the distribution of tourist trails against the background of bioclimatic conditions in forest communities. Obtained from Kampinos National Park, these data are part of the KNP conservation plan (KNP DIGITAL DATA). According to these digital sources, 17 forest communities were distinguished within the Park. For the purposes of this study, they were combined into 9 groups based on similarities of habitat and bioclimate. The obtained data were processed using ArcGIS 10.1 software, thanks to which a detailed digital map of the Park's forest communities was generated.

Table1

Characteristic of the bioclimate of recreational layer of groups of the forest communities of the KNP

Groups of forest communities	Partici-pation in the forest area [%]	Insolation	Humidity	Ventilation	Oxygen production	Ozone production	Air ionization	Aeroplankton**	Phytoaerosols	Bioclimate index
Subcontinental pine-oak forest (*Querco roboris-Pinetum, Qr-P molinietosum, Serratulo-Pinetum*)	**51.61**	0.5	0.5	0.5	0	0.5	1	0.17	0.5	0.46
Subatlantic and subcontinental fresh pine forests (*Peucedano-Pinetum, Leucobrio-Pinetum*)	**8.57**	1	1	1	-0.5	1	-1	0.66	1	0.52
Subatlantic wet pine forests (*L-P molinietosum, Molinio-Pinetum*)	**3.82**	0	-0.5	0	-0.5	1	-1	0.17	0.5	-0.04
Pine peatland wood (*Vaccinio uliginosi-Pinetum*)	**0.02**	-1	-1	-1	-1	–	-1	-0.33	0.5	-0.69
Riparian forest (*Fraxino-Alnetum, Salici-Populetum, Ficario-Ulmetum, Fraxino-Alnetum / Querco roboris-Pinetum molinietosum*)	**11.04**	-1	-1	-1	0	–	0	-1	1	-0.43
Alder woods (*Ribeso nigri-Alnetum, Sphagno-Alnetum*)	**4,29**	-1	-1	-1	0	–	0	-1	1	-0.43
Oak-hornbeam forest (wet-ground variety) (*Tilio-Carpinetum*)	**6.99**	1* -1	-0.5	-0.5	0.5	1	0	0.33	0	0.23* -0.02
Oak-hornbeam forest (dry-ground variety) (*Tilio-Carpinetum*)	**12.29**	1* -1	0	-0.5	0.5	1	0	0.33	0	0.29* 0.04
Light oak forest (*Potentillo albae-Quercetum*)	**1.36**	0.5	0	0	0.5	-	0.5	0.33	1	0.40

* – period before the development of leaves (≈spring), ** – averaged assesment for fungi, bacteria and plant pollen, - – no data

Coniferous forest communities predominate in KNP (about 64% of the Park's forests). The largest area is covered by subcontinental pine-oak forests (*Querco roboris-Pinetum* and *Serratulo-Pinetum*) – 51%, and fresh pine forests (*Peucedano-Pinetum, Leucobrio-Pinetum*) – nearly 9%. Among the deciduous forests (36%), oak-hornbeam forests *Tilio-Carpinetum* (19%) and riparian forest *Fraxino-Alnetum, Salici-Populetum* and *Ficario-Ulmetum* (11%) predominate. Other communities do not account for more than 5% of the Park's area (Table 1).

KNP's forest communities, combined into groups, were described using 8 characteristics (parameters) of the bioclimate of the recreational layer. Each characteristic was then assessed in terms of its impact on human health and well-being. Based on the quantitative data and descriptions presented in a study by KRZYMOWSKA-KOSTROWICKA (1997), each of the eight parameters was given an impact score: "1" (favourable impact); "-1" (unfavourable impact); "0" (neutral impact). Intermediate values were also possible: "0.5" (favourable/neutral) and "-0.5" (unfavourable/neutral) (Table 1). The weight of the parameters was equal. The final score reflecting the assessed impact of a given community on people (i.e. the bioclimate index) was calculated as the mean value of all the parameters. The assessment of aeroplankton took into account the content of plant pollen, fungus spores and bacteria in the air. The possible values of the index of bioclimatic conditions ranged from -1 (extremely unfavourable bioclimate) to 1 (very favourable bioclimate). It was assumed that an index value above 0.2 means a positive impact on the human organism while below -0.2 means a negative impact. Values ranging from -0.2 to 0.2 indicate a neutral impact. For oak-hornbeam forest, separate values were calculated for spring (before the development of leaves) and summer because the sun exposure assessment for these communities is fundamentally different in these seasons of the year (Table 1).

After assessing the impact of each community on human health, maps of bioclimatic conditions in KNP were prepared, separately for the spring and summer season. Against this background, the routes of the tourist trails were presented.

Results and Discussion

Based on the digital data obtained, a map of KNP forest communities was created (Figure 1). It shows the arrangement of the Park's plant communities in the form of alternating, east-west oriented strips. The predominant forest communities are pine-oak forests extending over large, higher-lying sand dune areas. They are accompanied by a small number

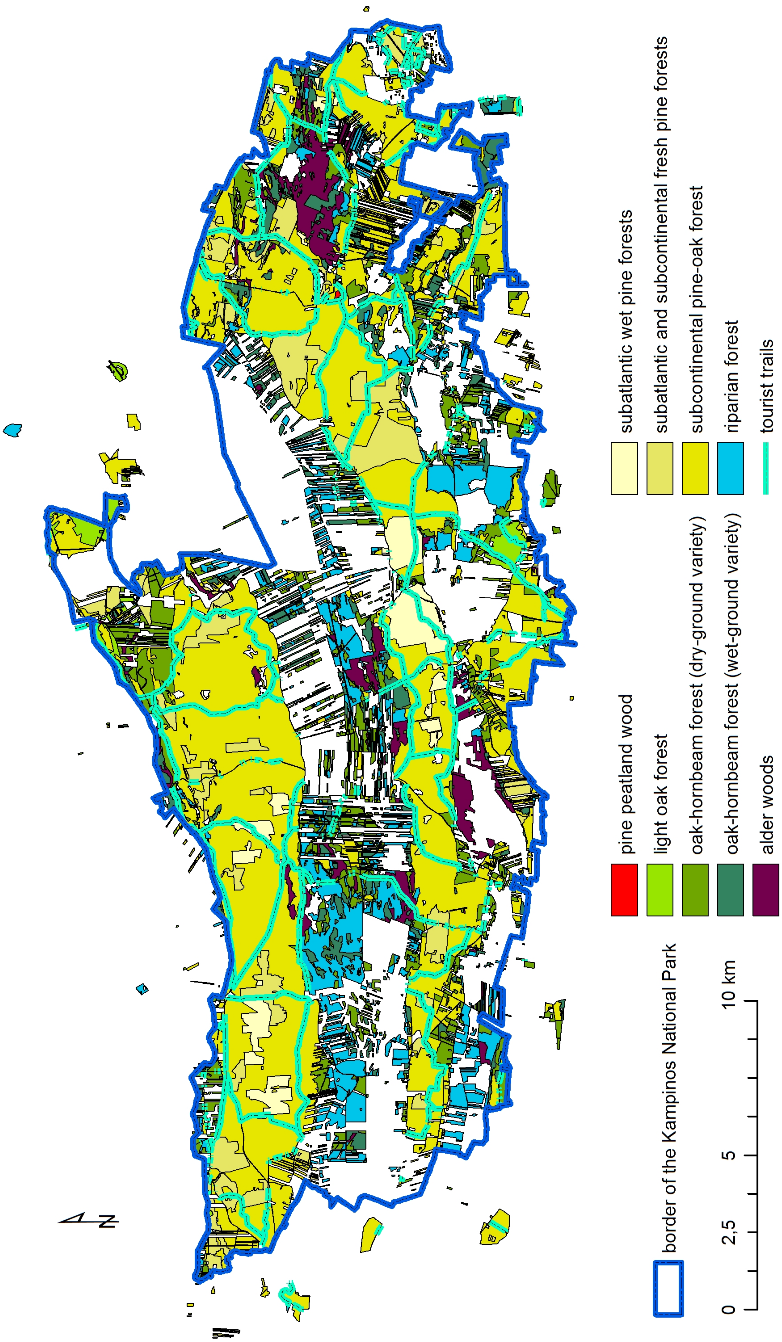

Fig. 1. Forest communities and tourist trails in KNP

of large patches of fresh and wet pine forests. Oak-hornbeam forests grow in the lower-lying, wetter and more fertile areas. In the most humid habitats, in the valleys of the Łasica, Zaborowski and Olszowiecki canals, there are riparian forests and alder woods as well as oak-hornbeam forests (KNP digital data, MOSZYŃSKA 2002). They form a mosaic of numerous small patches of forest communities with the accompanying shrub, meadow and reed communities (Figure 1).

The score of the bioclimate of the recreational layer of KNP forests is in the -0.69 to 0.52 range (Table 1). The best bioclimatic conditions occur in fresh pine forests (sub-oceanic *Leucobryo-Pinetum* and sub-continental *Peucedano-Pinetum*; bioclimate index: 0.52) and pine-oak forests (*Querco roboris-Pinetum typicum, Querco roboris-Pinetum molinietosum, Serratulo-Pinetum*; bioclimate index: 0.46). These communities are characterised by good sun exposure and ventilation, and low humidity. There are no pathogenic bacteria, and the amount of plant pollen is small. On the other hand, there is a large amount of disinfecting essential oils (phytoaerosols). The bioclimate of subcontinental oak-hornbeam forests (typical and wet) varies depending on the season of the year. In spring, their bioclimate is rated as favourable (0.29 and 0.23), while in summer, due to the highly shaded forest floor, the score drops to neutral (0.04 and -0.02) – Table 1. Wet pine forests, *Leucobryo-Pinetum molinietosum* and *Molinio-Pinetum*, also received a neutral score with regard to the bioclimatic conditions (-0.04). Unfavourable bioclimatic conditions occur in wet habitats: pine peatland wood *Vaccinio uliginosi-Pinetum* (-0.69) as well as riparian forest (*Fraxino-Alnetum, Salici-Populetum, Ficario-Ulmetum typicum*) and alder woods (*Ribeso nigri-Alnetum, Sphagno-Alnetum*) communities with a score of -0.43. The conditions in these forests are not suitable for longer stays due to high air humidity, limited ventilation, high concentration of volatile substances as well as animal-related nuisance (mainly bothersome insects) – Table 1.

However, forests with favourable bioclimatic conditions predominate in KNP, accounting for 81% of its forest area in spring and 62% in summer. This variation is related to oak-hornbeam forests whose score in the summer season drops to the neutral value range. 4% of the forests in spring and 23% in summer have a bioclimate that is neutral to human health and well-being. Regardless of the season, communities with an unfavourable bioclimate cover 15% of the Park's forest area (Figure 2).

A spatial analysis of the distribution of bioclimatic conditions in KNP shows their characteristic strip-like pattern resulting from the distribution of the communities and, indirectly, the humidity level in a particular area. Unfavourable conditions occur in depressions between the strips of sand dunes (Figure 3).

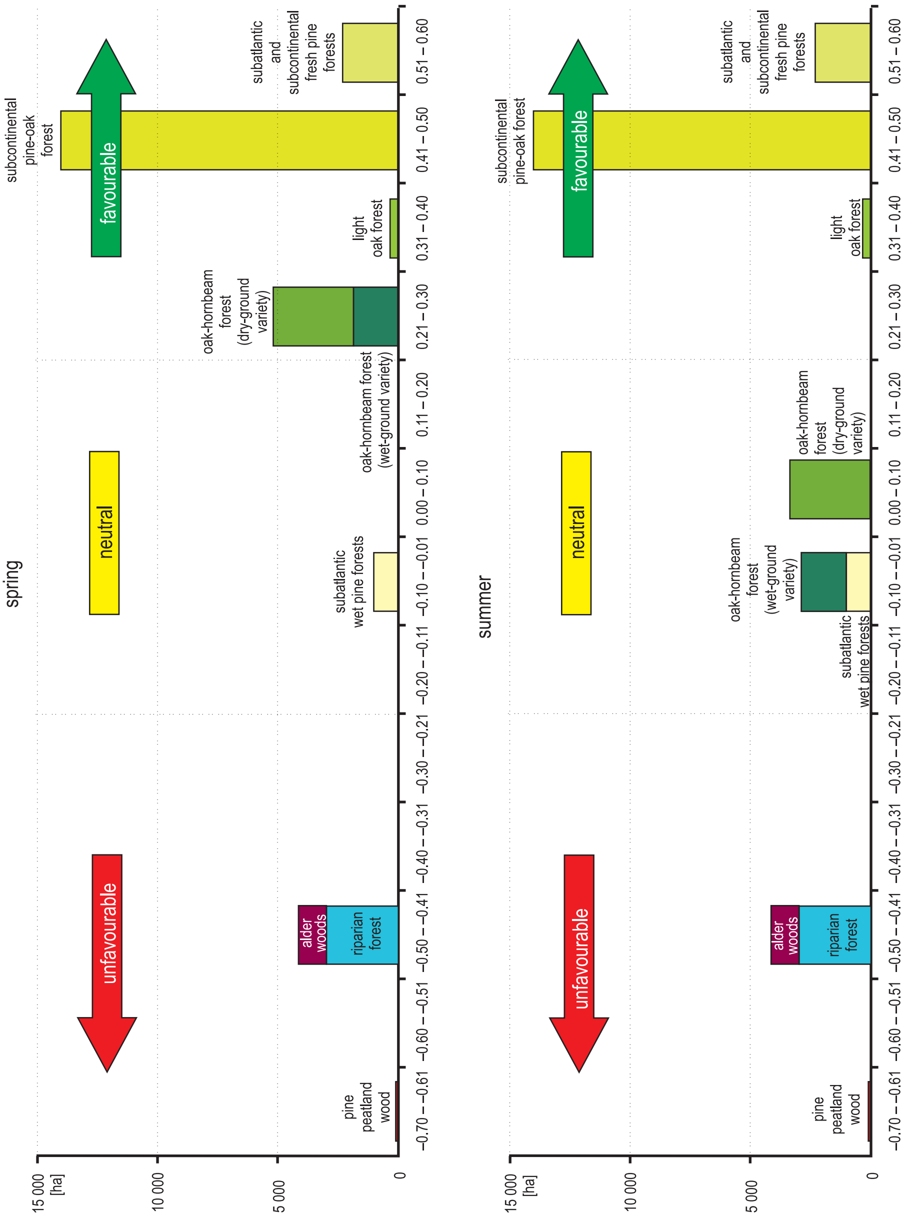

Fig. 2. Bioclimate score ranges for KNP forest communities in spring and summer

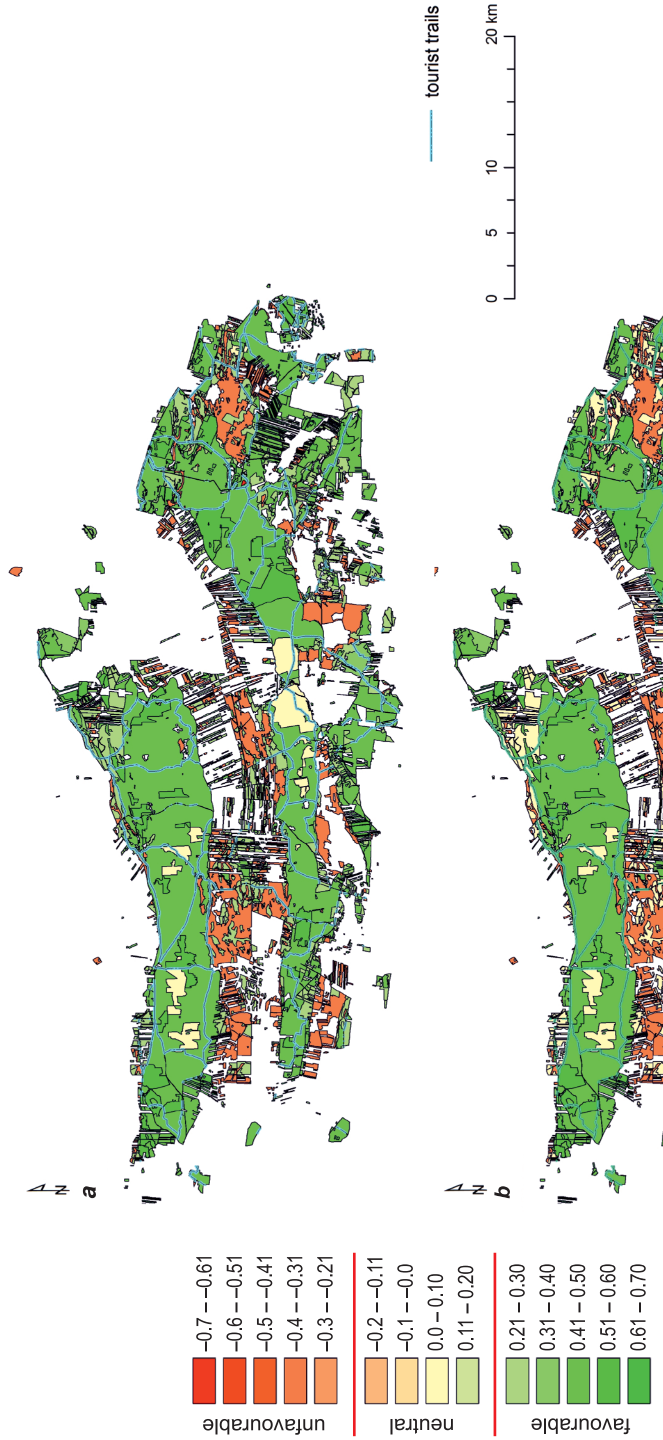

Fig. 3. The distribution of tourist trails in relation to the bioclimatic conditions of KNP forest communities in: *a* – spring and *b* – summer

For the purposes of this study, 270 km of tourist trails passing through forest communities (with forest on both sides of the trail) were analysed. The trails are distributed quite evenly across the entire area of the Park, even though they mostly run across drier, higher-lying areas (Figure 3). The distribution of the trails corresponds to the strip-like pattern of Kampinos landscapes. Most of the east-west oriented trails lead through the most attractive sites in the Park, while the others have a connecting function. The trails pass through 8 out of 9 groups of communities distinguished (all except pine peatland wood). Subcontinental pine-oak forests occur along most of the forest stretches of the trails (67% of their length) (Figure 1). Its bioclimatic conditions were rated as favourable (0.46). However, fresh pine forests, occurring along nearly 8% of the length of the trails, received the highest bioclimate score (0.52) – Table 1. These two coniferous forest communities with the highest score have a slightly different impact on human health and well-being. The climate of pine forests is definitely unifunctional and has a therapeutic effect on respiratory tract diseases. The numerous essential oils occurring there have disinfecting properties, thanks to which the air is free of pathogenic factors. Furthermore, staying in pine forests reduces blood pressure and tones up the nervous system. Thus, long stays in these communities are not recommended to older people, persons with low blood pressure, hypothyroidism, or prone to migraines (particularly on hot and windless days). Bioclimate in pine forests also has a short-term numbing effect and temporarily slows down reflexes (KRZYMOWSKA-KOSTROWICKA 1997, MOSZYŃSKA 2000, FORNAL 2004). Pine forests occur along more than 20 km of KNP trails. The bioclimate of pine-oak forests received a slightly lower score, but it is much more universal. This community has a soothing effect in terms of biotherapeutic and psychoregulatory properties. Large amounts of essential oils, including phytoncides, occur there. Furthermore, allergens and animal-related nuisance pose a low risk. Owing to the above properties and lower intensity of stimuli, the bioclimate of pine-oak coniferous forests is suitable for persons of different age and in different health condition (KRZYMOWSKA-KOSTROWICKA 1997, MOSZYŃSKA 2000, BASTEK and PŁOSZAJ-WITKOWSKA 2016). It is important particularly because these communities received a high recreational usefulness score in surveys based on the age of the tree stand and forest habitat type (CZUBASZEK et al. 2014). 180 km of tourist trails pass through the pine-oak forests in KNP.

A high rating of bioclimatic conditions was also given to light oak forests that occur only along short stretches of the trails (less than 8 km). The bioclimatic conditions of the oak forests are universally favourable to everyone regardless of age and health, except for people allergic to pollen.

Oak forests have a strong bactericidal and stimulating effect, and strengthen the human immune system (KRZYMOWSKA-KOSTROWICKA 1997, BASTEK and PŁOSZAJ-WITKOWSKA 2016). Furthermore, thanks to their strong filtering and detoxifying properties, these forest communities absorb particulate matter and heavy metals (MOSZYŃSKA 2000).

77% of KNP's tourist trails pass through the described communities with favourable bioclimatic conditions in summer. In spring, the length of trails with favourable conditions increases to 94% thanks to the numerously represented oak-hornbeam forests (45 km) – Figure 4. The subcontinental oak-hornbeam forest (typical and wet) is a community where bioclimatic conditions change from favourable in spring to neutral in summer. In spring, the effect of these forests is universally positive. Above all, they have a stimulating and antiseptic effect, enhancing the immunity of the human organism, supporting blood circulation, and increasing blood pressure. However, owing to the high intensity of stimuli, longer stays in oak-hornbeam forests are not recommended to people with hypertension, hyperthyroidism, and those in a state of emotional agitation (MOSZYŃSKA 2000, FORNAL 2004, BŁAŻEJCZYK and KUNERT 2011, FALENCKA-JABŁOŃSKA 2012). In summer, the comfort of staying in oak-hornbeam forests is reduced due to the dense tree crowns inhibiting ventilation and access of solar radiation as well as the presence of ticks, mosquitoes and gadflies. In addition, neutral bioclimatic conditions occur all year around in sub-Atlantic wet pine forests that grow along circa 8 km of the tourist trails in KNP. In total, bioclimate rated as neutral occurs along 3% of the Park's trails in spring and 20% in summer (Figure 4).

The bioclimate of communities occurring in the wet habitats of riparian forests and alder woods was rated as unfavourable to human health and well-being. The conditions in these forests are not suitable for longer stays due to high air humidity and limited ventilation, i.e. conditions that have a strong straining effect on heart action. Furthermore, high concentrations of volatile substances, pollen and fungus spores, and large numbers of insects occur there (KRZYMOWSKA-KOSTROWICKA 1997, MOSZYŃSKA 2000). CZUBASZEK et al. (2014) report that areas with alder woods, particularly those of lower age classes, are unsuitable for rest and recreation. Despite the unfavourable bioclimatic conditions and poor suitability for recreation, riparian forest and alder woods communities show the highest efficiency in absorbing particulate matter, gases and heavy metals; they also have very strong bactericidal properties (MOSZYŃSKA 2012). Short stretches of KNP trails, totalling just over 8 km (only 3% of the total length of the Park's forest trails) pass through the wet communities mentioned above.

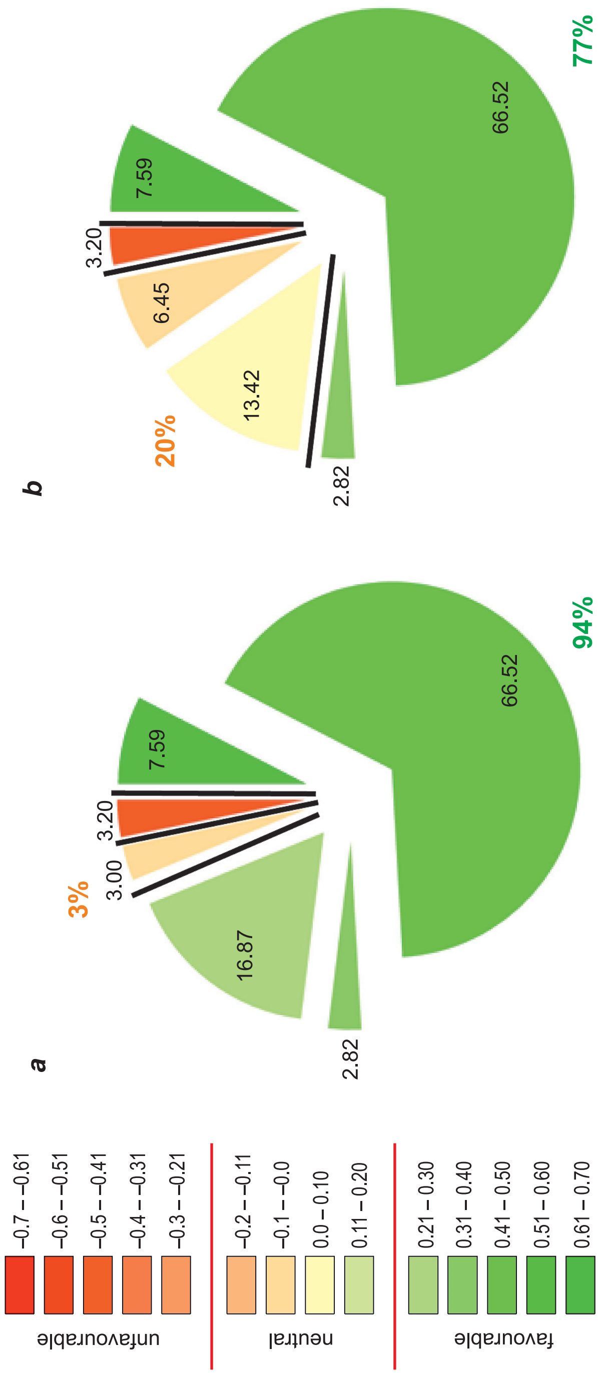

Fig. 4. Percentage share of the length of tourist trails in bioclimate ranges for KNP forest communities *a* – spring, *b* – summer

Thus, the distribution of tourist trails in relation to the bioclimatic conditions of KNP forest communities is very advantageous in terms of their close proximity to Warsaw as well as intensive tourist and recreational use. The proximity of sites conducive to leisure and enabling contact with nature and a clean environment makes suburban forest areas very attractive from the perspective of recreation (PAWŁOWICZ and SZAFRANKO 2014). By improving the microclimate conditions, green areas within residential areas have a positive influence on human health, which is confirmed, for example, in studies by KARDAN et al. 2015. However, not every town or housing development has a sufficient share of green areas. In such cases, the proximity of forest areas that people can visit frequently is a great asset. Along with the growing awareness of the benefits of contact with a forest environment, its therapeutic properties conducive to the development of tourism and recreation are also gaining importance (GRZYWACZ 2011, MARSZAŁEK 2010).

Furthermore, large urbanised areas such as the Warsaw metropolitan area face the problem of environmental pollution. Particulate matter, gas pollutants and noise are the biggest nuisance to people. Forest areas located close to cities enable an improvement of air parameters as well as the health and mental state of residents. Alongside its recreational and tourist function, KNP plays the role of "Warsaw's green lungs". Therefore, a greater emphasis should be placed on the development of health-promoting nature tourism. KNP's tourism offer should be expanded to include education and promotion of the health benefits of staying in forest communities. The importance of the awareness of the health-promoting values of this area in the context of tourism was discussed by MOSZYŃSKA (2000, 2012), among other authors. The society should be made aware of how forest therapy can benefit human health and well-being, and the forms and possibilities of using this kind of therapy on an everyday basis (walking, cycling, roller-skating, Nordic walking) should be indicated. Based on the health benefits and attractiveness of the forest environment where people can restore their vitality and improve their health, it is possible to develop forest tourism products used in forest therapy.

Conclusions

In Kampinos National Park, the best bioclimatic conditions occur in the following coniferous forest communities: fresh pine forests (*Peucedano-Pinetum*, *Leucobryo-Pinetum*) and pine-oak forests (*Querco roboris-Pinetum typicum*, *Querco roboris-Pinetum molinietosum*, *Serratulo-Pine-*

tum). In total, they cover more than 60% of the Park's area. Unfavourable bioclimatic conditions occur in marshy coniferous forests (*Vaccinio uliginosi-Pinetum*) as well as riparian forest (*Fraxino-Alnetum*, *Salici-Populetum*, *Ficario-Ulmetum typicum*) and alder woods (*Ribeso nigri-Alnetum*, *Sphagno-Alnetum*) communities. These communities cover a small area accounting for 15% of the Park's forests. Most of the tourist trails in KNP pass through communities with favourable bioclimatic conditions: most of them (67%) pass through the subcontinental pine-oak forest. Bioclimate beneficial to people occurs along 94% of the length of KNP trails in spring and 77% of the trails in summer. Unfavourable bioclimatic conditions occur along 3% of the length of KNP trails regardless of the season during which the investigation is conducted. The distribution of tourist trails in relation to the bioclimatic conditions of KNP forest communities is very advantageous from the perspective of tourism and recreation. The proximity of sites conducive to leisure and enabling contact with nature and a clean environment is extremely important for residents of large urbanised areas. Therefore, emphasis should be placed on the development of health-promoting nature tourism. KNP's tourism offer should be combined with education and promotion of the health benefits of staying in the particular forest communities. Based on the attractiveness of the forest environment where people can restore their vital strength and improve their health, it is possible to develop forest tourism products used in forest therapy.

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