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TABLE OF CONTENTS

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EVALUATION OF QUANTITATIVE, QUALITATIVE TRAITS AND COMPATIBILITY OF RICE GENOTYPES IN TWO REGIONS OF IRAN

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Key words: adaptation, amyloze, grain yield, rice.

Abstract

In order to evaluate quantitative and qualitative traits and compatibility of rice cultivars, an experiment was conducted with 30 rice genotypes in a randomized complete block design with four replications in two regions of Mazandaran Province (Amol and Sari) in 2018–2019. The results of composite variance analysis of data showed that the interaction between genotype and location on all quantitative and qualitative traits was significant except for grain yield. A significant difference was found between the studied genotypes in terms of grain yield, indicating genetic diversity of the studied genotypes. The results showed that Amol II, Dasht and Keshvari cultivars had acceptable grain yield and desirable quality characteristics. The grain yield had a positive and direct correlation with traits of the plant height, panicle length, number of fertile tillers per hill and Thousand grain weight, but this correlation was not significant.

Introduction

Rice is the staple food of more than 2 billion people in Asia, providing about 80% of energy needs from rice. Rice contains 80% carbohydrates, 7–8% protein, 3% fiber and 3% fat (KADAM et al. 2018). The interaction between genotype and locations refers to the relative yield of cultivars among different locations, which indicates differences in the ranking of genotypes, or in other words, differences in the level of expression of genetic differences between locations (LI et al. 2017). In order to identify rice genotypes with relatively wide compatibility, studies on the amount and patterns of interaction between genotype and locations are of great importance (SHARIFI et al. 2017). In order to select genotypes to increase grain yield, yield-related traits should be considered due to the complexity

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and relationship between yield and yield components (OLADOSU et al. 2018). Yield is a complex trait that is controlled by many factors such as polygenesis, locations, and genetic diversity (USMAN et al. 2017).

The yield of rice genotypes will fluctuate significantly with changing environmental conditions (BOSE et al. 2014). The choice of genotype is necessary based on the evaluation of yield stability from the mean yield under different environmental conditions (ISLAM et al. 2016). Determining the yield of a genotype is determined by the effects of the genotype and locations and their interaction (YAN et al. 2007). By evaluating the compatibility of 30 rice genotypes to the climatic conditions of West Gilan during the two years of cultivation, Saeedzadeh reported that a significant difference was found between the genotypes in terms of most of the studied traits. Among the studied genotypes, the traits of the number of tillers per plant, the yield of each plant and harvest index showed a positive and significant correlation with grain yield (SAEEDZADEH 2010).

Other researchers studied improved rice genotypes in different parts of Indonesia and found that grain yield was affected by the effects of genotype, location, and interaction between genotype and location (TARIKU 2017). MOMENIZADEH et al. (2015) in the study of the interaction between rice genotype and locations in Mazandaran reported that the interaction between genotype and locations for the traits of the number of hollow grains per ear, the capacity of hollow grain, total number of grains, total grain capacity and harvest index was significant, indicating different reactions of genotypes from one place to another.

The researchers by evaluating the interaction between genotype and locations on grain yield of promising rice improved lines in different regions of Mazandaran Province during three years reported that a significant difference was found between genotypes in terms of studied traits such as the plant height, number of ears per plant, number of grains per ear and grain yield in most places (MOMENI et al. 2018). By examining the compatibility of different rice genotypes to different locations in southern China and Laos, ZHANG et al. (2017) reported that PR23 genotype with the highest yield and compatibility to the locations was selected as the top genotype for cultivation in humid and semi-humid areas as well as for grain production and forage in intercropping systems. In general, identifying high and stable yield genotypes in different locations, locations, seasons, and years can be of great help to breeders in recommending identified genotypes to farmers for cultivation. Therefore, this experiment was performed with the aim of evaluating the compatibility of 30 rice genotypes in two regions of Mazandaran Province to identify the best genotypes in terms of grain yield and quality.

Material and Methods

Experimental characteristics

In order to evaluate quantitative and qualitative traits and compatibility of rice genotypes, we compared 30 rice genotypes in the form of complete randomized design with four replications in two regions of Mazandaran Province (Field of the Department of Rice Research Institute of the country-Amol and Research Field of the Institute of Genetics and Biotechnology, University of Agricultural Sciences and Natural Resources of Sari) in 2018–2019 (Figure 1 and Figure 2). Amol Rice Research Institute is located between (36°28'N, 52°23'E), and at an altitude of 23 m above sea level. Sari University of Agricultural Sciences and Natural Resources is located between (36°39′ N, 53°4′E) and at an altitude of 11 m above sea level.

Fig 1. Cultivation of genotypes in University of Agricultural Sciences and Natural Resources

Fig 2. Cultivation of genotypes in Amol Rice Research Institute

Before performing the experiment, the soil of the experimental regions was sampled, the results of which were presented in Table 1. The profile of the genotypes examined in this experiment is also shown in Table 2.

Table 1

The names of the rice cultivars studied in the experiment

No	Genotypes	No	Genotypes
16	Doiar	1	Amol 1
17	IR42	2	Sepidrud
18	Fuji Minori	3	Khazar
19	Onda	4	pnd160-2-1
20	1-2-299-13429	5	Amol 2
21	IR36	6	Amol 3
22	Tabesh	7	Fajr
23	IR24	8	Usen
24	Pouya	9	Line 101
25	Keshvari	10	Nemat
26	Taichung 65	11	346
27	Senyu-285	12	Dasht
28	Neda	13	Bijar
29	Champa	14	BA370
30	IR56	15	Tetep

Table 2

Physico-chemical properties of soil Experimental areas at a depth of 0 to 30 cm soil

Parameter	Location	
–	Amol	Sari
Soil texture	Lum-Silti	Clay-Silti
[%]		
Clay	27	35
Silt	45	49
Sand	28	16
[mg kg^{-1}]		
P	8.5	8.7
K	100	109.7
([%]		
Organic matter	2.8	2. 1
[pH]		
Acidity total saturation	7	1.44
EC [dS m^{-1}]		
Electrical conductivity	7.2	1.84

During physiological maturity, 12 plants were randomly selected from each experimental plot and traits such as the plant height, panicle length, number of effective tillers per hill, number of filled grains, 1000 grain weight and grain yield were measured. Grain yield was calculated by harvesting an area of 4 square meters from each experimental unit after removing the marginal effects and based on 14% humidity. Some physical and chemical quality traits were also measured in the laboratory. The conversion efficiency was calculated using the ratio of the amount of white rice to the total initial rough rice. In order to measure the determinant traits of grain quality, such as amylose content, the methods of Juliano (JULIANO 1971), gelatinization temperature by LITTLE et al. (1958) and gel consistency, CAGAMPANG et al. (1973) were used. Data analysis of compound variance was performed using software MSTATC and mean comparison (LSD) based on the test of the least significant difference at the probability level of 5%. The correlation coefficient of the traits as well as the cluster analysis of the studied genotypes were performed using software SPSS.

Results

Plant height

The results showed that in general, the genotypes in Sari had a higher plant height compared to Amol, which indicates a different reaction of the genotypes studied in the two experimental sites. It seems that the better condition of the soil in Sari in terms of percentage of nutrients and organic matter has led to improved plant growth compared to Amol. The minimum plant height in Amol and Sari was observed with means of 65.75 and 97.75 cm, respectively, in genotypes 26 and 1 (Table 3). Long-legged varieties are more sensitive to weeds, so in addition to problems with crop harvesting, yield of these varieties is reduced. Diversity in plant height of rice is considered as one of the most important basic factors due to the interaction between genotype and location (NASSIR ARIYO 2011, SANDHU et al. 2019).

Panicle length

The length of the panicle in Sari was higher than in Amol (Table 3). The significant interaction between genotype and location for the length of the panicle indicates different reactions of genotypes to factors such as soil physical and chemical properties, latitude and longitude and altitude, which caused differences in the length of the panicle in the studied genotypes.

Table 3

Comparison of average quantitative traits of rice cultivars in two regions of Mazandaran province

No	Plant height		Panicle length		Number of effective tillers		Number of filled grains		Thousand grain weight		Grain yield		Average grain yield
	Amol	Sari	Amol	Sari	Amol	Sari	Amol	Sari	Amol	Sari	Amol	Sari	Amol and Sari
1	$114.5^{k–n}$	$97.75^{q–v}$	28.50^{hij}	19.50^{z}	$18.50^{h–n}$	12.50^{st}	174.3^{b}	78.50^{wx}	$24.09^{a–i}$	$24.41^{a–h}$	$7260^{b–g}$	$6971^{b–j}$	7116^{bc}
2	$108.5^{m–p}$	$109.0^{m–p}$	$25.13^{o–r}$	$26.00^{l–q}$	$22.00^{c–g}$	8.250^{u}	157.3^{cd}	141.5^{efg}	$24.08^{a–i}$	18.23^{p}	$6024^{i–r}$	$5766^{l–v}$	$5895^{e–j}$
3	$108.0^{m–q}$	175.3^{ab}	$26.25^{l–p}$	32.25^{de}	$22.25^{c–f}$	$16.00^{m–r}$	169.0^{bc}	143.0^{efg}	$25.00^{a–g}$	18.66^{op}	$5804^{l–u}$	$5572^{n–w}$	$5688^{f–k}$
4	$115.0^{k–n}$	119.5^{jkl}	$25.63^{m–q}$	35.75^{a}	$17.75^{i–p}$	$17.75^{i–p}$	149.8^{def}	$95.25^{s–v}$	27.05^{a}	$20.44^{l–p}$	$6987^{b–i}$	$6680^{c–m}$	$6834^{b–e}$
5	121.3^{ijk}	127.0^{ij}	$26.38^{l–o}$	35.00^{ab}	$17.25^{j–p}$	$17.00^{k–q}$	$114.5^{k–o}$	138.8^{fgh}	$24.75^{a–g}$	$21.16^{h–p}$	7553^{abc}	$7335^{a–f}$	7444^{ab}
6	$112.3^{k–o}$	177.0^{a}	$27.50^{i–l}$	28.25^{ij}	25.50^{ab}	26.25^{ab}	$98.75^{r–u}$	$119.8^{j–n}$	$25.24^{a–e}$	25.75^{abc}	7026b–i	$6755^{c–l}$	$6890^{b–e}$
7	122.0^{ijk}	134.3^{hi}	$24.50^{q–t}$	32.25^{de}	$19.00^{g–m}$	13.50^{rst}	79.50^{wx}	$121.0^{i–m}$	$24.75^{a–g}$	$23.10^{c–m}$	$7235^{b–g}$	$6890^{c–k}$	7063^{bcd}
8	$97.0^{r–w}$	121.3^{ijk}	$26.38^{l–o}$	30.00^{gh}	$16.50^{l–r}$	$17.50^{i–p}$	88.00^{uvw}	$117.5^{j–o}$	$23.86^{a–k}$	$20.35^{l–p}$	$6926^{c–j}$	$6723^{c–l}$	$6825^{b–e}$
9	$105.3^{n–s}$	155.3^{c-f}	23.88^{r-u}	28.50^{hij}	16.00^{m-r}	27.00^{a}	115.0^{k-o}	111.5^{m-q}	24.25^{a-h}	20.60^{k-p}	5829^{k-t}	5568^{n-w}	5699^{f-k}
10	133.5^{hi}	154.5^{def}	$23.38^{s–v}$	32.50^{cde}	$22.00^{c–g}$	$21.00^{d–h}$	74.00^{x}	127.0^{hij}	$23.88^{a–j}$	$22.02^{e–o}$	$7265^{b–g}$	$6960^{c–j}$	7112^{bc}
11	137.5^{gh}	148.0^{ef}	$27.13^{j–m}$	32.50^{cde}	25.75^{ab}	$17.00^{k–q}$	$97.75^{r–u}$	158.5^{cd}	$24.00^{a–j}$	$20.92^{i–p}$	$6117^{i–q}$	$5906^{j–s}$	$6011^{d–i}$
12	128.8^{hij}	134.5^{hi}	$26.13^{l–p}$	$27.50^{i–l}$	$19.75^{f–k}$	$15.25^{o–s}$	$119.3^{j–n}$	140.8^{efg}	$21.88^{f–o}$	$24.67^{a–g}$	$7482^{a–d}$	$7198^{b–h}$	7340^{ab}
13	88.75^{vw}	149.8^{ef}	21.00^{xyz}	30.50^{fg}	$18.00^{h–o}$	$14.75^{p–t}$	138.0^{fgh}	$121.8^{i–m}$	$25.63^{a–d}$	$24.38^{a–h}$	$5780^{l–v}$	$5605^{m–w}$	$5693^{f–k}$
14	$106.5^{m–r}$	$105.0^{n–s}$	$24.50^{q–t}$	34.00^{bc}	$17.50^{h–p}$	$20.50^{d–i}$	147.3^{def}	144.5^{ef}	$24.75^{a–g}$	$23.90^{a–j}$	$6326^{e–o}$	$6191^{g–p}$	$6259^{c–g}$
15	$102.8^{o–t}$	157.0^{cde}	$24.80^{p–s}$	$27.25^{i–l}$	$18.00^{h–o}$	$19.50^{f–l}$	93.25^{tuv}	$97.00^{r–v}$	$24.99^{a–g}$	$23.39^{c–m}$	$4959^{r–w}$	4721^{vw}	4840^{ijk}
16	$113.0^{k–o}$	122.0^{ijk}	$23.88^{r–u}$	34.25^{ab}	$17.50^{i–p}$	$17.75^{i–p}$	128.8^{hij}	$120.0^{i–m}$	$21.75^{g–o}$	$23.39^{c–m}$	$4825^{t–w}$	4713^{vw}	4769^{k}
17	$96.75^{r–w}$	145.0^{fg}	21.50^{wxy}	$26.00^{l–q}$	$16.50^{l–r}$	$21.75^{c–g}$	$102.3^{p–t}$	$100.8^{q–t}$	$22.00^{e–o}$	$22.42^{d–n}$	$5709^{l–v}$	$5483^{n–w}$	$5596^{f–k}$
18	$93.75^{t–w}$	$110.3^{l–p}$	23.13^{tuv}	31.25^{efg}	$16.50^{l–r}$	$15.75^{n–r}$	92.25^{tuv}	$120.8^{i–m}$	$22.62^{c–n}$	$23.00^{c–m}$	$5104^{q–w}$	$4969^{r–w}$	5036^{ijk}
19	92.25^{uvw}	165.5^{bc}	$22.38^{u–x}$	$26.00^{l–q}$	$15.75^{n–r}$	$20.25^{e–j}$	$119.8^{j–n}$	$108.0^{n–r}$	$24.25^{a–h}$	$23.28^{c–m}$	$5286^{o–w}$	$5146^{p–w}$	$5216^{g–k}$
20	$104.0^{o–t}$	115.8^{klm}	22.88^{uvw}	$25.00^{o–r}$	$18.00^{h–o}$	12.5^{0st}	$120.3^{i–m}$	85.50^{vwx}	$25.66^{a–d}$	$21.91^{f–o}$	$5764^{l–v}$	$5614^{m–w}$	$5689^{f–k}$
21	90.0^{vw}	149.0^{ef}	$23.38^{s–v}$	$27.00^{j–n}$	24.25^{abc}	$17.50^{i–p}$	131.8^{ghi}	$100.5^{q–t}$	27.00^{ab}	$23.40^{c–l}$	$6356^{e–o}$	$6125^{h–q}$	$6240^{c–g}$
22	$95.0^{s–w}$	183.8^{a}	22.88^{uvw}	33.75^{bcd}	$22.00^{c–g}$	$16.25^{m–r}$	177.3^{ab}	127.3^{hij}	$23.75^{b–k}$	$23.12^{c–m}$	$6304^{f–o}$	$6071^{i–q}$	$6188^{c–h}$
23	$103.3^{o–t}$	$101.5^{p–u}$	$26.50^{k–o}$	$27.50^{i–l}$	$16.50^{l–r}$	11.75^{t}	125.5^{ijk}	138.8^{fgh}	$24.65^{a–g}$	$23.31^{c–m}$	$6656^{c–m}$	$6425^{d–n}$	$6540^{b–f}$
24	87.0^{w}	$103.0^{o–t}$	22.00^{vwx}	$25.50^{n–q}$	24.75^{abc}	$14.00^{q–t}$	$125.0^{i–l}$	$96.50^{r–v}$	$25.13^{a–f}$	$20.14^{m–p}$	$5343^{o–w}$	$5211^{p–w}$	$5277^{g–k}$
25	90.0^{vw}	156.8^{cde}	22.13^{vwx}	33.50^{bcd}	27.25^{a}	13.75^{rst}	148.3^{def}	188.8^{a}	$24.75^{a–g}$	$20.80^{j–p}$	7648^{abc}	$7396^{a–e}$	7522^{ab}
26	65.75^{x}	161.8^{cd}	20.25^{yz}	28.75^{hi}	$23.25^{b–e}$	$15.75^{n–r}$	93.00^{tuv}	$101.3^{q–t}$	$24.75^{a–g}$	$21.76^{g–o}$	$5197^{p–w}$	$5056^{q–w}$	$5126^{h–k}$
27	89.50^{vw}	175.0^{ab}	19.00^{z}	31.75^{ef}	$21.00^{d–h}$	$17.25^{j–p}$	$99.50^{r–u}$	$113.3^{l–p}$	$23.71^{c–k}$	19.68^{nop}	$5371^{n–w}$	$5150^{p–w}$	$5260^{g–k}$
28	$101.0^{p–u}$	154.3^{def}	$25.63^{m–q}$	$26.50^{k–o}$	23.50^{bcd}	24.50^{abc}	91.50^{tuv}	79.25^{wx}	$23.91^{a–j}$	$23.42^{c–l}$	8353^{a}	8043^{ab}	8198^{a}
29	$101.8^{p–u}$	$112.5^{k–o}$	$25.63^{m–q}$	$24.50^{q–t}$	24.25^{abc}	26.00^{ab}	172.8^{b}	150.8^{de}	$23.60^{c–l}$	$22.92^{c–n}$	4738^{uvw}	4583^{w}	4660^{k}
30	105.0^{n-s}	151.5^{def}	28.00^{ijk}	31.75^{ef}	24.25^{abc}	$17.75^{i–p}$	174.8^{b}	$106.5^{o–s}$	$24.65^{a–g}$	$23.54^{c–l}$	$5175^{p–w}$	$4912^{s–w}$	5043^{ijk}

Similar letters in each column shows non- significant difference according to Duncan test at 5% level

Increasing the length of the panicle alone cannot be an important advantage unless it is accompanied by an increase in the number of florets and grains in the panicle (MENG et al. 2015). Studies by ZHANG et al. (2017) also show that a statistically significant difference was found in the length of rice panicle between the different genotypes studied and different experiment sites.

Number of effective tillers

The results showed that different experimental sites had different effects on the studied genotypes in terms of the number of effective tillers per hill, so that genotype 2, which had the lowest number of tillers among genotypes in Sari, had increased number of tillers by about 62.5% in Amol (Table 3). This diversity may be due to changes in topography (PENG et al. 2006), soil type, fertility and soil organic matter (GAO et al. 2006), as well as irrigation regimens, nutrient rounds, accessibility and nutrient uptake (KREYE et al. 2009). The researchers stated that the interaction between genotype and location was significant for the number of tillers, and that different genotypes had different numbers of tillers in experimental sites, which was consistent with the results of this experiment (POLI et al. 2018). The number of tillers per hill is directly related to grain yield per hectare, so that breeders focused on increasing the number of tillers per hill to increase grain yield of early matured genotypes in rainfed and irrigated regions (OLADOSU et al. 2018).

Number of filled grains

The results of mean comparison of the interaction between genotype and location showed that the highest number of filled grains with a mean of 188.8 grains related to genotype 25 (Keshvari cultivar) in Sari with genotype 22 (Tabesh cultivar) in Amol had no statistically significant difference (Table 3). The researchers reported that the effects of the genotypes under study, the environment and the interaction of the genotype and the environment on the number of feathers in the cluster were significant at the 1% probability level (OLADOSU et al. 2018). The researchers added that the number of filled grains is one of the criteria for selecting a genotype to improve rice grain yield. In similar results to the present study, SHARIFI and AMINPANAH (2017) reported that a significant difference was found between the studied genotypes in terms of the number of filled grains. The researchers stated that among 9 genotypes studied, genotype 6 was selected as the best genotype with a mean of 139.89 number of filled

grains. High genetic diversity coefficients have been reported by researchers for the number of filled grains (BITEW 2016, RUKMINI DEVI et al. 2016).

Thousand grain weight

The results of mean comparison of the interaction between genotype and location showed that the maximum Thousand grain weight with a mean of 27.05 g belonged to genotype 4 (pnd160-2-1) in Amol, which with other genotypes in this region except genotypes 12, 16, 17, 18, 22, 27 and 29 showed no statistically significant difference (Table 3). The highest 1000 grain weight in Sari (25.75 grams) was related to genotype 6 (Amol III) which was grouped with statistical genotypes in this region. The minimum 1000 grain weight with a mean of 18.23 g belonged to genotype 2 (Sepidroud) in Sari (Table 4). ZHANG et al. (2017) and OLADOSU et al. (2018) reported similar results with this study on the significant difference in 1000-grain weight between different genotypes and experimental sites. 1000 grain weight is a trait that has a lot to do with grain yield (BALAKRISHNAN et al. 2016).

Grain yield

The results of mean comparison of data showed that the maximum mean grain yield with a mean of 8198 kg/ha belonged to genotype 28 (Neda cultivar) which was not statistically different from genotypes 5 (Amol II cultivar), 12 (Dasht cultivar) and 25 (Keshvari cultivar). Neda cultivar produced a high number of fertile tillers in both Amol and Sari, which could be a reason for the increase in grain yield in this genotype (Table 3). The number of fertile tillers per hill is one of the important components determining grain yield, and paying attention to this functional component is one of the basic issues before successful breeding plans (OLADOSU et al. 2018). A significant difference was found in yield between the studied genotypes, which indicates high genetic diversity for the selection of genotypes. Other researchers also found that a statistically significant difference was found between different genotypes studied in terms of grain yield (ZHANG et al. 2017), which was consistent with the study results. Different genotypes have high diversity in terms of mean production yield (NASSIR ARIYO 2011, POLI et al. 2018). Significant differences between genotypes in terms of the amount of crop produced have also been reported in the results of other researchers (NASSIR ARIYO 2011, POLI et al. 2018).

Table 4

Comparison of average quality characteristics of rice cultivars in two regions of Mazandaran province

No	Amylose content		Consistency gel		Gelatinization temperature	
–	location					
	Amol	Sari	Amol	Sari	Amol	Sari
1	$23.00^{l–q}$	$21.35^{s–w}$	60.38^{rst}	59.00^{st}	$3.685^{n–t}$	2.670^{z}
2	$24.63^{d–i}$	$22.88^{m–q}$	60.50^{rst}	60.25^{rst}	2.883^{xyz}	$3.200^{t–y}$
3	20.25^{x}	20.40^{wx}	69.25^{ij}	72.00^{gh}	2.543^{z}	$3.415^{o–w}$
4	$25.17^{b–e}$	25.73^{bc}	56.00^{u}	55.25^{u}	$3.050^{w–z}$	$3.000^{w–z}$
5	$23.55^{k–p}$	$25.00^{c–f}$	70.75^{hi}	$62.50^{m–q}$	$3.105^{v–z}$	$3.365^{p–x}$
6	$22.70^{o–r}$	$22.80^{m–r}$	38.50^{z}	42.50^{z}	$3.152^{u–z}$	$3.315^{q–y}$
7	$21.83^{r–u}$	$23.02^{l–q}$	82.35^{c}	72.95^{fg}	5.925^{de}	$4.520^{g–j}$
8	$21.20^{t–x}$	$22.85^{m–r}$	63.50^{lm}	65.50^{k}	$3.890^{l–o}$	$3.822^{l–p}$
9	$23.95^{g–l}$	$24.30^{e–k}$	$61.00^{o–r}$	63.00^{mn}	$3.780^{m–r}$	$4.250^{i–m}$
10	$25.13^{b–f}$	$24.88^{c–g}$	45.00^{y}	48.75^{wx}	$3.250^{s–y}$	$3.625^{n–u}$
11	$22.10^{q–t}$	22.25^{qrs}	50.50^{vw}	50.25^{vw}	6.150^{cd}	5.970^{d}
12	$24.13^{f–k}$	$23.77^{h–n}$	89.13^{a}	81.50^{c}	$3.188^{u–y}$	$3.307^{r–y}$
13	$21.20^{t–x}$	$23.38^{k–p}$	87.15^{b}	74.25^{ef}	6.550^{abc}	6.088^{cd}
14	$20.83^{u–x}$	$21.20^{t–x}$	72.25^{gh}	70.75^{hi}	6.673^{ab}	6.963^{a}
15	20.55^{vwx}	20.70^{vwx}	65.50^{k}	$62.50^{m–q}$	6.250^{bcd}	5.963^{d}
16	$21.52^{s–v}$	22.67^{pqr}	68.80^{j}	68.25^{j}	2.638^{z}	$3.370^{p–w}$
17	$20.92^{u–x}$	20.23^{x}	28.75^{z}	35.25^{z}	$3.273^{s–y}$	$3.017^{w–z}$
18	26.13^{b}	$24.60^{d–j}$	34.65^{z}	36.60^{z}	6.368^{bcd}	4.787^{gh}
19	$20.98^{u–x}$	27.17^{a}	65.50^{k}	66.03^{j}	5.000^{fg}	4.505^{hij}
20	$23.73^{i–o}$	$24.67^{d–i}$	44.75^{y}	50.00^{vw}	$4.000^{k–n}$	$4.050^{j–n}$
21	$23.58^{j–p}$	20.55^{vwx}	48.03^{x}	51.30^{v}	$3.175^{u–y}$	$3.800^{m–q}$
22	$24.75^{c–i}$	$23.83^{h–m}$	60.50^{rst}	$62.55^{m–p}$	$3.410^{o–w}$	$3.705^{n–s}$
23	20.48^{wx}	20.77^{vwx}	58.70^{t}	60.65^{qrs}	4.550^{ghi}	$4.000^{k–n}$
24	$25.13^{b–f}$	$20.98^{u–x}$	$60.75^{p–s}$	60.55^{rst}	2.832^{yz}	$3.080^{w–z}$
25	$25.05^{c–f}$	$24.73^{c–i}$	86.15^{b}	81.57^{c}	$3.938^{k–n}$	$3.585^{n–v}$
26	$23.02^{l–q}$	$23.75^{i–n}$	63.70^{klm}	65.30^{kl}	$4.050^{j–n}$	$4.052^{j–n}$
27	$21.20^{t–x}$	$23.00^{l–q}$	34.50^{z}	30.75^{z}	$3.210^{t–y}$	$3.938^{k–n}$
28	$24.80^{c–h}$	25.45^{bcd}	77.35^{d}	75.32^{e}	6.700^{ab}	6.875^{a}
29	$22.77^{n–r}$	$23.73^{i–o}$	62.70^{mno}	$61.38^{n–r}$	5.300^{f}	5.450^{ef}
30	$24.60^{d–j}$	$25.20^{b–e}$	41.70^{z}	47.30^{x}	$4.300^{i–l}$	$4.400^{h–k}$

Qualitative characteristics

The highest amylose content with a mean of 27.17% was related to genotype 19 (Onda cultivar) in Sari, which had a significant difference with other genotypes in both studied locations. The maximum content of amylose in Amol (26.13%) was observed in genotype 18 (Fuji Minori cultivar) – Table 4. According to the method of Juliano (JULIANO 1971), rice varieties based on the content of amylose are classified as waxy (0 to 2%), very low in amylose (3 to 9%), low in amylose (10 to 19%), moderate amylose (20 to 25%) and high amylose (more than 25%). According to this classification, in addition to genotypes 4, 18, 24 and 25 in Amol and genotypes 4, 19 and 28 in Sari which were in the category of high amylose rice, other genotypes studied in two locations were classified as moderate amylose.

Moderate-amylose cultivars are separated after cooking and remain soft for a long time, while high-amylose cultivars are dry (RAY and HILLER-ISLAMBERS 1989) and low-amylose cultivars are glazed and sticky (JULIANO 1971).The researchers examined the interaction between genotype and locations on the quality of rice grain and stated that in multi location experiments, the stability of grain quality is considered as a criterion for selecting the best genotype (PADMAVATHI et al. 2013). The results showed that the maximum gel consistency with a mean of 89.13 mm belonged to genotype 12 (Dasht cultivar) in Amol and the lowest gel consistency in this region with about 67.7% reduction was related to genotype 17. The maximum gel consistency in Sari with a mean of 81.57 mm was related to genotype 25 (Keshvari cultivar) – Table 4. The highest gelatinization temperatures, with means of 6.963 and 6.875º C, were related to genotypes 14 (BA370) and 28 (Neda cultivar) in Sari, which did not have a statistically significant difference with genotypes 13, 14 and 28 in Amol (Table 4). The results showed that BA370 and Neda cultivar genotypes had high gelatinization temperature in both locations (Table 4). The minimum gelatinization temperature with about 63.4% reduction was related to genotype 3 in Amol. In rice quality evaluations, scores of 3 to 5 were desired for rice gelatinization, the most native and high-quality Iranian genotypes are in this range. High gelatinization temperature causes cooked rice to harden and dry, and low gelatinization temperature causes rice to soften and stick after cooking. The low and medium rice gelatinization temperatures compared to high rice gelatinization temperature need less water and time to cook, which is a good feature (KASAI et al. 2001).

The study results showed that in Sari, apart from genotypes, other genotypes studied had a good gelatinization score. Also in Amol, with the exception of genotypes, other genotypes had a desired range in terms of

gelatinization temperature. The results of the present study showed that genotypes in both experimental sites had similar degrees of gelatinization temperature, which were in the range of high rice gelatinization temperature. By investigating cooking quality of grains of different rice genotypes in Mazandaran to select the best genotypes, the researchers reported that among the studied genotypes, genotypes 7302 and 7304 were suitable for planting in northern Iran due to good cooking quality and were selected as the best genotypes (RAHIM SOUROSH et al. 2007).

Correlation coefficient

In the present experiment, the correlation between grain yield and traits of plant height, ear length, number of fertile tillers per hill and 1000 grain weight was positive but not significant. Grain yield showed a positive and significant correlation at the probability level of 1% with the qualitative traits of amylose content and gel consistency. The plant height and ear length were very strongly correlated. Also, the length of the ear and the number of fertile tillers per hill were positively correlated with amylose content, especially the number of tillers, which was significant at the probability level of 1% (Table 5). By examining the interaction between genotypes in the locations, ZHANG et al. (2017) reported that a positive and significant correlation was found between grain yield and number of grains per square meter for the studied genotypes. Other researchers also found that grain yield was positively correlated with traits such as the plant height, panicle length, and 100-grain weight (SHARIFI and EBADI 2018), which was consistent with the study results.

Table 5

Correlation coefficients of the studied traits in two test sites (Amol and Sari)

Plant height	Panicle length	Effective Tillers	Total number of grains	Thousand grain weigh	Grian yield	Amylose content	Consistency gel
1	–	–	–	–	–	–	–
0.579	1	–	–	–	–	–	–
-0.090	-0.169	1	–	–	–	–	–
-0.059	0.190	0.048	1	–	–	–	–
-0.246	-0.283	0.251	0.015	1	–	–	–
0.004	0.070	0.070	-0.011	0.107	1	–	–
0.090	0.144	0.212	0.037	0.008	0.183	1	–
-0.015	0.044	-0.059	0.206	0.033	0.280	-0.002	1
-0.010	0.031	0.100	-0.105	0.091	-0.051	-0.037	0.218

Discussion

In the present experiment, the correlation between grain yield and traits of plant height, ear length, number of fertile tillers per hill and 1000 grain weight was positive but not significant. Grain yield showed a positive and significant correlation at the probability level of 1% with the qualitative traits of amylose content and gel consistency. The plant height and ear length were very strongly correlated. Also, the length of the ear and the number of fertile tillers per hill were positively correlated with amylose content, especially the number of tillers, which was significant at the probability level of 1% (Table 5). By examining the interaction between genotypes in the locations, ZHANG et al. (2017) reported that a positive and significant correlation was found between grain yield and number of grains per square meter for the studied genotypes. Other researchers also found that grain yield was positively correlated with traits such as the plant height, panicle length, and 100-grain weight (SHARIFI and EBADI 2018), which was consistent with the study results. The results showed that a significant difference was found between the experiment sites only in terms of the plant height, ear length, number of fertile tillers and thousand grain weigh. A significant difference was found between the genotypes in terms of all quantitative and qualitative traits. The interaction between genotype and location was significant except for grain yield on other studied traits. The genotypes of Amol II, Dasht, and Keshvari had good physical and chemical quality properties, although the yield of Neda cultivar was higher than the mentioned cultivars. Grain yield had a positive and direct correlation with traits of the plant height, ear length, number of fertile tillers per hill and Thousand grain weight, but this correlation was not significant.

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NANO COPPER VERSUS COPPER SULPHATE SUPPLEMENTATION IN BROILER DIETS – EFFECT ON GROWTH RESPONSE, CARCASS TRAITS AND COPPER RETENTION

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Key words: carcass, copper sulphate, nano copper, performance, residue.

Abstract

A 42-day trial was conducted with 144 (arbor acre) day-old broiler chicks to evaluate the growth response and carcass yield of broiler chickens to dietary supplementation of copper sulphate and nano copper and consequently copper retention. Birds were divided into four groups and assigned to the dietary treatments; 250 ppm $CuSO_4$, 225 ppm Cu-NP, 275 ppm Cu-NP supplementation and control (antibiotics). Data collected were subjected to one - way Analysis of Variance. Results indicated that birds fed dietary nano copper had similar ($P < 0.05$) growth and dress yield [%] compared to other groups. Supplemented birds at higher dosages (250 mg/kg and 275 mg/kg of $CuSO_4$ and Cu-NP, respectively) exhibit high faecal copper excretion and retention in liver compared to the control group. The study therefore concludes that copper residue in faeces and liver increased with dietary copper supplementation, however, nano copper is better available to the birds than $CuSO_4$. Hence, supplementation of 225 ppm of nano copper is recommended for less liver copper load and excretion.

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Introduction

For over a decade now, the edict in European nations on the use of antibiotics in livestock production arising from several challenges associated with its use both in animals and human as final consumers continuously increased researches on alternatives to it. Several studies (OBERDORSTER et al. 2005, RICHARDS et al. 2010) have evaluated the use of trace element especially copper as an effective growth promoter in poultry with encouraging results.

RICHARDS et al. (2010) and SAMANTA et al. (2011) reported that copper is efficient in promoting growth and health as well as reducing meat cholesterol in poultry. Various studies have recommended copper inclusion at high levels (CHORI and PARK 1989, BAKER et al. 1991, SAMANTA et al. 2011) while some other authors obtained excellent result with lower dosages (GONZALES-EGUIA et al. 2009, LEESON 2009, ZHAO et al. 2010, KARIMI et al. 2011). These conflicting results necessitate more studies to have the knowledge of the best level(s) that would yield optimum growth with less adverse effect on the health of the animals. Copper (Cu) is mostly supplied in the diet of broiler chickens in form of copper sulphate (RICHARDS et al. 2010, SCOTT et al. 2018), however there have been reports that an average of 250 mg/kg recommended (CHORI and PARK 1989, BAKER et al. 1991) are not readily absorbed and are thus excreted causing environmental pollution while some are retained in the muscle and organs of the birds (GONZALES-EGUIA et al. 2009, LEESON 2009, ZHAO et al. 2010, KARIMI et al. 2011). Hence, the reason for increased investigation on the availability and absorption of its nano particles in poultry which have vast socio-economic and environmental benefits (*National Nanotechnology Initiative* 2011). According to HILL and LI (2017), the choice of nano copper (Cu-NP) as an alternative growth and health promoter in animal feed is to reduce the population of harmful bacteria and enhance the growth of beneficial bacteria for improved animal performance. The catalytic or biological roles of nano minerals are greatly dependent on their particle size (DICKSON and LYON 2000, LEWIS and KLOIBANOV 2005).

Nano materials are easily absorbed in the small intestine and further distributed into the blood, brain, lung, heart, kidney, spleen, liver, intestine, and stomach (HILLYER et al. 2001). Hence, it becomes imperative not to focus only on growth response of the birds to dietary copper inclusion ($CuSO_4$ or Cu-NP) but also investigate the residue in these organs at the various levels of dietary inclusion to deduce the possible influence on their integrity. This study therefore focused on the comparison between these

two forms of copper (CuSO4 and Cu-NP) as regards their influence on the growth, carcass yield and their retention in organs of broiler chickens.

Materials and Methods

Site of study

The study was carried out at the poultry facility of A-cube Farms, Apakila, Camp, Abeokuta, Ogun State, Nigeria. It is located in climate with the derived savannah zone of South-Western Nigeria. It has a humid climate with mean annual rainfall of 1037 mm and temperature of about 34.7°C.

Source of test materials

Management of birds and experimental design

Nanocopper (Cu-NP) and copper sulphate ($CuSO_4$) were procured from Acrochem store in Lagos, Nigeria.

One hundred and forty-four (144) day old arbor acre strain of broiler chicks were sourced from a reputable hatchery in Ibadan, Nigeria. The pen and all necessary equipment were thoroughly washed and disinfected prior to the arrival of the birds. On arrival, birds were divided into four groups of 36 birds each. Each group was assigned to one of the four dietary treatments as follows; 250 ppm $CuSO_4$, 225 ppm Cu-NP, 275 ppm Cu-NP supplementation and control (without dietary copper supplementation but antibiotics). The groups were replicated three times (housed in separate pens) to consist 12 birds each. Brooding was carried out for two weeks. Feed and water were provided *ad libitum* and birds were raised on deep litter for 6 weeks. Commercial broiler starter (22% CP and 12.72 MJ/kg) and finisher (20% CP and 11.72 MJ/kg) diets were supplemented with the various copper types and levels, mixed evenly and fed to the birds at the starter (0–4 weeks) and finisher (4–6 weeks) phases, respectively (Table 1). All necessary vaccinations and medications were done except that the birds on dietary copper supplementation were not administered antibiotics.

Table 1

Nutrient composition of commercial feed

Parameter	Starter diet	Finisher diet
Crude protein [%]	22.00	20.00
Fat [%]	6.00	6.00
Crude fiber [5%]	5.00	5.00
Calcium [%]	1.00	1.00
Available phosphorus [%]	0.45	0.40
Lysine [%]	1.00	0.85
Methionine [%]	0.50	0.35
Salt [%]	0.30	0.30
Metabolizable energy [MJ/kg]	12.72	11.72

Data collection

Growth performance evaluation

Feed intake. The amount of feed given to the birds and the left-over were measured weekly to determine the feed intake.

$$\text{Feed intake [kg]} = \text{feed given [kg]} - \text{feed leftover [kg]}$$

Body weight and weight gain. The birds were weighed on replicate basis at the commencement of the experiment and subsequently on weekly basis.

$$\text{Body weight [g]} = \text{total weight of birds [g]} / \text{total no of birds}$$

$$\text{Weight gain [g]} = \text{final weight [g]} - \text{initial weight [g]}$$

Average daily weight gain. Average daily weight gain = (final weight - initial weight) / number of birds.

Feed conversation ratio. This is the proportion of feed that was converted into flesh in the birds. It was calculated as daily feed intake divided by weight gain.

$$\text{FCR} = \text{total feed intake [g]} / \text{total weight gain [g]}$$

Mortality rate. This is the measure of the number of deaths recorded in birds. It was calculated as the total number of dead birds divided by the total number of birds and expressed in percentage.

$$\text{Mortality [\%]} = (\text{total number of dead birds} / \text{total number of birds}) \cdot 100$$

Carcass yield evaluation

At the end of the 6th week, two (2) birds with body weight equal to average of the birds in each replicate were selected to evaluate carcass traits. Feed was removed 4 hours before slaughtering. Each bird was slaughtered via the jugular vein and allowed to bleed for 2 minutes followed by scalding at 60°C, and then removal of feathers.

The head and shank were removed and weighed. The visceral were removed and the dressed weight was determined. The weight of the cut parts (thigh, breast, neck, back, wings, and drumstick), organs (kidney, lungs, liver, and gizzards), offal (intestine) and the abdominal fat were determined and expressed as a percentage of the live weight according to ODUNSI et al. (1999).

Samples collection

Blood collection

At the 42nd day of the experiment, two birds were randomly selected from each replicate for bleeding with a 5 ml syringe fitted with a 24-gauge sterile hypodermic needle, 3 ml of blood was carefully drawn from the left wing at the point of bifurcation of the vein and put into a sterilized plain bottle for copper content analysis in the laboratory.

Muscle and organs collection

At the 42nd day of the experiment, two (2) birds were randomly selected from each replicate, fasted overnight, culled and sacrificed. The birds were opened and meat sample was taken from the breast muscle. Manual evisceration was done carefully to avoid rupturing of the organs and the liver, spleen and heart were removed for copper content analysis in the laboratory.

Faecal collection

Two birds per replicate were chosen at random, housed in a different pen and their droppings were collected into a plain bottle. The collected droppings were taken to the laboratory for assessment of the copper content.

Sample analysis

The meat, organs and faecal samples collected were dried in an oven at 65°C for 36 hours to remove moistures. Then 2 grams of each dried sample was put in a flask and digested with a mixture of HNO_3 and H_2SO_4 (3:1 v/v). The digestion process was continued until the solution became clearer. The samples were transferred into another flask and diluted to 25 ml with distilled water. Copper residue was estimated by Atomic Absorption Spectrophotometer. Copper residue in blood was also estimated using the Atomic Absorption Spectrophotometer.

Analysis of data

Data collected were subjected to one-way ANOVA in a Completely Randomized Design using SPSS version 23. Significant difference among means was separated at 5% level of significance using Tukey's test.

Results

Effect of dietary copper supplementation on the growth performance of broiler chickens

Effect of dietary copper supplementation on the growth performance of broiler chickens is presented in Table 2. All parameters measured were similar ($P > 0.05$) across the dietary treatments. Final weight across the treatments were 2096.33, 2060.00, 1996.33 and 2100.33 g for control,

Table 2

Effect of copper supplementation on the performance of broiler chickens

Parameters [g]	Copper supplementation					
	antibiotics	$CuSo_4$ (250 ppm)	Cu-NP (225 ppm)	Cu-NP (275 ppm)	SEM	*P*-Value
Initial weight g]	38.72	39.06	38.91	39.00	0.07	0.81
Final weight [g]	2096.33	2060.00	1996.33	2100.33	24.08	0.91
Weight gain [g]	2057	2020.94	1957.41	2061.33	24.02	0.91
Daily weight [g]	48.99	48.11	46.61	49.08	0.57	0.91
Total feed [g]	6056.33	6286.33	5968.00	5933.33	79.45	0.78
Daily feed [g]	144.19	149.68	142.09	141.27	1.89	0.75
FCR	2.95	3.10	3.08	2.87	0.05	0.75
Mortality [%]	0.33	0.67	0.00	0.00	0.16	0.68

250 ppm $CuSO_4$, 225 ppm Cu-NP and 275 ppm Cu-NP, respectively. Feed intake per day ranged from 141.27 g (control) to 149.68g (250 ppm $CuSO_4$). Feed conversion ratio ranged from 2.87 to 3.10 across the treatments.

Effect of dietary copper supplementation on carcass characteristics of broiler chickens

Effect of dietary copper supplementation on the carcass characteristics of broiler chickens is presented in Table 3. All parameters measured for the effect of dietary copper supplementation did not vary significantly ($P > 0.05$) except for back, spleen and intestine weight that were statistically ($P < 0.05$) differed. Back and intestine weight (13.00% and 5.05% respectively) were least in birds given antibiotics and was increased in birds fed diet containing 275 ppm/kg of Cu-NP (16.08% and 6.35%, respectively). Spleen weight of birds fed diet containing 225 ppm/kg of Cu-NP and 275 ppm/kg of Cu-NP (0.09 and 0.11) were the highest across the treatment.

Table 3

Effect of dietary copper supplementation on the carcass characteristics of broiler chickens

Copper supplementation						
Parameters	control	$CuSO_4$ (250 ppm)	Cu-NP (225 ppm)	Cu-NP (275 ppm)	SEM	*P*-value
Live weight [g]	2266.67	2133.00	2032.67	2187.00	49.11	0.25
*Dressed weight [%]	59.15	63.95	65.84	62.50	1.41	0.23
***Cut parts [%]**						
Head	2.34	2.32	2.22	2.34	0.03	0.94
Neck	3.21	3.71	3.63	3.20	0.14	0.58
Breast	22.13	21.85	24.39	20.55	0.80	0.28
Back	13.07^{c}	15.01^{bc}	13.77^{ab}	16.08^{a}	0.67	0.03
Wings	6.56	7.54	7.35	6.75	0.23	0.38
Thigh	9.51	10.33	10.04	9.42	0.22	0.80
Drumstick	8.17	9.03	9.57	9.77	0.36	0.64
Shank	3.13	3.53	3.54	3.60	0.11	0.68
***Internal organs [%]**						
Liver	1.91	1.65	2.25	1.79	0.13	0.25
Lungs	0.51	0.62	0.58	0.56	0.02	0.49
Spleen	0.04^{b}	0.06^{b}	0.09^{a}	0.11^{a}	0.02	0.01
Empty gizzard	1.74	1.80	1.73	0.02	1.77	0.98
Intestine	5.05^{b}	5.73^{ab}	5.92^{ab}	6.35^{a}	0.27	0.01
Heart	0.39	0.57	0.52	0.49	0.04	0.62

$^{a–c}$ Means with different superscripts across the rows differs significantly ($P < 0.05$)

* All cut parts, organs and dressed weight were expressed as a percent of live weight

Effects of dietary copper supplementation on copper retention in the blood, meat, organs and droppings of broiler chickens

The effect of dietary copper supplementation on copper retention in blood, meat, organs and droppings of broiler chickens is presented in Table 4. Dietary copper supplementation had significant ($P < 0.05$) effect on the copper retention in faeces, meat and liver, however, no significant ($P > 0.05$) variations were noted in heart, spleen and blood.

Table 4

Effects of dietary copper on copper retention in blood, muscle, organs and droppings of broiler chickens

Copper sources						
Parameters [mg/kg]	control	$CuSO_4$ (250 ppm)	Cu-NP (225 ppm)	Cu-NP (275 ppm)	SEM	*P*-value
Faeces	202.6^b	268.9^a	189.5^b	290.5^a	24.69	0.00
Muscle	20.7^b	30.1^{ab}	33.4^a	23.8^{ab}	2.89	0.04
Heart	7.6	8.5	6.7	8.8	0.47	0.15
Liver	50.3^b	62.6^a	58.8^{ab}	67.9^a	3.70	0.04
Spleen	3.3	3.9	3.7	3.6	0.13	0.47
Blood	0.5	0.4	0.4	0.4	0.03	0.76

$^{a–b}$Means with different superscripts across the rows differs significantly ($P < 0.05$)

Faecal copper content was similar in birds supplemented with 250 ppm $CuSO_4$ (268.9 mg/kg) and 275 ppm Cu-NP (290.5 mg/kg) but significantly ($P < 0.05$) higher than values obtained in other groups. Comparable higher ($p < 0.05$) muscle copper content was recorded in birds fed 225 ppm Cu-NP (33.4 mg/kg), 250 ppm $CuSO_4$ and 275 ppm Cu-NP while the least was observed in birds fed the control diet (21.8 mg/kg).

The mean copper concentration in the liver was similar in birds supplemented with 250 ppm $CuSO_4$ (62.6 mg/kg) and 275 ppm Cu-NP (67.9 mg/kg) but significantly ($P < 0.05$) higher than values obtained in the control groups. However, birds supplemented with 225 ppm Cu-NP (58.8 mg/kg) had comparable values with other groups.

Discussion

In this study, similar growth performance indices among the treatment groups agrees with the report by SARVESTANI et al. (2016) stating that poultry feed supplemented with nano copper (Cu-NP) had comparable growth performance to other groups. REFAIE et al. (2015) also observed

similar effect of Cu-NP on the performance of rabbits. Contrarily, PESTI and BAKALLI (1996) indicated that supplementation of either 125 mg/kg or 250 mg/kg of Cu as copper sulphate improved growth and the feed efficiency, but no further growth promoting effect was elicited at higher levels (375 mg/kg) for broiler chickens. However, numerical better performance (highest final weight, weight gain, least feed intake and best FCR) noted in birds supplemented with 275 ppm Cu-NP indicates its potential to improve broiler growth. PRESCOTT et al. (1993) reported that using Cu-NP as a supplement in chicken diet might have beneficial effects on growth, feed efficiency and chicken health by causing damage to pathogens, with a resultant reduction in the production of bacterial toxins, increased synthesis of vitamins and other growth factors, and improved the absorption of nutrients. Similar effect of Cu-NP (275 ppm) could also be as a result of short duration of the study (6 weeks).

Similar feed intake among the birds on various treatment in this study is in consonance with the result reported by HUANG et al. (2015) using weanless piglets. HUANG et al. (2015) stated that average daily gain and feed intake were not affected by concentration of Cu, however, CROMWELL et al. (1998) reported that Cu supplementation at 100 to 250 mg/kg diet increases growth and feed intake in swine. In a related study, SKRIVAN et al. (2002) reported that broilers fed on diets containing greater concentrations of copper had reduced feed intake. Though not significant, no occurrence of mortality among birds supplemented with Cu-NP is an indication that Cu-NP has the potential to support survivability of broiler chickens.

Similar carcass trait among birds supplemented with Cu-NP and $CuSO_4$ corroborated the report of AKINSANMI and IGBASAN (2011) stating that similar carcass trait was observed among broiler chickens fed diet supplemented with copper from different sources ($CuSO_4$, CuO and Copper acetate).

Excess copper in animal diet could result in low digestibility and absorption in poultry and cause more copper to be excreted in the faeces as reported by GONZALES-EGUIA et al. (2009), LEESON (2009), ZHAO et al. (2010) and KARIMI et al. (2011). DOZIER et al. (2003) asserts that a minimum of 95% of pharmacological levels of Cu are excreted as against 85% at the normal levels.

This is in tandem with the high level of copper excreted by the birds in this study. Feeding 240 ppm rather than 9 ppm Cu increased the excreta content from 25 to 400 ppm Cu (SKRIVAN et al. 2006). The report of this study agrees as posited by the authors as there was increase in faecal copper with increase in dietary copper level irrespective of the source. However, similar faecal copper content observed in this study; Cu-NP

(275 ppm) vs $CuSO_4$ (250 ppm) despite the higher dosage of the former indicates that nano copper is better absorbed in the body of broiler chickens than $CuSO_4$.

Low copper residue found in the liver of the control birds reflects that no additional copper ($CuSO_4$ or Cu-NP) was included in their diet. Higher residues noted in other copper supplemented groups is in harmony with the report of UNDERWOOD and SUTTLE (1999) who posited that the main target organ for copper is the liver and its concentration in the liver is based on dietary intake. KARIMI et al. (2011) also reported that high level inclusion in diets increases its concentration because it is readily retained and form residues in the liver. This result is also consistent with the report of GUO et al. (2001) and JEGEDE et al. (2011). Similar concentration in liver of birds supplemented with 250 ppm $CuSO_4$ and 275 ppm nanocopper is an indication that nanoparticles are better absorbed in birds than their salts.

High doses of dietary supplementation of copper lead to copper toxicity in the body because when the liver reaches its storage limit, it releases copper which is then accumulated in different organs. Non-significance in the copper residue in the heart and spleen depicts that the sources as well as levels of dietary copper adopted in this study did not reach threshold levels in the liver. Variation in copper content in meat contradicts the report of HAMDI et al. (2018) that copper content of the breast muscle increases with increase in dietary copper level. JEGEDE et al. (2011) reported that supplementation levels of copper did not vary the copper content in blood, heart, lung, thigh, breast and bone except the liver. MROCZEK et al. (2014) reported that copper was highest in liver and the lowest in breast muscle which confirms the result in this study as copper content in meat is lower than in liver. OGNIK et al. (2019) reported that increase in the addition of Cu, by 54 or 96% in relation to the amount recommended in NRC (1994), did not cause a linear increase in the content of this element in the liver or breast muscle. The differences in residue in meat could neither be adduced to copper types (copper sulphate and nanocopper) nor inclusion levels as no definite trend was observed, hence could not be explained. UNDERWOOD and SUTTLE (1999) reported that copper dietary intake influences its concentration in the liver and kidney but no relationship was found in muscles.

Conclusion

The use of copper sulphate and varying levels of nanocopper in this study yielded similar effect on the growth performance and dressing percentage of broiler chickens though administration of nanocopper at the rate of 275 ppm demonstrated a slightly promising result in terms of weight gain, feed conversion ratio and survivability. It was also deduced from this study that the liver is a major organ for copper retention and increased with increasing levels of supplementation regardless of the copper type. Copper excretion was also relatively better in birds supplemented with dietary nanocopper. Hence, nanocopper can be adopted as a viable alternative growth promoter without posing environmental challenges arising from excretion.

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AQUATIC BEETLE ASSEMBLAGES RESPONSE TO DROUGHT IN SMALL WOODLAND STREAMS

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Key words: Coleoptera, biomass, density, flow intermittency, Borecka Primeval Forest.

Abstract

The effect of an unstable hydrological regime on aquatic beetle (Coleoptera) assemblages in intermittent streams (with periodic summer droughts in some segments) was investigated in two successive years with differing climatic conditions. Samples were taken after complete flow resumption in October in each of four unnamed streams flowing into and out of Lake Łękuk Wielki (northeastern Poland). Nine taxa representing five families of aquatic beetles were identified. Seasonal changes in the density and biomass of aquatic beetles were similar in both years of the study. Maximum values of those parameters were noted in the fall followed by strong, distinct decreases in January. The null hypothesis that there was no difference between dry and wet years for taxon richness, mean biomass, and mean density was tested. No significant differences for these attributes were noted, except biomass at one station. Significant differences (ANOSIM, $p < 0.05$) were determined in beetle assemblages between wet and dry years.

Introduction

Flow-generated disturbances that periodically disrupt stable conditions in streams vary greatly in duration, spatial extent, and predictability (LAKE 2000). Perturbations in the ecosystems of flowing waters can destroy habitat patches and create new ones that are then colonized and inhabited by biota with the return of stable flow conditions. Global climate change scenarios predict more frequent, extended droughts of flowing waters (BOULTON and LAKE 2008). During droughts, macroinvertebrate communities often shift toward populations more capable of withstanding harsh conditions associated with drought, including decreased water quality and higher water temperatures (BECHE et al. 2006). In beetle communities there is a reconstruction of the species composition, in which the

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species are beginning to have the greatest quantitative significance (PAKULNICKA et al. 2015, WESTVEER et al. 2018). Taxonomic diversity over time can be enhanced in temporary streams as compared with perennial systems because of fluctuations in community composition between lotic, lentic, and terrestrial phases (BOGAN and LYTLE 2007).

Perennial streams are common in Central Europe and the drying up of this stream type is unpredictable and can occur in extremely dry years (ŘEZNÍČKOVÁ et al. 2007). Streams that maintain any year-round flow are common in the postglacial area of northern Poland. Most streams in this region are perennial, and they account for 44% of the drainage network length (MAZUREK 2010), although streams with intermittent or ephemeral flows also exist. The Lake Łękuk Wielki watershed is located in the Puszcza Borecka and is one of the seven Base Stations of Integrated Monitoring of the Environment in Poland that was created to monitor the conditions of and changes in natural ecosystems in Poland (KOSTRZEWSKI et al. 1995) where human impact is not intense.

Coleoptera fauna in perennial streams was studied by ZAŤOVIČOVÁ et al. (2004), and in intermittent streams by PAVIĆEVIĆ and PEŠIĆ (2012), while other researchers focused on comparisons between these two stream classes (ŘEZNÍČKOVÁ et al. 2013) or between perennial and temporary streams (STUBBINGTON et al. 2009). This study included observations of assemblages of aquatic beetles inhabiting a few perennial streams located in the same catchment area in two years in which the hydrological regimes differed.

The aim of this study was to determine the species composition of aquatic Coleoptera occurred in perennial streams, to compare beetle assemblages among all the sampling sites, and to estimate biomass. Another purpose of the study was to identify possible differences between coleopterans from wet and dry years distinguished mainly using precipitation parameters. Therefore, the null hypothesis that there was no difference between dry and wet years for taxon richness, mean biomass, and mean density was tested.

Material and Method

Research subject

The study was performed in the catchment area of Lake Łękuk Wielki in the Borecka Primeval Forest located in the Great Mazurian Lakeland of northeastern Poland (22°02'E, 54°08'N). The western part of the catchment area is formed by a vast dead-ice depression in which Lake Łękuk

Wielki (23.7 ha) is located. Four small, unnamed streams (0.3–4.2 km long) flow directly into the lake and drain a catchment area of 13.5 km^2 (Figure 1). Land uses in the Łękuk Wielki catchment include 66% forest (mainly spruce, *Picea abies*, beech, *Fagus silvatica*, oak, *Quercus robur*, and alder, *Alnus* sp.), 25% pastures, and 6% wetlands (mainly peats and swamps) with very little human impact.

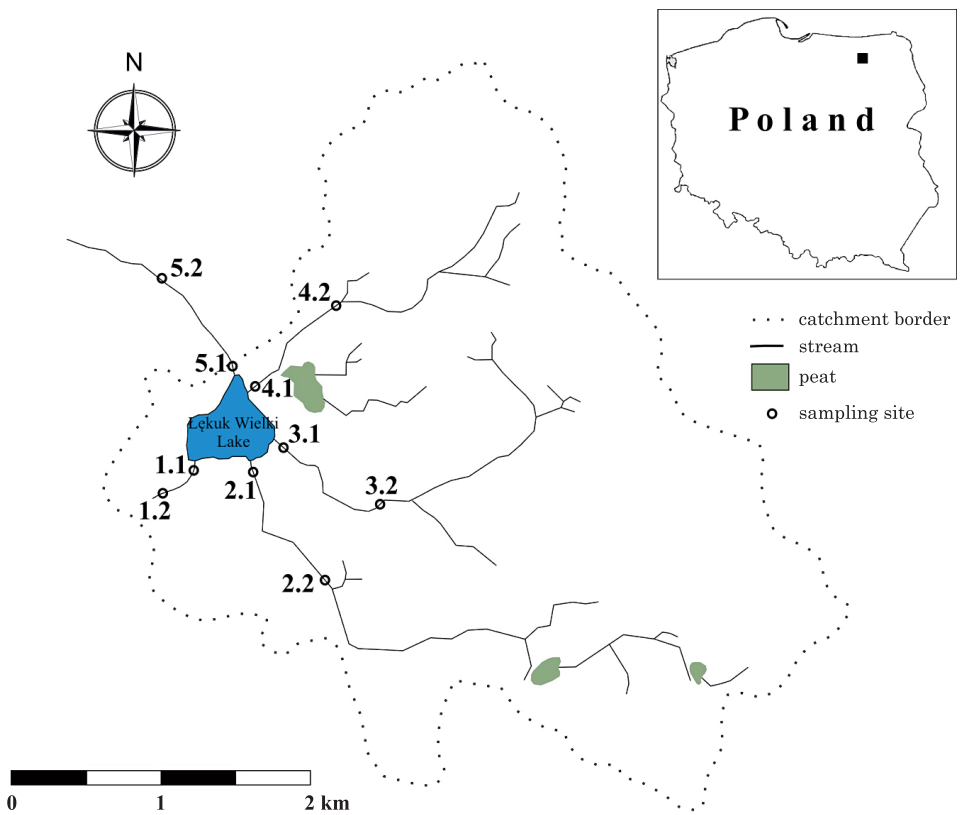

Fig. 1. Study area and location of 10 sampling sites

The lake tributaries run through a forest covering the catchment, and originate from a broken drain-pipe (stream 1) or have no well-defined sources and originate from several permanent springs situated between the moraine hills at an altitude of 180 m (streams 2, 3, and 4). The upper and middle courses of the streams (except for stream 1) are in flat areas, and their gradients do not exceed 1%. Along most of its length, stream 1 is fed from a partly damaged drainage system located nearby. At the boundary between the morainic upland and the dead-ice depression the streams flow in ravines and their gradients reach about 3%. The final segments of

the streams are in a flat area formed of alluvial deposits, and their gradients are again low (SMOLEŃSKI and SIEKLUCKI 1997). The outflow from the lake (stream 5) has regulated banks at a distance of about 1 km, while the remaining parts are of a natural character. *Ulothrix zonata* (Chlorophyta) occurs in the streambed from late winter to mid spring at a distance up to 20 m below the lake. Periphyton (represented almost exclusively by diatoms) was very weakly developed in the inflows (DUMNICKA and KOSZAŁKA 2005).

In the streams studied, the discharge varied from high in spring to very low or completely absent in summer. Very low precipitation over several weeks resulted in the stream beds drying up (SMOLEŃSKI and SIEKLUCKi 1997). Drying proceeded along the stream courses from evaporation and infiltration through the sandy sediment to the hyporheic zone. The outflow from the lake could also dry up. The inflows were typical woodland streams, where leaf breakdown was a very important component of the allochthonous organic matter. Marginal vegetation was limited to the relatively short final segments of the streams (DUMNICKA and KOSZAŁKA 2005).

The hydrochemical properties of the water in all the streams studied were similar, but seasonal changes at the same stations were high and were correlated with discharge values. During the study period, stream waters had an alkaline pH range of 7.5–8.2, medium values of conductivity (322–600 μS cm^{-1}), and a low to medium nutrient content ($P–PO_4$ 1–131 μg dm^{-3}; $N\text{-}NO_3$ 0.1–6 mg dm^{-3}) (ŚNIEŻEK et al. 2000).

Methodology

The streams were studied over a 24-month period, from October 1997 to September 1999. All the streams dried up nearly completely from July to mid October, before the sampling period. The first samples were collected four weeks after the streams filled with water, and the last samples were collected from the dried stream beds. Surface water disappeared in the lower segments of streams 1, 2, and 3 and the outlet. Only stream 4 was considerably diminished, but its bed in the lowest segment remained moist. Two stations were selected at each stream - on the alluvial deposits near the lake (site codes – stream number and n. 1) and at the sector flowing through the forested slopes (sites codes – stream number and n. 2). A pair of stations was also located in the outlet of the lake (Figure 1).

Aquatic Coleoptera were sampled with a core sampler (a steel tube with an area of 29.2 cm^2), and each sample comprised ten sub-samples, for a total of 221 samples and 2210 total sub-samples. The sub-samples were

taken across the stream from each station at each sampling, and they were distributed proportionally to the area occupied by the habitats (silt and clay, sand, gravel and pebbles, cobbles, woody debris, and algae). Live coleopterans were picked from the sediments under a stereoscopic microscope after washing them through a sieve (mesh size 0.25 mm) and were preserved in formalin (4%).

Coleoptera larvae were identified to the genus or species, and adults were identified to the species mainly according to GALEWSKI and TRANDA (1978), ROZKOŠNY (1980), GALEWSKI (1990) and KLAUSNITZER (2009). All specimens identified were weighed to determine wet mass to the nearest 0.1 mg on an analytical scale.

Statistical analysis

To characterize the structure of the beetle assemblages found in the streams, we used species/genus composition, biomass, and density data collected at each sampling site. Analysis of similarity (UPGMA, Bray-Curtis distance metric) was used to compare differences in the coleopteran communities among sites. Cluster analysis dendrogram classification was produced with the MultiVariate Statistical Package 3.13 (KOVACH 2007).

To verify pattern occurrence in the aquatic beetle communities, Non-metric Multidimensional Scaling (NMDS) analysis was performed using aquatic beetle density, with each point as a sampling unit and the year (dry or rainy) as the group variable. The metaMDS function from the vegan package was used (OKSANEN et al. 2020) with the Bray-Curtis distance measure. The ordination stress statistic was used as a measure of goodness of fit. To compare beetle community structures in wet versus dry years, while pooling across seasons, one-way analysis of similarity (ANOSIM; CLARKE and WARWICK 1994) was conducted using the Bray Curtis similarity matrix. Non-metric multidimensional scaling (nMDS; 100 restarts) plots were used to create graphic representations of the ANOSIM results. The analyses were performed in the R statistical environment (*R Core Team* 2021).

The Wilcoxon rank-sum test was used to detect whether there were significant differences ($p < 0.05$) in taxon richness, mean biomass, and mean density for each month sampled between dry and wet years. The analysis was performed with the TIBCO Software Inc. statistical package (2017).

Results

Nine taxa of aquatic beetle were confirmed in the material collected. Among the larvae collected, two were identified to the species and four to the genus, and they belonged to five families. One Coleoptera in the imago stage was identified as *Scarodytes halensis*. Within the genus *Elodes* two forms were designated with numbers. Additionally, larvae that were not classified to the genus were also observed. Overall, 186 individuals were caught. Representatives of the order Coleoptera were collected at all of the sampling sites. The greatest taxon richness was confirmed at site 4.1, where six taxa were observed. The highest mean density and biomass of beetles was confirmed at site 4.2 at 122 ind. m^{-2} and 1.37 g m^{-2}. The highest share in the number (49%) and biomass (57%) of the order Coleoptera was of larvae from the genus *Elodes*, which were distinguished with the number 1. Larvae of the genus *Agabus* were the only aquatic beetles that inhabited all of the streams studied, and they were noted at all eight sampling sites. The list of all the taxa recorded, including data on their density (%) share of the samples, is presented in Table 1.

Table 1

Percentage share of aquatic Coleoptera taxa at sampling sites

Specification	1.1	1.2	2.1	2.2	3.1	3.2	4.1	4.2	5.1	5.2
Dytyscidae										
Agabus sp.	67	40	–	67	50	60	54	2	–	2
Platambus maculatus (Linné)	–	–	–	–	–	–	17	–	–	2
Scarodytes halensis (Fabricius) – imago	–	–	–	–	–	–	8	–	–	4
Hydraenidae	–	–	–	–	–	–	–	–	–	–
Hydraena sp.	–	–	50	–	–	–	4	–	–	–
Hydrophilidae										
Helophorus sp.	–	–	50	–	50	–	4	–	–	–
Elmidae										
Oulimnius tuberculatus (Müller)	8	–	–	–	–	–	–	–	100	87
Scirtidae										
Elodes sp. 1	17	50	–	–	–	40	13	95	–	4
Elodes sp. 2	8	10	–	–	–	–	–	2	–	–
Coleoptera n.d.	–	–	–	33	–	–	–	–	–	–
Average density [ind. m^{-2}]	20	16	3	5	3	8	36	122	2	70

The density of Coleoptera changed significantly during the study period. The highest density values were recorded at the beginning of the study (in the first wet season) just after the dry period and in October and in December in the second year of the study (Figure 2).

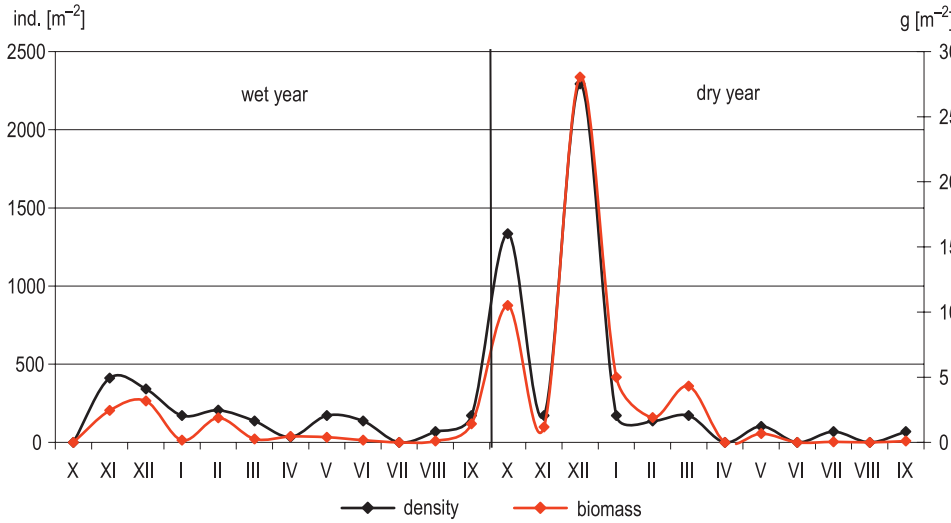

Fig. 2. Seasonal variation in the density (ind. m^{-2}) and biomass (g m^{-2}) of all aquatic beetle taxa (larvae and adults) at sampling sites

Based on the taxonomic structure of the individual assemblages, cluster analysis distinguished several groups of sites (Figure 3). The beetle assemblages at both sites in stream 1 and at site 3.2 were similar.

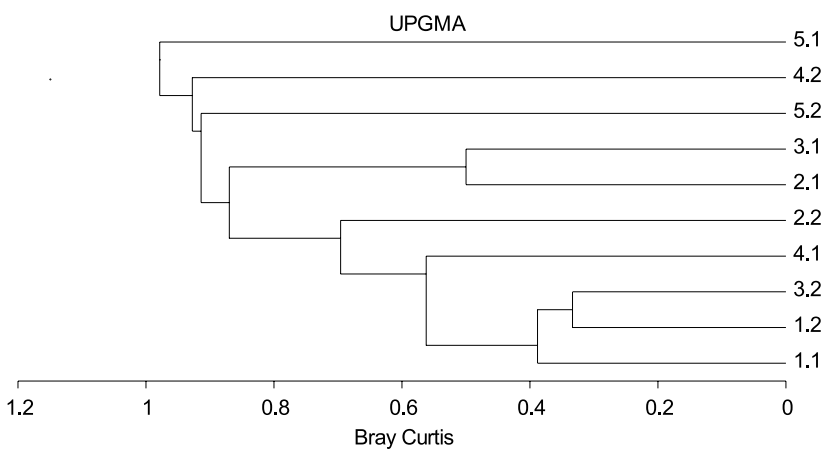

Fig. 3. Dendrogram of cluster analysis based on the aquatic beetles assemblages

The assemblages inhabiting the remaining sites did not exhibit much similarity, and the most differentiated assemblages occurred at both of the sites at the outflow and at site 4.2.

The nMDS plot using the Bray-Curtis index showed clear separation among sampling sites located in the inflowing streams and the outflow with 0.8 stress. The distribution of the sites by season was apparent at sites 2.1 and 3.1 (wet) and also at sites 1.1 and 3.2 (dry) – Figure 4. On the graph, the distribution scheme in stream 4 was in agreement with the sites independently of season.

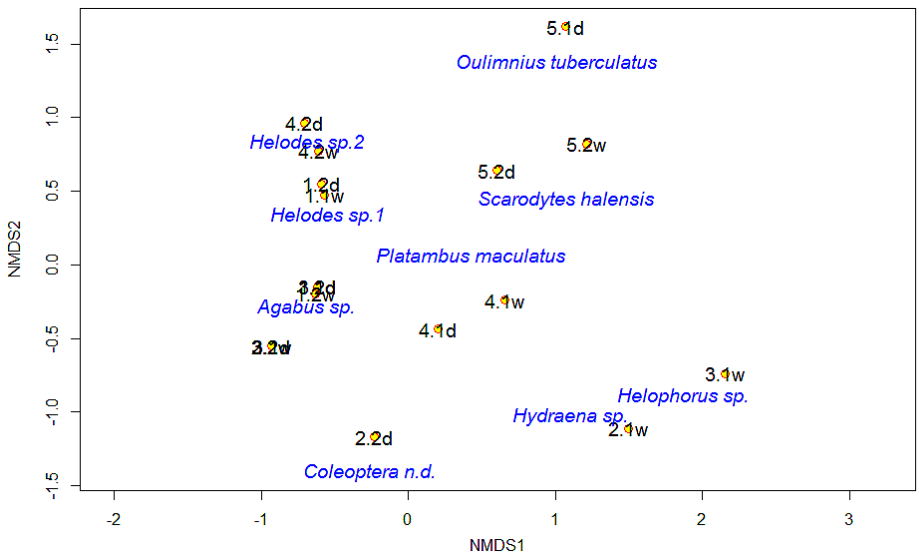

Fig. 4. Graphical representation of the non-metric dimensional scaling (NMDS) with Bray-Curtis similarity and ANOSIM. nMDS plots comparing beetle community structure between wet and dry year (site number.w and site number.d, respectively)

In the first year of the study, seven taxa were confirmed in the streams, which was exactly the same number as was confirmed in the following year. Among the Coleoptera collected, only larvae of the species *Platambus maculatus* occurred for the first time in the streams studied in the second season of the study. The occurrence of larvae of the genus *Helophorus* and *Scarodytes halensis* imagines was not noted in the second year of the study. Higher density values of Coleoptera were confirmed in the second, dry year of the study, and only at sites 1.1 and 2.1 was the opposite noted. At site 3.1 mean density values were the same in each of the two years compared. The comparison of the mean beetle biomass differed; at sites 1.1 and 2.1 higher values were noted in the first, wet study year, while at the other sites higher biomass was noted in the second year. Taxa

richness was higher at sites 1.1 and 2.1 in the first year of the study, while at sites 3.1 and both of the sites in stream 4 it was the same. At the remaining sampling sites taxonomic differentiation varied, but it was higher in the second year of the study.

Significant differences were noted in beetle assemblages between wet and dry years (ANOSIM Global $R = 0.285$, $p = 0.022$). While, there was no significant difference in aquatic beetle density between the wet and dry years at the stations studied, there was significant variation in biomass between the wet and dry years at station 4.1 (The Wilcoxon rank-sum test, $p < 0.05$) – Table 2, but these differences were not statistically significant at the other stations.

Table 2

Density, biomass and taxa richness of the beetles collected at the sampling sites

Sites	Density				Biomass				Richness	
	wet	dry	z	*p* value	wet	dry	z	*p* value	wet	dry
1.1	25.7	8.6	1.527	0.127	0.298	0.159	0.676	0.499	3	2
1.2	5.7	22.8	1.930	0.054	0.098	0.289	1.521	0.128	2	3
2.1	5.7	0.0	1.414	0.157	0.003	0.000	1.342	0.180	2	0
2.2	2.9	5.7	0.577	0.564	0.035	0.194	1.069	0.285	1	2
3.1	2.9	2.9	0.000	1.000	0.002	0.051	0.447	0.655	1	1
3.2	5.7	8.6	0.577	0.564	0.013	0.084	0.730	0.465	1	2
4.1	22.8	45.6	1.156	0.248	0.144	0.881	2.366	0.018*	4	4
4.2	22.8	211.0	0.738	0.461	0.138	2.493	1.363	0.173	3	3
5.1	0.0	2.9	1.000	0.317	0.000	0.002	1.000	0.317	0	1
5.2	59.9	68.4	1.539	0.124	0.138	0.158	1.491	0.136	3	4

Comparison of density and biomass was carried out using the non-parametric Wilcoxon matched--pairs test (exact *p* values)

* Orders with significant differences ($p < 0.05$)

Discussion

Aquatic beetles quickly recolonized dried-up streams, and they were observed in all of them as soon as the first month of the study. The highest taxa richness was recorded at site 4.1, where six of the nine beetle taxa occurring in the streams studied were confirmed. This could have stemmed from the conditions in stream 4 that were the most stable in terms of water flow. This stream was also the least likely to dry up during dry periods (SMOLEŃSKI and SIEKLUCKI 1997), and when the surface flow stopped, the bottom sediments were kept moist by the water table that was at a depth of 20–50 cm beneath the stream bed (SOSZKA et al. 1992). The bee-

tles inhabiting stream 4 had the most advantageous conditions for waiting out dry periods in the interstitial waters, wet sediments, and fallen leaves on the bottom and banks of the stream. This is how beetles of the genus *Elodes* were able to recolonize this stream from the hyporheic zone (DURKOTA et al. 2019). Representatives of this widely distributed genus (KLAUSNITZER 2009) had the greatest effect on the seasonal fluctuations observed in Coleoptera assemblage density. These beetles inhabit areas adjacent to typical water bodies such as rivers (SZAWARYN et al. 2021), oxbows (PAKULNICKA et al. 2016) and streams (RUTA et al. 2003) and also ones with more extreme hydrological conditions such as streams that dry up (MOTH IVERSEN et al. 1978) and even tree holes (BALTZLEY et al. 1999), and they appear to be well adapted to life in perennial streams in which drought conditions occur sporadically. Their numerous density in fall was correlated with the availability of leaves that fall into the water from trees growing near the stream.

Beetles can also recolonize streams by aerial routes, which is limited, according to WILLIAMS and HYNES (1976), in the temperate climate from spring to fall. Adult Coleoptera either inhabit or lay eggs in steams. The occurrence of only *Scarodytes* imagines noted during the first year of the study in the runoff and at stream 4 sampling station at the outflow could suggest that they originated from the lake. This species is one of the first beetles to colonize newly-formed water bodies (KEHL and DETTNER 2003, PAKULNICKA 2008).

Although the streams studied were similar in type and physical, chemical, and geological conditions, the water beetle assemblages differed at the sampling sites. Only three of the sites exhibited any distinct taxonomic similarity, and these were characterized by the occurrence nearly exclusively and in very similar proportions of larvae of the genera *Agabus* and *Elodes*.

Studies conducted simultaneously on permanent and intermittent (the one that regularly dries up in the summer) streams showed that the former was inhabited by six taxa of aquatic beetle and the latter by just two (ŘEZNÍČKOVÁ et al. 2013). In their studies of streams in southern England, both CASEY and LADLE (1976) and FURSE (1977) reported significant differences in invertebrate assemblages between periodically dry and permanent sites. The results of the current study were different in that there was a lack of differences in the density of taxa between the seasons with different hydrological conditions. This was consistent with BOTTORFF and KNIGHT (1988), who investigated two first order streams in California, one of which was a periodic stream while the other had year-round flow, and they confirmed that the macrozoobenthos assemblages were similar with regard to the number of taxa in the main benthic taxonomic groups.

Changes in Coleoptera density and biomass in the streams studied can also be confirmed by WARD's (1992) hypothesis that nearly all species of aquatic beetle occurring in the temperate zone produce one generation annually. Seasonal changes in beetle density and biomass were similar in both seasons of the study. Maximum values were observed in fall followed by distinct, sharp declines in January. Despite the notable higher overall beetle density observed in the second, dry year of the study, differences between the seasons were statistically insignificant at each of the sites sampled.

Conclusions

Precipitation deficits led to abnormally low streamflow and drought. Such extreme conditions led the aquatic beetles to recolonize, and the number of taxa did not differ in the streams studied in the following season when water flowed continuously. Seasonal changes in beetle density and biomass were similar regardless of hydrological regime, except significant variation in biomass between the wet and dry years at station 4.1 ($p < 0.05$). The results of the study indicated that the taxa best adapted to variable conditions in the streams were the beetles of the genus *Elodes*.

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SUB-LETHAL TITANIUM DIOXIDE NANOPARTICLES (TiO_2 NPs) INDUCES DISRUPTION OF CHLOROPHYLLS AND SELECTED ANTIOXIDANTS ACTIVITIES ON *CHLOROIDIUM ELLIPSOIDEUM* (GERNECK)

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Key words: pollutant, *Chlorella* sp. stressors, antioxidant.

Abstract

The increasing production of manufactured product containing TiO_2 NPs is a global threat that raises concern for freshwater biodiversity and human health. The objective of this study was to examine the chronic effects of TiO_2 NPs on the biomass (chlorophyll *a*, *b*) and antioxidant activities (catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GRx) and malondialdehyde (MDA) of *C. ellipsoideum* exposed to sublethal doses of TiO_2 NPs. The initial, exposure of *C. ellipsoideum* to acute doses of TiO_2 NPs demonstrated toxicological response with EC_{50} 69.90 mg L^{-1}. However, the effects of sub-lethal concentrations on the microalgae showed significant reduction ($p < 0.05$) of Chlorophyll a and b with the increase of sub-lethal concentrations of TiO_2 NPs. The percentages of increment on selected markers of oxidative stress in this study increased compared to the control; Catalase (18.30–58.66%), SOD (2.68–16.85%), GRx (26.50–86.67%) and MDA (40.00–106.00%). This study suggests that sub-lethal exogenous concentrations are disruptive to the physiology of *C. ellipsoideum*. Therefore, care should be taken when handling and disposing of manufactured products containing TiO_2 NPs. This study is useful for understanding the potential harmful effects of TiO_2 NPs bioaccumulated in aquatic ecosystems. Further studies are recommended on other commonly used nanomaterials and their physiological influence on microalgae.

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Introduction

Engineered nanoparticles (ENPs) are increasingly found in manufactured agricultural, pharmaceutical, cosmetics, sunscreens, paints, drugs, medical, recreational industrial and house hold products (CONTADO 2015, KESSLER 2011). The presence of nanomaterials in a large number of manufactured products likely leads to their increasing release into the environment. Therefore nanomaterials entering the aquatic environments through wastewater and effluents have certainly increased in bottoms and sediments of freshwater ecosystems (WU et al. 2015). The presence and impact of large amount of nanomaterials in aquatic ecosystems have become a great concern for the welfare of aquatic biodiversity, more especially the primary consumers inhabiting freshwater ecosystems.

Approximately five million tons of titanium dioxide were consumed in 2009; 1.5 million tons are produced annually in the European Union and it is expected to continue to increase further globally (LANDSIEDEL et al. 2010, ORTLIEB 2010). Titanium dioxide (TiO_2) has become part of our everyday lives. It is found in various consumer goods and products of daily use such as cosmetics, paints, dyes and varnishes, textiles, paper and plastics, food and drugs (WEIR et al. 2012).

Nanoparticles have wide applications in various fields due to their small size (LAURENT et al. 2010). TiO_2 NPs are bright with high refractive index ($n=2.4$) which makes them suitable for industry dealing with toothpaste, pharmaceuticals, coatings, papers, inks, plastics, food products, cosmetics and textile (WEIR et al. 2012). Three crystalline phases of titanium dioxide, are anatase (tetragonal), rutile (tetragonal), and brookite (orthorhombic) in which brookite has no commercial value (PAOLA et al. 2013).

TiO_2 NPs has a potential role in photocatalytic degradation of pollutant in water, and also their catalytic activity could be attributed to a larger area per unit mass (CHEN and MAO 2007). The TiO_2 NPs have wide applications, viz., reducing toxicity of dyes and pharmaceutical drugs; waste water treatment; reproduction of silkworm; space applications; food industries; etc., and so have immense industrial importance. Due to their self-cleaning and antifogging property, they are used in the preparation of cloths, windows, tiles and anti-fogging car mirrors (EPIFANI et al. 2008).

The careless handling of TiO_2 NPs result in their unintended release into industrial and non-industrial waste streams discharged inadvertently into natural aquatic environments and becoming a menace for aquatic biodiversity including human (OZAKI 2013).

The presence of TiO_2 nanoparticles into the environment affects phytoplankton and coastal ecosystems that support fishing and recreational

activities (*Titanium dioxide*… 2012). Algae are primarily used as biological marker in aquatic system to study biological toxicity of pollutants. TiO_2 nanoparticles inhibit algae by causing membrane structure deformation due to increased lipid peroxidation (OZKALELI and ERDEM 2018). TiO_2 NPs exhibit more cellular toxicity in anatase due to increased amount of intracellular reactive oxygen species (ROS). Growth rate of freshwater green algae predominant in North America viz., *Scenedesmus quadricauda*, *Chlamydomonas moewusii* and *Chlorella vulgaris*, was found to be inhibited due to the presence of TiO_2 NPs in freshwater microcosms (CARDINALE et al. 2012). Combination of anatase and rutile showed more toxicity and antagonistic effect on freshwater algae Chlorella (MUKHERJEE et al. 2015). Elsewhere, some authors demonstrated the adverse effects of TiO_2 NPs on the growth rate, biomass and antioxidant of some Chlorella species (MATOUKE et al. 2018, MUKHERJEE et al. 2015, KULACKI and CARDINALE 2012). In Natural aquatic ecosystems TiO_2 nanoparticulate are toxic and disruptive with negative impact on aquatic ecosystems (WU et al. 2015). This nanomaterial has been reported in United Kingdom (UK) rural, agricultural and urban industrial rivers with an average concentration of 2.10 $\mu g\ L^{-1}$ (NEAL et al. 2011). In freshwater surface and sediments from Xiamen Bay in China with a concentration of 2.74 $g\ kg^{-1}$ (LUO et al. 2011).

In natural environment TiO_2 NPs may interact with organisms, natural organic matter and other naturally occurring geogenic and biogenic colloids because of its peculiar properties such as size, shape (ADELEYE and KELLER 2016). However, with the increasing production of TiO_2 NPs it is expected that many natural aquatic ecosystems may be polluted through water drainages into sewages, lakes, ponds, rivers, estuaries and the fate of aquatic organisms compromised.

Despite the suspected galloping growth of TiO_2 NPs in our environment and coupled with the fact that it could be harmful to freshwater aquatic biodiversity; studies on the impact of TiO_2 NPs on primary producers in freshwater ecosystems are sparse. Moreover, in many countries where TiO_2 NPs is highly consumed, there are scanty legislative measures for the monitoring of this metallic compound in aquatic environment. Therefore, the study of the impact of TiO_2 NPs on primary producer (microalgae) is of great concern for aquatic food web because it provides oxygen and nutrient for consumers (MUKHERJEE et al. 2015, BAJGUZ 2012).

Some studies on microalgae exposed to TiO_2 NPs showed a wide range of toxicological effects on cells growth, but focused only on the acute effects (OZKALELI and ERDEM 2018, KULACKI and CARDINALE 2012). However, studies on the sub-lethal effects of TiO_2 NPs on growth rate, photosynthe-

sis are still limited (WANG et al. 2014, MUKHERJEE et al. 2011). In this study, we attempted to assess the impact of sub-lethal treatments of TiO_2 NPs on microalgae and additionally focus on the response of anti-oxidative stress commonly used as bioindicators of pollution. More knowledge on the impact of TiO_2 NPs on antioxidants is needed to assess the stress factor of TiO_2 NPs on freshwater organisms but also, to enhance awareness, safety and management. *Chloroidium ellipsoideum* was chosen because it is a primary producer in freshwater and it is very important for the survival and equilibrium of aquatic food web.

This study was aimed at evaluating the impact of sublethal TiO_2 NPs on photosynthesis and antioxidant activity in *C. ellipsoideum*.

Materials and Methods

Chemicals

Powder Titanium (IV) oxide nanoparticle (99.50%) of particle size 21 nm, CAS Number 13463-67-7, Pcode: 1002000564 was obtained from Sigma-Aldrich (St. Louis, MO 63103, USA). All other chemicals were of analytical grade. Stock solution was prepared in de-ionized water at a concentration of 1g L^{-1}. TiO_2 NPs was sonicated (600w, 40 KHz, 25ºC) for 30 min to enable full dispersion of NPs. Serial dilution of the stock solution was used to obtain the expected concentrations for chemicals tests.

Characterization of TiO_2 NPs

The characterization of TiO_2 NPs was previously reported in our study (MATOUKE et al. 2018). The determination of phase purity of NPs was carried out with X-ray diffractionometer (XRD) using an Empyrean XRD (Panalytical, The Netherlands) equipped with filtered Cu K λ radiation (λ = 1.5418 Å) that operated at 40 K_v and 40 mA. The XRD patterns were recorded from 10 to 80 2θ degree with a scanning speed of 0.526º per minute. The determined sizes of nanoparticles were confirmed using the Sherrer equation. In addition, image of powder nanoparticles of 0.1 mg L^{-1} was observed using a scanning electron microscope (Zeiss model), Germany. We also determined the zeta-potential in the medium (0.1 mg L^{-1}) using Nanobrook ZetaPlus Brookhaven 220001, USA.

Algal culture

All toxicity tests were conducted using freshwater *C. ellipsoideum* provided by the National Institute for Freshwater and Fisheries Research (NIFFR), Kainji, New Bussa, Nigeria.

The provided axenic cultures of microalgae *C. ellipdoideum* were grown under controlled sterile conditions in 250 mL Erlenmeyer fibre glass flask containing 100 mL of sterilized modified B11 medium (STANIER et al. 1971). In order to determinate the algal inhibition (EC_{50}), alga cultures were maintained at 25±2ºC on a 14: 10-h: light: dark cycle with a light intensity of 100 $\mu Em^{-2}\ s^{-1}$, and continuous shaking (100 rpm) for 72 h. *C. ellipdoideum* cells at exponential growth phase were inoculated in the Elenmeyer flasks containing fresh medium. The exponential growth phase had a density of $2 \cdot 10^3$ cells mL^{-1} and were enriched in triplicate with varying TiO_2 NPs concentrations (10, 20, 40, 60, 80 and 100 mg L^{-1}) according to the Organization for Economic Cooperation and Development (*Proceedings*… 1984) 201 algal growth inhibition test guidelines.

For further study, nominal concentrations of TiO_2 NPs in culture media were: 1.85, 3.88, 6.06, 8.39 and 10.9 mg L^{-1} obtained from the result of algal inhibition (EC_{50}) and represent sub-lethal doses of EC_5, EC_{10}, EC_{15}, EC_{20}, EC_{25}, respectively with the control (without dosage). The experimental culture in Erlenmeyer flask (250 mL) contained initial cell density of $2 \cdot 10^3$ cells mL^{-1} inoculated in media (100 mL of BG 11) at exponentially growing phase. The cultures lasted for 72 h and were kept under 25±2ºC on a 14: 10-h: light: dark cycle with a light intensity of 100 $\mu Em^{-2}\ s^{-1}$, and continuous shaking (100 rpm). Three experimental replicates were performed and growth, chlorophyll and antioxidants were monitored.

Growth determination

Growth of algal cells was monitored by direct count of viable cells under microscope using a Neubauer haemacytometer. Percentage inhibition of growth was calculated as (ADELEYE and KELLER 2016) [6]:
where:

$$GI = \frac{N_c - N_t}{N_c} \cdot 100$$

GI – the percent inhibition in average cell density

N_c – the average cell density in the control group,

N_t – the average cell density for the treatment group.

The EC_{50} values, which represent the concentrations of the test substances leading to 50% reduction in the algal growth compared to the control, were calculated from the dose-response curve Weibull model (MONTEIRO et al. 2011) analysis on Regression toxicology software for Excel. For further analysis, sub-lethal concentrations (EC_5, EC_{10}, EC_{15}, EC_{20}, EC_{25}) derived from the acute concentrations of TiO_2 NPs were used.

Chlorophyll *a* and *b* determination

The extraction and analysis of chlorophyll a were done according to the procedure described by (AMARAL 2012). Chlorophyll *a* and *b* extracted using absolute methanol. The chlorophyll *a* (Chl*a*) concentration was calculated using the equation:

$$\text{Chl}a\ [\text{mg L}^{-1}] = (11.47 \cdot \text{OD}_{664}) - (0.4 \cdot \text{OD}_{630})\ x/y$$

Where *x* is the total volume of extraction solvent used and *y* represents the volume of culture filtered.

Chlorophyll *b* (Chl*b*) was calculated according to the formula (LICHTENTHALER and WELLBURN 1985):

$$(\text{Chl}b)\ [\text{mg L}^{-1}] = 27.05\ \text{OD}_{653} - 11.21\ \text{OD}_{666.}$$

OD_{630}, OD_{653}, OD_{664}, and OD_{666}, is optical density at a wavelength of 630, 653, 664 nm and 666 nm, respectively. OD was determined using a UV-2600 spectrophotometer (Shimadzu Scientific Instrument, China).

Antioxidant bioassays

Microalgal cells (50 mL) of each culture were centrifuged at 100 rpm for 10 min. Centrifuged algal cells were ground in 1 mL of 20 mM phosphate buffer (pH 7.4) , 0.1 g of white quartz sand in a chilled tissue grinder was added to the mixture. The mixture was centrifuged at 12000 g for 10 min at 4ºC to obtain the supernatant for further analysis. The supernatant was stored as aliquot for antioxidant estimations. Protein measurements were performed according to LOWRY et al. (1951).

Glutathione peroxidase (GPX, EC 1.11.1.7)

Peroxidase activity was measured using guaiacol and H_2O as the donor as substrate. The substrate mixture contained 10 mL 1% guaiacol, 10 mL 0.3% hydrogen peroxide and 100 mL 0.05 M sodium phosphate buffer (pH 6.5). The mixture in the cuvette was made up of 2.87 mL substrate, 0.1 mL of crude extract, and 0.03 mL antioxidant solution in a total vol-

ume of 3 mL (HEMEDA and KLEIN 1990). The control contained 0.03 mL of ethanol. Peroxidase activity was determined spectrophotometrically at 25ºC and 470 nm. The result was expressed as µg mg^{-1} FW min^{-1}.

Superoxide dismutase (SOD, EC. 1.15.1.1)

Superoxide dismutase (SOD) activity followed the method described by (GAO 2005) which consisted of the reduction of tetrazolium. One unit is the amount required to inhibit 50% of NBT photoreduction. The control tube, the light tube and the measuring tube were divided for each sample. Each tube contained 550 mmol L^{-1} potassium phosphate buffer (pH 7.8), 130 mmol L^{-1} methionine solution, 750 µmol L^{-1} NBT solution, 20 µmol L^{-1} riboflavin solution,100 µmol L^{-1} EDTA-Na_2, distilled water, and the enzyme solution was added to the measuring tube, the same amount of distilled water was added to the other tubes. The experiment was conducted under 1000 Lx Fluorescent color reaction for 15 min, the dark control tube was used as a blank. The absorbance was read at 560 nm and the result expressed as U mg^{-1}FW h^{-1}.

Glutathione reductase (GRx, EC 1.6. 4.2)

Glutathione reductase (GRx, EC 1.6. 4.2) activity was determined according to (SCHAEDLE and BASSHAM 1977). GR catalyzed following reaction:

$$GSSH + NADPH \rightarrow GSH + NADP+.$$

GR activity was evaluated by measuring the change of NADPH. 1 mL reaction mixture containing 50 mmol L^{-1} potassium phosphate buffer (pH 7.8), 20 mmol L^{-1} EDTA, 1.5 mM NADPH, 5 mM GSSG, 200 µL enzyme solution, and measured the change of absorbance at 340 in 1 min under 20°C immediately (extinction coefficient is 6.2 mmol L^{-1} cm^{-1}). The result was expressed in U mg^{-1} protein.

Catalase (CAT, EC 1. 11.1.6)

Catalase activity was determined using UV absorption method (GAO 2005). Sample was divided into two tubes added with live enzymes in one and another with dead enzymes, then Tris-HCl buffer (pH 7.0), distilled water were added in each tube and the mixture in each tube was pre-heated with 200 mmol L^{-1} H_2O_2 for 3min using a water bath at 25°C. The absorbance was measured immediately at 240 nm. The result was expressed in U mg^{-1} FW min^{-1}.

Malondialdehyde (MDA)

Malondialdehyde (MDA) measurements, used to estimate the level of lipid peroxidation in algal cells, were carried out according to (HEATH and PACKER 1968). 2 mL of 10% trichloroacetic acid (TCA), containing 0.5% thiobarbituric acid (TBA) was added to 1 ml of the microalgal suspension. The mixture was then heated in a water bath for 15 min and allowed to cool in an ice bath then, centrifuged at 3000 rpm for 5 min and the absorbance of the supernatant was read at 532 nm and 600 nm. Lipid peroxidation was expressed as the MDA content in nanomoles per 10^5 cells (nmol mg^{-1} FW) based on the difference between absorbance at 535 and 600 nm, using a molar extinction coefficient of 155 mM^{-1} cm^{-1}.

Data analysis

For the algal growth inhibition tests, the EC_{50} values (metal concentration required to cause a 50% reduction in growth) were computed using curve Weibull model analysis on Regression toxicology software for Excel. The data obtained from the study was normalized and subjected to Levene's test for homogeneity of variance and one way analysis of variance (ANOVA) was used to determine the differences in means parameters (chlorophyll and antioxidant activities) using GraphPad Prism 8.3 for windows. 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 or 0.01.

Results

The results of the x-ray Diffractogram (XRD) for TiO_2 NPs indicated eleven (11) diffracted peaks that reckon their tetragonal structure. From the diffractogram we derived the two main textures Anatase (82%) and rutile (31%) phase. The pictogram TiO_2 NPs powder with the Scanning electron microscope (SEM) showed an agglomeration of particles of 21 nm according to the manufacturer. However, 1 mg L^{-1} of prepared TiO_2 NPs revealed an average zeta potential of 0.19 mV informing us of an unstable mixture (Figure 1).

The 72 h EC_{50} values obtained from inhibition of cells of *C. ellipsoideum* exposure to TiO_2 NPs was 69.90 mg L^{-1}. The assay revealed sub-lethal concentrations with EC_5, EC_{10} EC_{15}, EC_{20} and EC_{25}: 1.85, 3.88, 6.06, 8.37 and 10.90 respectively showed TiO_2 threshold concentrations on microalgae cells (Table 1).

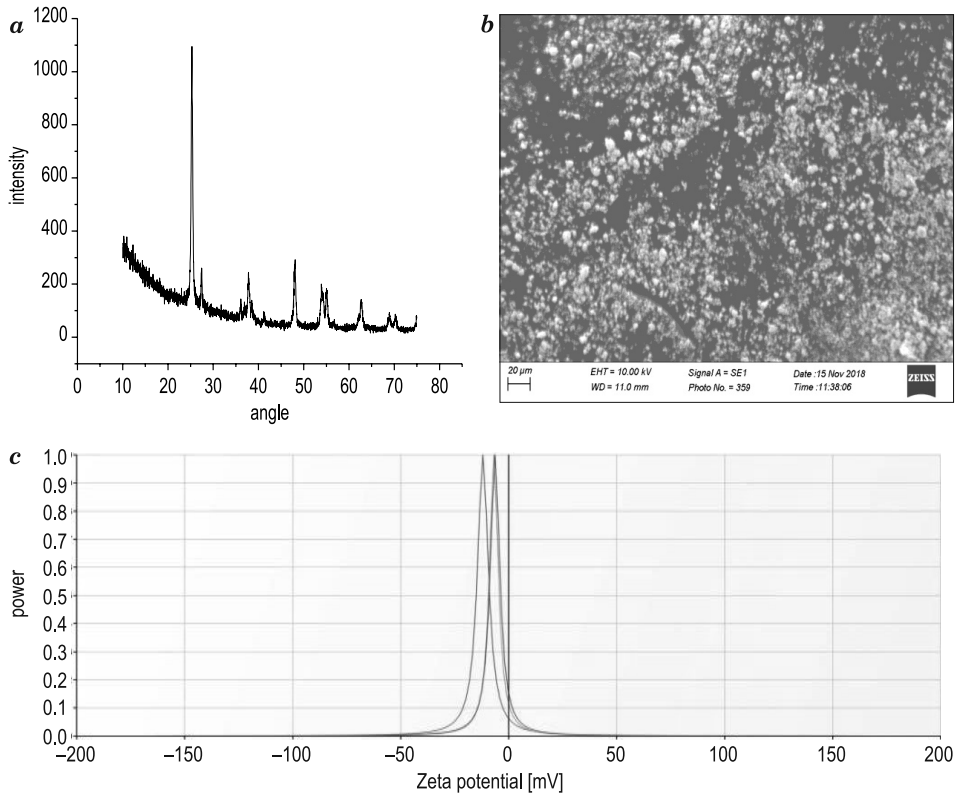

Fig. 1. Characterization of titanium dioxide nanoparticles (TiO2 NPs): a – X-ray diffraction pattern; *b* – Scanning electron micrograph; *c* – Zetapotential of 1 mg L^{-1} TiO_2 NPs. Scale bar represents 30 µm. Images at a magnification of 2000× in culture medium. Scale bar 10 µm. Images at a magnification of 2000× in culture medium. Scale bar 10 µm. Images at a magnification of 2000×

Table 1

Effective concentration (EC_{50}) after 72 h of exposure of TiO_2 NPs to *C. ellipsoideum*, Estimation of parameters of Weibull model

Effective concentration [mg L^{-1}]	TiO_2 NPs
EC_{50}	69.9
EC_{25}	10.9
EC_{20}	8.37
EC_{15}	6.06
EC_{10}	3.88
EC_5	1.85

Chlorophyll *a* and *b* exposed to sub-lethal concentration of TiO_2 NPs decreased significantly ($p < 0.01$) with increasing concentrations of TiO_2 NPs compared to the controls (Figure 2), and the highest inhibition of chlorophyll *a* and *b* were observed after 72 h in cells exposed to 10.90 mg L^{-1} of TiO_2 NPs. Chlorophyll *a* and *b* are inversely proportional to the level of concentrations. The decreases in chlorophyll *a* were 38.76, 53.37, 67.98, 70.78 and 76.68% compared to control; while chlorophyll *b* decreased as follows: 87.64, 88.20, 91.57, 92.97 and 93.25% compared to the control. This indicates that chlorophyll b was highly decreased compared to chlorophyll *a*.

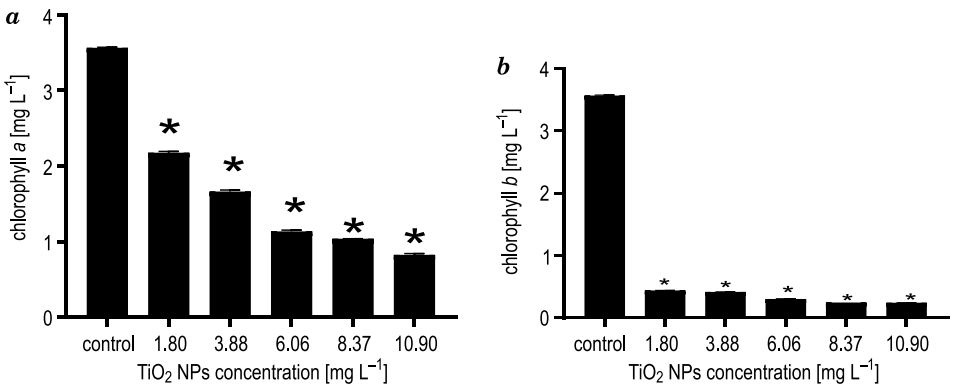

Fig. 2. Comparison of Chlorophyll *a* and *b* levels in *C. ellipsoideum*: *a* – comparison of Chlorophyll *a*; *b* – chlorophyll *b* levels in *C. ellipsoideum* with exposed concentrations (control 0.0, 1.80, 3.88, 6.06, 8.37 and 10.90 mg L^{-1}) of TiO_2 NPs after 72 h. Data were expressed as mean ± SD of three replicate samples. $^*p < 0.01$ indicate significant differences between exposure group and the corresponding control group (ANOVA followed by Tukey's test)

All antioxidant enzymes activities (Catalase, SOD and GRx) on the algal cells exposed to TiO_2 NPs (Figure 3) significantly increased ($p < 0.01$). The range of percentages increment compared to the control was between: 22–58.66, 2.68–16.85, 26.50–86.67 and 33.33–100 for Catalase, SOD, GRx and MDA respectively. However, lipid peroxidation (MDA) increase in this study, demonstrated a high fluctuation with the concentrations 6.06 and 8.37 mg L^{-1}.

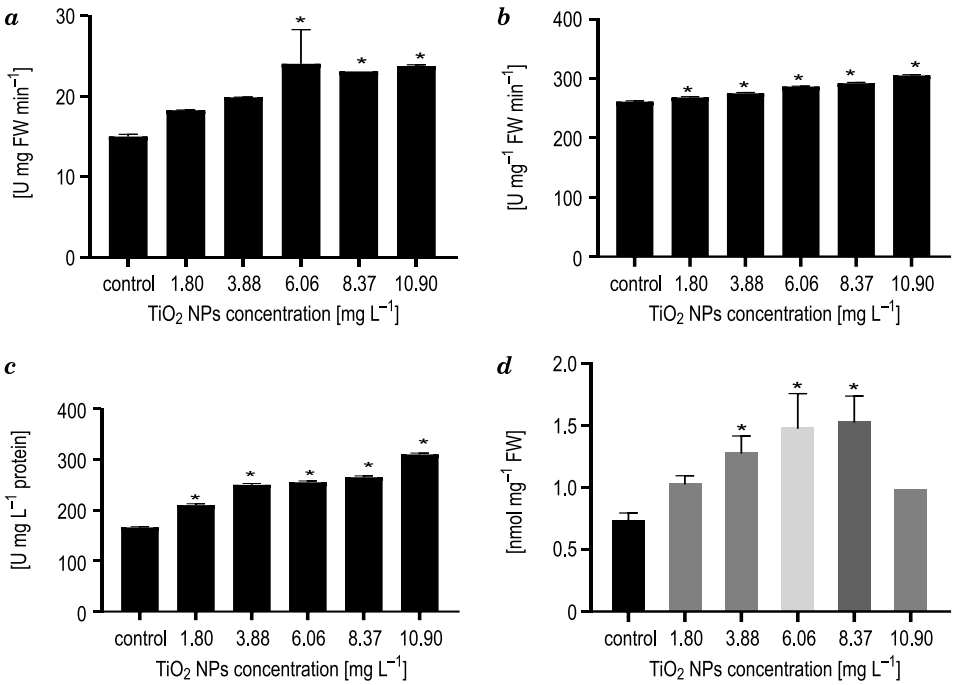

Fig. 3. Antioxidant enzymes concentrations in *C. ellipsoideum*: *a* – catalase activity; *b* – SOD activity; *c* – GRx activity; *d* – MDA activity with exposed concentrations (control 0.0, 1.80, 3.88, 6.06, 8.37 and 10.90 mg L^{-1}) of TiO_2 NPs after 72 h in *C. ellipsoideum*. Data are expressed as mean ± SD of three replicate samples. $^*p < 0.01$ indicate significant differences between exposure group and the corresponding control group (ANOVA followed by Tukey's test)

Discussion

In this study, the acute concentration of TiO_2 NPs was sufficient to induce toxicity to *C. ellipsoideum* with harmful effects on its physiology. This pollutant in aqueous solution probably released free radicals capable of scavenging for microalgal cells and forms ligands that chelate and alter the survival of the exposed microalgae. The effective concentration (EC_{50}) in response to stress caused by TiO_2 NPs is probably due to their toxicity. However, based on the EC_{50} value recorded (69.90 mg L^{-1}), exposures of microalgae showed that *C. ellipsoideum* is intolerant to TiO_2 NPs compared with the recorded values of 9.10 mg L^{-1} to *C vulgaris*, 4.90 mg L^{-1} to *C. pyrenoisa* and 10.91 mg L^{-1} to *Phaeodactylum tricornutum* (DAOHUI et al. 2018, HUREL et al. 2012, ZHU et al. 2010).

To understand the action of TiO_2 NPs on *C. ellipsoideum*, chlorophyll content was evaluated and significant decrease was recorded in this study. Similar changes have been identified in other findings on microalgae

Scenedesmus obliquus (YUAN et al. 2010), implying that this decrease in chlorophyll content commonly occur in microalgae exposed to TiO_2 NPs. The changes observed due to the selected sub-lethal concentrations used in the present study are probably linked to the adsorption of TiO_2 NPs on algal cells. The bioaccumulation of this chemical in *C. ellipsoideum* cells could be responsible for the disruption and impairment of photosynthesis electron transfer system (HUREL et al. 2012) involved in the mechanisms pathways of photosynthesis. Moreover, the decrease of chlorophyll levels in this study may be ascribed to the presence of TiO_2 NPs into algal cells that are known to interfere with chlorophyll synthesis either through direct inhibition of an enzymatic step or by inducing deficiency of an essential nutrient during photosynthesis (QIAN et al. 2009). Furthermore, *C. ellipsoideum* exposure to TiO_2 NPs could block light penetrating the cells and induce disruptive effects in the cells thereby causing decreasing chlorophyll content. Recently, the presence of TiO_2 NPs on *C. vulgaris* was characterized by cell wall/membrane damage, plasmolysis and internalization of TiO_2 NPs with critical effects on photosynthesis (HUREL et al. 2012).

The mechanism involved for the prevention and inhibition of cells damage due to environmental stressors is the synthesis of antioxidant activities. These stressors enhanced the ability of the organism to accumulate hydrogen peroxide and superoxide anion commonly known as reactive oxygen species (ROS). Previous studies have reported the increase of ROS production on algae following the exposure of nanomaterial (CHENG et al. 2016, ZHONG et al. 2014, RAI et al. 2013). In this study, *C. ellipsoideum* after exposed to TiO_2 NPs demonstrated an alteration of antioxidants. This probably suggests that antioxidants could act synergistically to mitigate the effect of toxicity. Similar results have been reported in the effect of cadmium on the growth and antioxidant response of freshwater *C. vulgaris* (CHENG et al. 2016, ZHU et al. 2007).

Catalase, SOD and MDA activities were significantly increased in this study. This shows the tendency of TiO_2 NPs to alter the functioning ability of H_2O_2 as reported by (SHARMA et al. 2012) [29]. Catalase promotes the dismutation of H_2O_2 into H_2O and O_2. H_2O_2 which is a product of SOD is detoxified by CAT, which is one of the main ROS-scavenging enzymes. SOD plays protective roles against oxidative damage and its increase might be due to the direct effect of the metal-oxide on the SOD genes. The great induction of SOD by TiO_2 NPs in this study could be due to the enhancement of ROS.

The alteration of MDA activity in this study was observed to vary with the change in concentrations; however exposure of *C. ellipsoideum* to TiO_2

NPs significantly increased MDA activity. The increase of secondary end-product of oxidation which is eventually considered as biomarker of lipid peroxidation in this study could be attributed to the effect of TiO_2 NPs which could have stressed the algae thereby oxidizing polyunsaturated fatty acid with their free ions (Hemeda 1990).

The relatively significant decrease of GRx activity of *C. ellipsoideum* exposed to TiO_2 NPs was recorded. This decrease could be assigned to the sudden change on the specific gene expressed on the treated *C. ellipsoideum*. Similar depletion of GRx was reported on *C. ellipsoides* exposed to TiO_2 NPs and phosphorous (MATOUKE et al. 2018)

Conclusions

In this study, exposure of *C. ellipsoideum* to TiO_2 NPs indicated an alteration of the biomass (chlorophyll *a*, *b*) and antioxidant activities (catalase, superoxide dismutase, glutathione reductase and malondialdehyde). The study also demonstrated that *C. ellipsoideum* is sensitive to TiO_2 NPs therefore, monitoring of this microalga in natural ecosystem for this metallic compound is necessary for its conservation. Thus, research studies on the impacts of TiO_2 NPs on other freshwater plankton's organisms are relevant in order to have an insight on their challenges in aquatic ecosystems.

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ENVIRONMENTAL POLLUTION ASSESSMENT OF SURFACE WATER IN OMEGE COMMUNITY USING CHEMICAL DATA AND RISK-BASED EVALUATION

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Key words: mining, contamination, surface water, heavy metals, health risk.

Abstract

Mining of mineral resources creates economic backbone for many countries but its attendant consequences on the environment and human health is far-reaching, especially when the process is done without appropriate sustainability measures. In this study, physico-chemical parameters and heavy metal load of the upstream, midstream and downstream zones of Omege River, Ebonyi State Nigeria was determined according to standard procedures. USEPA risk model was applied to estimate health risks that could be associated with five heavy metals (Zn, Fe, Cu, Pb and Cr) in the three zones of the surface water in both children and adults. EC, TDS, TSS, TS, TH, Pb and Cr were found to be above the USEPA permissible limit in the three zones of the river. CI index based on zones of the surface water was in the order of upstream < midstream < downstream while it was Zn < Cu < Fe < Cr < Pb based on the heavy metals. There was extreme high contamination by Pb in the water samples from the study area particularly in the downstream with CI reaching up 600. HQ of Pb and Cr in the three zones of the surface water was > 1 for both children and adults with that of Pb (6.64–23.57) in threatening level. HI range from 14.06 in the upstream to 3.180 in the downstream for children and from 8.71 in the upstream to 19.72 in the downstream for adults. CRI estimated for Pb and Cr were not within the acceptable limit. These results suggest potential non-carcinogenic and carcinogenic health risk in both children and adults via oral consumption of water from Omege River. Finally, this study revealed that children were at higher health risk if exposed to the heavy metals via the consumption of water from Omege River than adults.

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Introduction

No doubt, for countries with natural mineral resources, mining is prevalent to exploit the resources to drive their economy. However, mining activities have created environmental stress particularly in developing countries like Nigeria where mining process is poorly carried out in an unsustainable manner (ADEWOYE et al. 2020). Toxic chemicals such as heavy metals are released to the environment at different stages of mining activities such as during extraction, beneficiation, and refinement. Impacts of mining during active mining period and after which the mining sites are abandoned can be felt significantly. For instance, ODUKOYA et al. (2017) reported contamination of surface water by toxic elements in adjoining active mining site; Antunes et al. (2017) documented toxic metals in an abandoned radium mine, and ADEWOYE et al. (2020) provided data of contamination of soil sample in an abandoned goldmine site.

Waste generated during mining including the mining water discharged into the environment are laden with toxic heavy metals in threatening concentrations which can leach and contaminate water resources such as surface and groundwater. Majority of heavy metals are non-essential and those that are essential can become toxic at elevated concentrations (USEPA 2014). Human exposure to these metals can elicit disease conditions and even death. In rural communities, there is poor access to portable drinking water and as such, people depend on natural water sources such as rain, groundwater and surface waters. However, surface water is the most sort-after among these water sources as they are almost without financial demand like digging a well or borehole to access groundwater and may not be seasonal like the rainwater. It was reported that 58% of households in rural communities in Nigeria lack access to safe water (WHO 2014).

Omege is a rural community situated in Ebonyi State in the southeast region of Nigeria. It is one among the several rural communities in the state that accommodates mining industries with evidence of environmental impacts from the mining activities. Recently, the use of health risk models in addition to physico-chemical indices of contaminated water has become an acceptable tool in ecotoxicology (SUNDARAY et al. 2011, OLANGUNJU et al. 2020). Unfortunately, data on the physico-chemical parameters and health risk associated with heavy metals in the major surface water of high socio-economic importance in the Omege community is lacking, hence the justification for this study.

Materials and Methods

Study area

Dwellers of Omege community in Ebonyi State, southeast Nigeria are mainly farmers, artisanal fishermen and workers in the mining industries. Omege River is a surface water of high socio-economic importance to the people of Omege community. The river serves various domestic and agricultural purposes including drinking, washing, recreation, irrigation, fishing and animal feeding. The community also accommodates First Patriot Limited, a mining industry that deals with extraction of lead and other useful metals.

Sampling and analysis

Sampling was carried out in August – October, 2019; transition months from rainy season to dry season. Three sampling zones; upstream, midstream and downstream designated as UPS, MDS and DST were established during the first sampling with three (that is 1, 2 and 3) sampling points each. The coordinates of the sampling points are presented in Table 1. Water samples were collected at depth 20–25 cm directly into prewashed 1 L polyethylene bottles covered with 0.45 μm mesh. Ultrapurified 6 M HNO_3 was added to the filtered water samples in-situ to keep the pH below 2 and transported to the laboratory at low temperature ($\leq 4^oC$) using ice packs for ex-situ analysis. pH, electrical conductivity, total dissolved solids (TDS) and total suspended solids (TSS) were measured in-situ using a digital multi-meter (TOPAC Instruments Inc.) while total solids (TS) was calculated as the sum of TDS and TSS. Total hardness (TH), total acidity and total alkalinity were determined according to APHA (2012) and titration method was used to analyse nitrate, sulphate, chloride, fluoride. Na, Ca, Zn, K, Fe, Cu, Pb and Cr were analysed using atomic absorption spectrophotometer (AAS) after digestion using aqua regia according to USEPA (1996) and APHA (2012) guidelines. Elemental quantification analysis was done at PRODA Laboratory, Enugu, Enugu State, Nigeria. The quality assurance of elemental analysis was based on reference material CASS-5 and all elements had good recovery rates with range of 90–100%.

Table 1

Coordinates of sampling points for surface water in the study area

Zones	Sample points	Coordinates
Upstream	UPS1	N 6º 10' 26.226"; E 8º 08' 42.936"
	UPS2	N 6º 10' 26.142"; E 8º 08' 42.996"
	UPS3	N 6º 10' 26.496"; E 8º 08' 43.332"
Midstream	MDS1	N 6º 10' 17.52"; E 8º 08' 54.108"
	MDS2	N 6º 10' 17.442"; E 8º 08' 54.216"
	MDS3	N 6º 10' 16.728"; E 8º 08' 56.886"
Downstream	DST1	N 6º 10' 21.204"; E 8º 09' 15.654"
	DST2	N 6º 10' 21.126"; E 8º 09' 15.378"
	DST3	N 6º 10' 21.624"; E 8º 09' 15.306"

*UPS – upstream; MDS – midstream; DST – downstream

Statistical analysis

Statistical analysis was done using SPSS (v. 20). Range (minimum and maximum) and means are reported.

Analysis of contamination index (CI)

Contamination index (CI) was used to evaluate elemental enrichment for the following heavy metals Zn, Fe, Cu, Pb and Cr in the surface water (upstream, midstream and downstream) using their mean values with respect to maximum permissible limit (MPL) set by USEPA (2006). CI was calculated as:

$$CI = \Sigma \frac{Cm}{MPL} \quad (1)$$

where:
CI – contamination index
Cm – concentration of metals in the sample
MPL – maximum permissible limit.

Contamination index is classified as: CI > 5 (contaminated) $1 \leq CI \leq 5$ (slightly contaminated) and CI < 1 (not contaminated).

Non-carcinogenic and carcinogenic health risk evaluation

Non-carcinogenic health risk assessment predicts the likelihood of an exogenous substance to elicit various health hazards that are non-cancerous in nature while carcinogenic health risk evaluation predicts the possi-

bility of a substance to cause cancer upon exposure (OLANGUNJU et al. 2020). Exposure to toxic heavy metals can be through ingestion, dermal or inhalation pathway. However, it has been shown that ingestion play the most important role in water contamination (GUO-LI et al. 2016, ODUKOYA et al. 2017, OLANGUNJU et al. 2020). USEPA health risk models (eqs. 2–5) were adopted and used to evaluate the risk of health hazard for water contamination via oral in children and adults. In this study, health risk estimation was done for five heavy metals which include Zn, Fe, Cu, Pb and Cr based on upstream, midstream and downstream zones of the surface water. Hazard quotient (HQ) and health index measures non-carcinogenic health risk while cancer risk index (CRI) assesses carcinogenic health risk.

$$\text{Hazard quotient (HQ)} = \frac{\text{CDI}}{\text{RFD}} \tag{2}$$

HQ > 1 means that non-carcinogenic health risk would be elicited upon exposure to a single metal:

$$\text{CDI}_{\text{Ingestion}} \frac{\text{Cm} \cdot \text{IngR} \cdot \text{EF} \cdot \text{ED}}{\text{BW} \cdot \text{AT}} \tag{3}$$

$$\text{Hazard Index (HI)} = \Sigma\text{HQ} \tag{4}$$

HI > 1 means that non-carcinogenic health risk would be elicited upon exposure to a myriad of stated metals

$$\text{CRI}_{\text{Ingestion}} = \text{CDI}_{\text{Ingestion}} \cdot \text{SF} \tag{5}$$

where:
CDI – daily chronic intake
RFD – oral reference dose based on USEPA (2009) as shown in Table 2.
Cm – concentration of heavy metals
IngR (ingestion rate of water) = 1 L for children and 2 L for adults
EF (exposure frequency) = 365 days/year
ED (exposure duration) = 6 years for children and 65 years for adults
BW (body weight) = 20 kg for children and 65 kg for adult
AT (average time) = 2190 for children and 23,725 days for adult.

SF (slope factor) via ingestion route = $8.5 \cdot 10^{-3}$ for Pb and $5.0 \cdot 10^{-1}$ for Cr. Slope factor which is the probability of developing cancer per unit exposure level of mg/kg/day has only been derived for Pb and Cr amongst the analysed heavy metals as presented in ADEWOYE et al. (2020) and as shown in Table 2. An acceptable value for cancer risk according to USEPA = $1 \cdot 10^{-6}$ to $1 \cdot 10^{-4}$.

Table 2

Physico-chemical and elemental analysis of water samples from the study area

Parameters	Unit	Upstream		Midstream		Downstream		USEPA Limit
		range (min–max)	mean	range (min–max)	mean	range (min–max)	mean	
pH		6.93–7.10	7.01	6.76–7.37	7.14	6.90–7.14	7.03	6.50–8.50
Conductivity	µs/cm	14610–14740	14666.67	14950–15650	15240	14850–15960	15453.33	500
TDS	mg/L	8740–21860	14393.33	16700–20260	18273.33	18200–22460	20220	250
TSS	mg/L	1080–1740	1406.67	1120–1280	1186.67	1220–1840	1466.67	30
TS	mg/L	10140–23600	15800	17860–21380	19460	19540–24300	21686.67	500
TH	mg/L	1000–3200	2066.67	1200–2350	1783.33	2500–3500	2766.67	250
Acidity	mg/L	30–57.5	42.5	15–20	17.5	12.5–30	22.5	–
Alkalinity	mg/L	150–250	216.67	300–400	350	250–600	450	20
Nitrate	mg/L	0.36–0.72	0.48	0.33–0.42	0.36	0.33–0.38	0.36	10
Sulphate	mg/L	67.01–101.44	85.15	122.73–208.49	162.98	35.02–69.85	48.74	250
Chloride	mg/L	6.62–10.02	7.96	7.94–12.08	9.61	8.38–16.04	11.23	250
Fluoride	mg/L	0.15–0.32	0.22	0.11–0.2	0.16	0.22–0.26	0.24	1.50
Na	mg/L	1.60–2.74	2.05	0.60–2.17	1.53	1–2.11	1.5	60
Ca	mg/L	0.85–1.21	0.97	0.60–0.94	0.8	0.61–0.94	0.75	60
Zn	mg/L	2.25–2.41	2.35	1.88–2.6	2.27	2.14–2.45	2.27	3
K	mg/L	1.38–1.52	1.43	0.58–2.23	1.51	0.35–1.32	0.93	> 200
Fe	mg/L	1.82–2.29	2	0.45–2.11	1.44	0.28–2.05	1.36	0.3
Cu	mg/L	0.68–0.82	0.76	0.55–0.8	0.67	0.09–0.96	0.66	1
Pb	mg/L	0.23–0.39	0.30	0.15–0.4	0.31	0.37–0.43	0.4	0.001
Cr	mg/L	0.01–0.19	0.1	0.14–0.52	0.33	0.19–0.38	0.36	0.005

Results and discussion

Physico-chemical parameters

The results of physic-chemical parameters and heavy metals in the water sample from the study are presented in Table 2. Generally, the magnitude of physico-chemical and elemental parameters in the water samples from the study area was in the order of upstream < midstream < downstream. The mean pH values of water samples in the upstream, midstream and downstream zones of the surface water are in the range of 7.01–7.14 which is within the USEPA limit, and which can be categorized

as neutral. There was elevated level of conductivity regardless of the sampling zone which is an indication of the presence of charged ions in the water. Furthermore, TDS, TSS, TS and TH were above the USEPA limit in the three sampling zones.

Mean concentrations of nitrate and sulphates were within the permissible limit which may be an indication that pollution of the surface water was not as a result of introduction of nutrients in form of fertilizer application or faecal particles in the adjoining areas. For nitrates, the range were 0.36–0.72 mg/L, 0.33 – 0.42 mg/L and 0.33–0.38 mg/L in the upstream, midstream and downstream respectively while for sulphate it was 67.01–101.44 mg/L, 122.73–208.49 mg/L and 35.02–69.85 mg/L respectively. The irregularities of values of nitrates and sulphates could be attributed to synthetic fertilizers used in the farms adjoining the river course. All the elements analyzed were within the limit except for Pb and Cr which could be associated with the intrusions from the mining process in the study area. Other researchers have also reported high level of Pb and other toxic metals in environmental matrices sampled from adjoining areas of mining site (LIM et al. 2008, SAHA et al. 2017, ODUKOYA et al. 2017). The mean value of lead in the water sample from the study area was 0.30–0.4 mg/L while that of Cr was 0.1– 0.36 mg/L.

Contamination index

Comparison of five heavy metals (Zn, Fe, Cu, Pb and Cr) with maximum permissible level (MPL) for drinking water according to USEPA (2006) is presented in Figures 1 to 5. Except for Zn and Fe whose values of CI were higher in the upstream than those of midstream and downstream, and for Cu with CI higher in the upstream than the midstream only; CI index for the heavy metals were generally in the order of upstream < midstream < downstream. Zn and Fe are naturally occurring metals whose presence in the environment may be due to geogenic release from the bedrock (KHAN et al. 2013). However, despite the fact that Zn and Fe are essential metals, at elevated concentrations, Fe can damage various organs (BABY et al. 2010) while Zn cause inhibition of normal respiratory function (COOPER 2008). Pb whose ore is primarily mined in the study area had the highest CI (300–660) among the other heavy metals analyzed.

The order of heavy metals based on CI was Zn < Cu < Fe < Cr < Pb. Pb is known to cause cognitive retardation children. Based on CI rating, it can be said that the surface water was not contaminated by Zn whether at upstream, midstream or downstream. There was slight contamination by Fe in midstream and downstream but significant in the upstream. CI due

to Cu was 0.76, 0.67 and 1.36 in the upstream, midstream and downstream respectively, suggesting that there was no contamination due to Cu in the upstream and midstream but slightly in the downstream. There was extreme contamination by Pb in the water samples from the study area particularly in the downstream with CI reaching up 600. In addition, significant contamination by Cr occurred in the surface water as CI value for upstream, midstream and downstream was 20, 66 and 72 respectively.

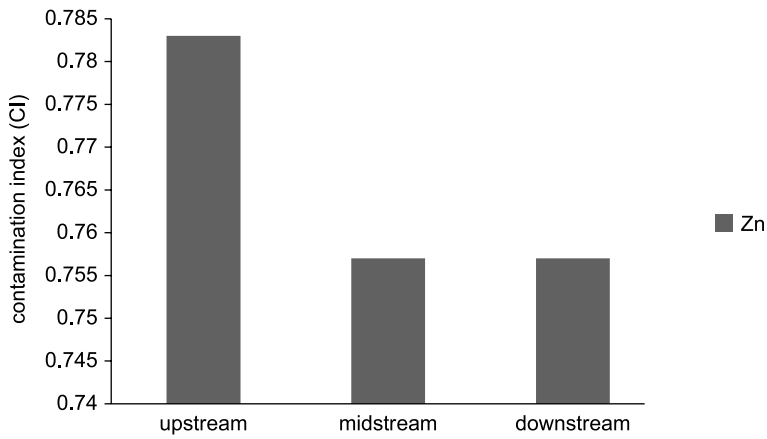

Fig. 1. Contamination index due to Zn in water sample of the study area

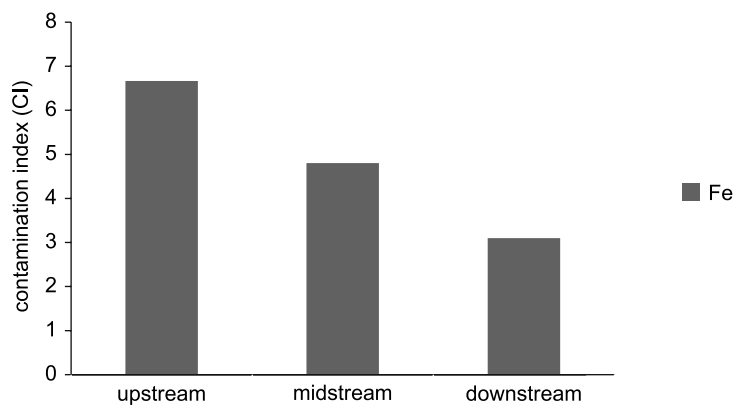

Fig. 2. Contamination index due to Fe in water sample of the study area

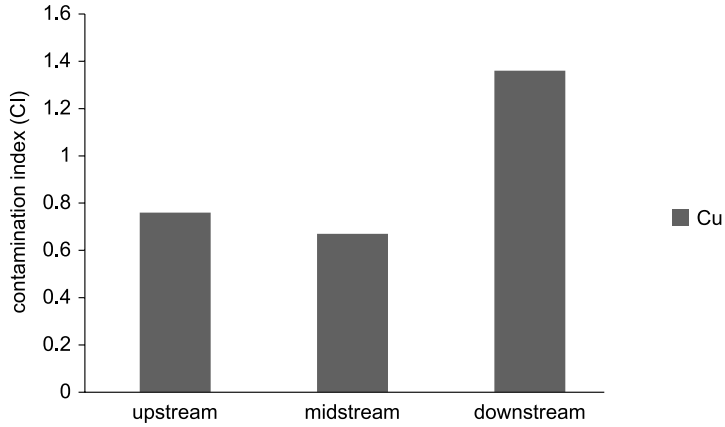

Fig. 3. Contamination index due to Cu in water sample of the study area

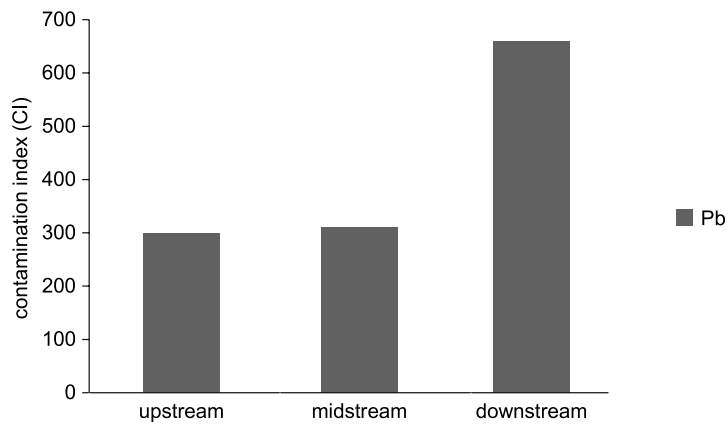

Fig. 4. Contamination index due to Pb in water sample of the study area

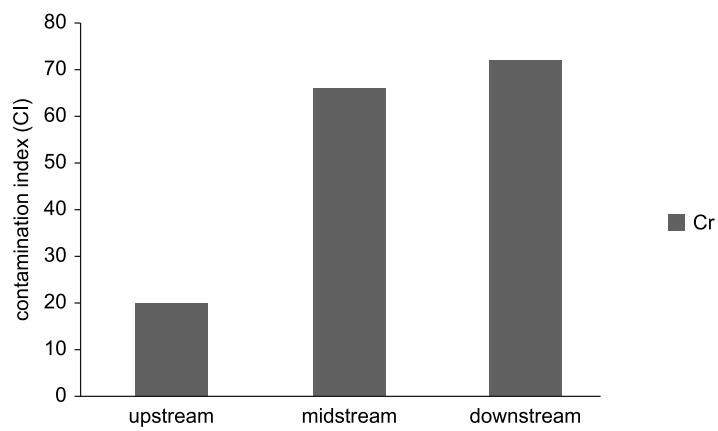

Fig. 5. Contamination index due to Cr in water sample of the study area

Chronic daily intake (CDI), hazard quotient (HQ) and health index (HI)

Non-carcinogenic risk which is the evaluation of propensity for an individual to experience health effects that are not carcinogenic was extrapolated from CDI estimation. Estimations for CDI is presented in Table 3 while HQ and HI are presented in Table 4. Despite the larger quantity of water taken by adults daily, CDI estimated for children was greater. This may be suggestive of children's vulnerability to toxic metals in their water as compared to adults. For Pb whose CI was greatest, the CDI for children was in the range of 0.015 to 0.033 while it was 0.0093 to 0.0205 for adults.

Table 3

Chronic daily intake (CDI) of heavy metals in water samples of the study area

Heavy metals	Chronic daily intake (CDI)						Reference dose (RFD)	Slope factor (SF) [mg/kg/day]
	children			adults				
	UPS	MDS	DST	UPS	MDS	DST		
Zn	0.1175	0.1135	0.1135	0.07285	0.07037	0.07037	0.3	–
Fe	0.1	0.072	0.0465	0.062	0.04464	0.02883	0.3	–
Cu	0.038	0.0335	0.068	0.02356	0.02077	0.04216	0.04	–
Pb	0.015	0.0155	0.033	0.0093	0.00961	0.02046	0.0014	0.0085
Cr	0.005	0.0165	0.018	0.0031	0.01023	0.01116	0.003	0.5

*UPS – upstream; MDS – midstream; DST – downstream

Table 4

Hazard quotient, health index and cancer risk index of heavy metals in water samples of the study area

Heavy metals	Hazard quotient (HQ)						Cancer risk index (CRI)					
	children			adults			children			adults		
	UPS	MDS	DST	UPS	MDS	DST	UPS	MDS	DST	UPS	MDS	DST
Zn	0.39	0.38	0.38	0.24	0.23	0.23	–	–	–	–	–	–
Fe	0.33	0.24	0.16	0.21	0.15	0.10	–	–	–	–	–	–
Cu	0.95	0.84	1.70	0.59	0.52	1.05	–	–	–	–	–	–
Pb	10.71	11.07	23.57	6.64	6.86	14.61	1.00E-04	1.32E-04	2.81E-04	7.91E-05	8.17E-05	1.74E-04
Cr	1.67	5.50	6.00	1.03	3.41	3.72	2.50E-03	8.25E-03	9.00E-03	1.55E-03	5.12E-03	5.58E-03
Health index (HI)	14.06	18.03	31.80	8.71	11.18	19.72	–	–	–	–	–	–

*UPS – upstream; MDS – midstream; DST – downstream

HQ for the five heavy metals (Zn, Fe, Cu, Pb and Cr) in the upstream, midstream and downstream revealed that Zn, Fe and Cu (except Cu at the downstream) posed no risk of health to both children and adults since their HQ was < 1. Pb and Cr had HQ in samples from the three zones of the surface water values > 1 in both children and adults. However, Pb had an extremely high HQ in the water sample from the study area. For instance, upstream, midstream and downstream sample had HQ of 10.71, 11.07 and 23.57 respectively in children while it was 6.64, 6.86 and 14.61 respectively in adults. This result further established that children are more predisposed to health risks compared to adults as submitted in the findings of OLANGUNJU et al. (2020). HI associated with water consumption regardless of the zones from which the water was collected in the study by children and adults with respect to Zn, Fe, Cu, Pb and Cr showed values outside the acceptable non-carcinogenic health risk. HI range from 14.06 in the upstream to 3.180 in the downstream for children and from 8.71 in the upstream to 19.72 in the downstream for adults. There is possibility of heavy metal release particularly Pb from the mining process in the adjoining area of the Omege River to the surface water and may be responsible for the possible non-carcinogenic health risk evaluated if water from the surface water is consumed by children and adults.

Cancer risk index (CRI)

CRI has been applied to determine the potential of developing cancer due to exposure to certain metals. CRI as estimated for upstream, midstream and downstream in children and adults as presented in Table 4 shows that Pb and Cr would elicit carcinogenesis in the two age groups irrespective of the zones of Omege River that they fetch water from for drinking. Furthermore, the results showed that drinking from downstream predisposes one more to cancer risk and children were at higher risk than adults. This is consistent with the report of ADEWOYE et al. (2020) that exposure to Pb outside the permissible cancer risk of would predispose individuals to risk of having cancer particularly in children. CRI due to Pb was in the range of 7.91E-05 in the upstream to 2.81E-04 in the downstream in both children and adults. Meanwhile, it was in the range of 1.55E-03 to 9.00E-03 for Cr in both age groups.

Conclusion

This research assessed the health status of Omege River in Ebonyi State, southeast Nigeria using physico-chemical parameters and health risk-based evaluation of five heavy metals. The study elucidated that even though mining may be a source of income for economic viability of a country, its impacts on the environment must be prioritised as the revenue generated from mining may not be enough to solve the consequential problems that will be posed to the environment as well as human health. Amongst Zn, Fe, Cu, Pb and Cr analyzed, Pb and Cr were above the permissible limit. HQ for the five heavy metals in the upstream, midstream and downstream zones showed that Zn, Fe and Cu (except for Cu at the downstream) posed no risk of health to both children and adults since their HQ was < 1. However, Pb and Cr had HQ in samples from the three zones of the surface water with values > 1 for both children and adults. It was noted that Pb had an extremely high HQ in the water sample from the study area. HI and CRI estimated for the selected heavy metals were not within the acceptable limit, suggesting potential non-carcinogenic and carcinogenic health risk in both children and adults via oral consumption of water from Omege River. Finally, this study revealed that children were at higher health risk if exposed to the heavy metals via the consumption of water from Omege River than adults.

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BIOFUNCTIONAL PROPERTIES OF MILK FAT GLOBULE MEMBRANE PHOSPHOLIPIDS

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Key words: milk, milk lipids, phospholipids, milk fat globule membrane, functional properties.

Abstract

The aim of the paper is to discuss the general properties of polar lipids present in the milk fat globule membrane (MFGM), their role in living organism and some benefits of consuming these components in the human diet.

Milk phospholipids contain glycerophospholipids and sphingolipids. These compounds perform a multitude of functions in living organisms: they serve as energy storage for the body, are a building blocks of cell membranes, they and their derivatives are bioactive molecules involved in the mediation and recognition of signals, and interact with other cell components. There has been an increasing number of reports documenting the health benefits of milk lipid consumption. Although they are present in milk in small quantities, their unique properties may help prevent and alleviate numerous diseases.

Introduction

There has been an increasing number of reports indicating that breast-feeding is vital for proper infant development (DONOVAN 2006, LEE et al. 2018). It also benefits the mother's health by reducing the risk of breast and ovarian cancers (JONES 2007). This synergistic interaction is the result of an evolutionary adaptation through which mechanisms advantageous for survival have emerged. The effects of milk on the child's health are extremely complex, and providing the infant with essential nutrients is only part of milk's functions. As such, milk continues to serve as a model food-stuff, while researches also draw upon it as an inspiration and a source of knowledge on the two-way links between humans and nutrition. Advancing

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the knowledge on the biological functions of milk constituents will help better harness the prospective properties of nutrients to develop biofunctional foods with distinct therapeutic values, designed to combat specific diseases or satisfy specific consumer needs.

Though scarce in the membrane, polar lipids play an important role in nutrition and normal cell function. Due to their unique properties, polar lipids perform numerous functions in living organisms: they serve as energy storage for the body, are a building block of cell membranes (isolating the cell from the environment and separating cellular compartments), they and their derivatives are bioactive molecules involved in the mediation and recognition of signals, they also interact with other cell components (NICOLSON and ASH 2014).

There has been an increasing number of reports documenting the health benefits of milk lipids in general, and the biological role of phospholipids in particular (AMBROZIAK and CICHOSZ 2013, SMOCZYŃSKI 2017, KUCHTA et al. 2012, EL-LOLY 2011). Consequently, the aim of the present paper is to discuss the general properties of polar lipids present in the milk fat globule membrane (MFGM), their role in living organism and some benefits of consuming these components in the human diet.

Lipid components of the milk fat globule membrane (MFGM)

Milk lipids are likely the most complex and least understood group of milk constituents. Lipid content depends on the species and ranges from 3.8 to 3.9% for woman's milk, and 3 to 5% for bovine milk. In mammal bodies, the lipids are formed in the epithelial cells of the mammary gland and secreted in emulsified form as milk fat globules coated with a phospholipid/protein trilayer. Secretion of fat globules is a process unique to the mammary gland and has been a subject of many studies (HEID and KEENAN 2005, MATHER and KEENAN 1998). In structural terms, fat globules consist of an apolar inner core mostly composed of triacylglycerols and cholesterol esters, surrounded by a membrane about 10–20 nm in diameter. This milk fat globule membrane has a very defined structure, making up 2–6% of the total globule mass, and consists mainly of proteins and phospholipids. The content of phospholipids in the membrane is estimated at 40 to 75% (DEETH 1997, SINGH 2006).

Milk phospholipids contain glycerophospholipids and sphingolipids. Glycerophospholipids consist of a glycerol backbone esterified with fatty acids at two positions, and a phosphate with a different organic group

(e.g. serine, choline) at the third position. For sphingolipids, the structure varies and is based on a sphingosine, i.e., a long-chain aliphatic amine (which additionally contains two or three hydroxyl groups). The attachment of a fatty acid to the amine group results in the formation of ceramide. If phosphocholine is attached to the ceramide, sphingomyelin is formed, whereas the attachment of a saccharide group produces a sphingoglycolipid (DEWETTINCK et al. 2008, VESPER et al. 1999).

Phospholipids account for up to 1% of milk fat (SÁNCHEZ-JUANES et al. 2009). In mammal cells, the majority of such lipids are produced in the endoplasmic reticulum (VANCE 2018). Table 1 presents the composition and content of the main lipid components of the milk fat globule membrane (based on FONG et al. 2007, SINGH 2006, SMOCZYŃSKI et al. 2012). Although the main fraction are triacylglycerols, the content of which varies in the literature between 20 to 80% of the membrane weight, the majority of triacylglycerols can originate from the contamination by the core of the fat globules during isolation of the membrane. Therefore, the literature shows a wide variation depending on the methods used to isolate the material (WALSTRA 1985).

Table 1

Lipid components of the milk fat globule membrane (FONG 2007, SINGH 2006)

Lipid group	Percentage [%]
Triacylglycerols	56.0–62.0
Diacylglycerols	2.1–9.0
Monoacylglycerols	0.4
Free fatty acids	0.6–6.0
Sterols	0.2–2.0
Phospholipids	26.0–40.6
including [% of phospholipids]:	
Sphingomyelin	20–22
Phosphatidylcholine	31–36
Phosphatidylethanolamine	27–30
Phosphatidylinositol	7–11
Phosphatidylserine	4–5
Lysophospatidylcholine	2
Lactocerebroside	3.4
Glucocerebroside	0.3

Characteristics of MFGM polar lipids

Phosphatidylcholine

Phosphatidylcholine (PC, also referred to as lecithin) is a phosphoglyceride in which the phosphate group is esterified with choline, and makes up 40 to 50% of the cell membrane phospholipids. PC is a zwitterion

over a wide range of pH values, owing to the presence of a positively-charged quaternary amine group and a negatively-charged phosphate residue. PC is biosynthesized in the endoplasmic reticulum (ER) through the so-called Kennedy pathway (KENNEDY and WEISS 1956), first by phosphorylating choline to form phosphocholine, then incorporating sn-1,2-diacylglycerol into the latter. The structure of PC is subject to changes through the exchange of fatty acids within the molecule, a process known as the Lands cycle (LANDS 1958). In the liver, phosphatidylcholine can also be synthesized through methylation of phosphatidylethanolamine (VANCE and RIDGWAY 1988).

Other PC constituents include: palmitic acid, stearic acid, oleic acid, linolenic acid, and arachidonic acid, with the saturated and unsaturated acids mostly occurring in the sn-1 and sn-2 positions, respectively. PC can be degraded by phospholipases, which exhibit specificity for the cleft bonds and thus can release certain metabolites important to signaling. The released choline is re-incorporated into new PC molecules (KRAHMER et al. 2011).

PC is involved in a number of important functions in the body. Together with other phospholipids, it plays a major role in lipid metabolism, being involved in the synthesis and transport of lipid-derived lipoproteins within the body. In combination with proteins and cholesterol, it forms a single-layer surrounding the hydrophobic core of lipoprotein particle composed of triacylglycerols and cholesterol esters. The essential role of PC in maintaining normal function of lipoproteins also relates to the presence of polyunsaturated carboxylic acids at the sn-2 position. The changes in the PC molecule brought about by the Lands cycle enable compounds, such as arachidonic acid, to be incorporated into the sn-2 position, modifying the biomembrane fluidity in turn (RAWICZ et al. 2000). Lower proportions of arachidonic acid in PC molecules have been shown to inhibit the synthesis and release of VLDLs (very low-density lipoproteins), leading to triacylglycerol accumulation in the liver (RONG et al. 2015). PC also indirectly regulates the synthesis and dynamics of intracytoplasmic lipid droplets. Suppressed PC biosynthesis in adipocytes leads to increased droplet size (KRAHMER et al. 2011).

Phosphatidylethanolamine

After PC, phosphatidylethanolamine (PE) is the second most abundant phospholipid in milk fat globule membranes and plant/animal cell membranes. It is mainly biosynthesized through the incorporation of cytidine diphosphate-ethanolamine into diglyceride – releasing cytidine

5`-monophosphate – or through the decarboxylation of phosphatidylserine (GIBELLINI and SMITH 2010).

Arachidonic and docosahexaenoic acids are major constituents of the PE, attached mainly at the sn-2 position, whereas saturated acids are the most common component at sn-1. In some types of cells (neurons, cells involved in the inflammatory response), a large proportion of PE (more than 50%) occurs as plasmalogen, i.e., the form containing an ether bond. By contrast, the plasmalogen form of PE is in trace amounts in the liver (HAN et al. 2001).

Unlike PC, PE – together with phosphatidylserine – occurs mainly in the inner part of the lipid bilayer, from the cell interior side. Being a polar head group, PE can form hydrogen bonds with other lipids or proteins, which stabilizes proteins within the membrane (YEAGLE 2014). The small size of the polar head can alter the curvature of the membrane surface and change the fluidity of the biomembrane (DAWALIBY et al. 2016).

PE also plays a major role in lipid metabolism and the formation of cytoplasmic lipid droplets. It is particularly associated with the function and metabolism of VLDL molecules – its presence in these molecules promotes their rapid take-up and removal from circulation. This may result in the lower risk of hypercholesterolemia (VEEN et al. 2017). Biomembranes also contain smaller quantities of phosphatidylcholine derivatives, such as lysophosphatidylethanolamine – which bears only one acyl residue – or mono-/dimethyl-phosphatidylethanolamine (GIBELLINI and SMITH 2010).

Sphingomyelin

Sphingomyelin (SM) is a sphingolipid, a complex group of lipid compounds, in which the long-chain amino alcohol sphingosine comprises the backbone. Sphingomyelin is formed through the binding of a fatty acid residue with an amide bond, after which a choline phosphate group is attached to the resulting ceramide. The biosynthesis and metabolism of sphingolipids are strictly controlled, influenced by multiple factors, and include many intermediate metabolites with their respective biological activities. By contrast, SM is predominantly formed by transferring phosphocholine from PC onto the ceramide (MERRILL 2011). The group was named in reference to the sphinx, which was intended to evoke their heretofore enigmatic properties. However, the biological role of its compounds has been increasingly documented in scientific literature (MERRILL 2011).

Like PC, SM is a zwitterion. It can account for up to half of the membrane lipids in some tissues, but in most cases PC exceeds it in proportion. Also, like PC, SM occurs mainly in the outer layer of the plasma mem-

brane. The SM molecule predominantly contains long-chain saturated fatty acids (palmitic and stearic) with a small number of monounsaturated fatty acids (RAMSTEDT et al. 1999).

Sphingolipids, including sphingomyelin (SM), are involved in a number of important functions in the body. One of the main functions of SM – resulting from its unique structure – is combining with cholesterol to form domains known as lipid rafts, i.e., special areas on the surface of the membrane that are slightly tighter packed than the others (SIMONS and IKONEN 1997). These domains serve as interaction sites for certain proteins. Since SM content correlates with cholesterol levels in membranes, SM can affect the levels and metabolism of cholesterol (VESPER et al. 1999). Moreover, SM is a major source of ceramides and other metabolites that act as signal transducers for growth regulation, cell migration, adhesion, apoptosis, and inflammatory response (HANNUN and OBEID 2018).

Phosphatidylinositol

Phosphatidylinositol (PI) is a glycerophospholipid that contains carboxyl acid in ester linkage with glycerol, as well as inositol (hexahydroxycyclohexane) bonded via a phosphoric acid residue. The PI molecule is negatively charged. This phospholipid has the unique characteristic of having high levels of stearic and arachidonic acids at sn-1 and sn-2, respectively (D'SOUZA and EPAND 2014). Biosynthesis of phosphatidylinositol is mediated by phosphatidylinositol synthase, which catalyzes the bond formation between CDP-diacylglycerol and inositol.

Apart from being a constituent of biomembranes, PI also facilitates the anchoring of various proteins to the membrane, while its metabolites also play a major role in signaling. The activity of phospholipase A_2 can stimulate the release of arachidonic acid, a main substrate in the biosynthesis of eicosanoids, such as prostaglandins, whereas phospholipase C activates the release of diacylglycerol. Diacylglycerol, in turn, regulates the entire enzyme family referred to as protein kinase C, a signaling pathway involved in a multitude of cell processes, including differentiation, proliferation, metabolism, and programmed cell death (DE CRAENE et al. 2017).

Phosphatidylserine

Phosphatidylserine (PS) is an essential bioactive anionic glycerophospholipid, containing two fatty acid residues, as well as serine attached to a phosphoric acid residue.

In animals, PS is biosynthesized through a calcium-dependent reaction, in which the polar head-group of a pre-existing phospholipid (PC or PE) is exchanged. A portion of the newly-synthesized phosphatidylserine, after transfer to the mitochondrion, can be decarboxylated, producing PE. Similar to other phospholipids, the fatty acid composition of PS can be modified through the Lands cycle (LANDS 1958, VANCE 2018). PS is usually present at low levels, though its concentration may be as high as 20% of the total phospholipids in plasma membranes and the brain (VANCE 2018).

As an active phospholipid, PS plays an important role in multiple signaling pathways. Since the PS molecule is charged, it greatly affects cell membrane structure, as well as the incorporation of certain proteins and ions (PS is mostly present on the cytoplasm leaflet of the bilayer) (VANCE 2018). These interactions and the incorporation of specific proteins or enzymes, often via calcium channels, are essential to specific activity and normal signaling. It is through these mechanisms that PS is directly involved in brain signaling, incorporating signal transduction proteins in neurons, and thus activating them (KIM et al. 2014).

PS is a key regulator of apoptosis. Increased concentration of Ca^{2+} activates scramblase, an enzyme that translocates PS to the outer leaflet of the lipid bilayer. Cell surface exposure of PS most likely enables immune system cells (e.g., macrophages) to bind onto a damaged cell through the proper receptors and remove it (BEVERS and WILLIAMSON 2016). PS also plays a major role in blood clotting by stimulating the activation of prothrombin to thrombin (LENTZ 2003).

Other phospholipids

Milk fat globule membranes also include other phospholipids and their derivatives, among them lysophosphatidylcholine, lactocerebroside, and glucocerebroside.

Lysophosphatidylcholine (LPC) can be produced from PC during digestion and absorbed. It can also be synthesized from PC in the body by phospholipase A_2. Lysophosphatidylcholine contains docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), and as such can deliver these acids to the brain using specific transport mechanisms (LAW et al. 2019). LPC may also exhibit antibacterial effects (RENSBURG et al. 1992).

Glucocerebroside (GluCer) and lactocerebroside (LacCer) are both sphingolipids. LacCer is produced when a galactose molecule (from the active form of UDP-galactose) is bonded onto the precursor (GluCer) by the β-galactosyltransferase enzyme. These phospholipids are important intermediates in the biosynthesis of more complex glycolipids, such as oli-

goglycosylcerebrosides or gangliosides, which incorporate additional carbohydrate residues into their structure (LINGWOOD 2011).

Glycolipids play an important role in the immune response and may induce the stabilization of lipid rafts. Glycolipids present on macrophage surfaces are involved in interactions between carbohydrate groups, facilitating the binding of bacteria and fungi and their subsequent elimination (LINGWOOD 2011). When present on the outer leaflet of the lipid bilayer, they form a structure known as a glycocalyx – a membrane coating covered with carbohydrate residues. This coating serves a protective function, but also plays a major role in intercellular communication, targeting of pathogens, and modulation of the inflammatory response (D`ANGELO et al. 2013).

Nutritional aspects of milk polar lipids

There is increasing number of scientific literature considering the health-promoting properties of the milk fat globule membrane components (TOKAS 2019). Many reports indicate a direct relationship between the consumption of membrane components, including polar lipids, and disease. Phospholipids and sphingolipids show a broad spectrum of activities as functional ingredients due to their regulatory properties, in addition to their structural functionalities. They are also effective at low concentrations (SCHMELZ 2000). The biological activity of phospholipids and their metabolites relates to such interactions as anti-carcinogenic, cognitive development, neurological diseases and aging (e.g. Parkinson, Alzheimer), reduction of cardiovascular risk and inflammation, liver recovery, neonatal gut development and gastroprotective role (DEWETTINCK et al. 2008, RODRIGUEZ-ALCALA et al. 2017, NICOLSON and ASH 2014).

DILLEHAY et al. (1994) treated mice with 1,2-dimethylhydrazine to induce colon cancer and showed that mice fed diets supplemented with milk sphingomyelin had a lower (20%) incidence of colon tumours compared with 47% in controls. These results showed that consumption of sphingomyelin affects the behaviour of colonic cells. In another study ZHANG et al. (2008) showed that dietary sphingomyelin inhibited the tumorigenesis and increased alkaline sphingomyelinase (a key enzyme responsible for sphingomyelin digestion in the gut) activity in the colon by 65%. SCHMELZ et al. (1996) reported that the administration of isolated dairy sphingomyelin in diet of CF1 mice, transformed malignant adenocarcinoma to benign adenoma.

CASTRO-GOMEZ et al (2016) investigated the in vitro effect of a concentrate of phospho- and sphingolipids obtained from buttermilk. The research

work showed antiproliferative effect on ovary cancer lines, which could be explained by the presence of sphingomyelin in the extract.

In the human body especially high concentration of phospholipids occurs in the brain, where together with long-chained polyunsaturated omega-3 fatty acids constitute the basic building blocks of the nervous system. So many research work relates to phospholipid role in the development of age-related diseases and Alzheimer's disease (SPITSBERG 2005). There are multiple studies indicating that phosphatidylserine supplementation may positively affect brain function (memory), improve health, slow ageing, and produce beneficial effects in Alzheimer's patients (DEWETTINCK et al. 2008, HASHIOKA et al. 2004). Short-term supplementation with PS was also shown to improve exercise capacity during high-intensity cycling, which might suggest an innovative application as a supplement (KINGSLEY 2006).

Phosphatidylcholine is essential for normal functioning of mitochondria, as well as protecting them against reactive oxygen species produced during ATP generation (HAILEY et al. 2010). In rodent studies, it has been proven that the introduction to the diet of milk-derived phospholipids has positive acting in the treatment of steatosis and liver enlargement (WAT et al. 2009). In addition, PC supports the regeneration of alcohol-damaged liver (KHARBANDA et al. 2006), and protects gastrointestinal cells from the harmful effects of toxins (ANAND et al. 1999).

Many studies have shown that consuming sphingomyelin, in addition to its anti-cancer effect, can have beneficial effects, including the regulation of cholesterol levels and protection against bacterial infections and mycotoxins (VESPER et al. 1999).

Dietary lipid supplementation is recommended to improve health by modifying and restoring the composition of cellular and intracellular (mainly mitochondrial) membranes (NICOLSON and ASH 2014). Defects in cell and intracellular membranes are common to all chronic conditions, including cancer and normal processes such as aging. Oxidative stress occurs when the production of reactive oxygen species (superoxide, hydroxyl radicals or hydrogen peroxide) is in excess to the cell's ability to destroy these molecules with its natural antioxidants (HALLIWELL 2006). Cellular targets of this reactive species can be nucleic acids, proteins and also lipids, and mitochondrial structures are especially sensitive to oxidative damage (WEI and LEE 2002). So phospholipid supplementation can have a number of positive effects and has been successfully used in many chronic diseases, without any toxic effects for human (NICOLSON and ASH 2014).

Milk and milk products can be a valuable source of bioactive membrane lipids (Table 2). Raw milk has a polar lipid content of 9.4–40 mg/100 g of milk (ROMBAUT and DEWETTINCK 2006). They are mainly associated with the milk fat globule membrane. Their content in various dairy products is associated with the fat content of the product, so the high-fat products are rich in phospholipids. The most polar lipids are concentrated in the butterserum, but the technological usefulness of this product for obtaining polar lipids is limited. Butter can be considered the best source of phospholipids, due to its high fat content. However, during churning, the membrane of milk fat globule is broken and largely migrates to the aqueous phase, partly due to their high affinity for proteins (YEAGLE 2014, AMBROZIAK and CICHOSZ 2013). So buttermilk is a low-fat product with high concentration of phospholipids. Considering the content of phospholipids in relation to the dry mass, buttermilk contains 4–5 times more than other products (ROMBAUT et al. 2005, AMBROZIAK and CICHOSZ 2013). Taking this into account, buttermilk can be considered as an interesting source for further purification and concentration of phospholipids to obtain a product with high functional and nutritional value.

Table 2

Polar lipid content of some dairy products (PIMENTEL et al. 2016, ROMBAUT and DEWETTINCK 2006)

Product	mg/100 g of product	g/100 g of fat
Raw milk	9–40	0.7–0.9
Cream	139–190	0.3–0.9
Butter	70–230	0.1–0.3
Buttermilk	9–160	4.5–33.1
Butterserum	660–1250	14.8–48.4
Yoghurt skimmed	18	5.5
Kefir semiskimmed	34	2.3
Ricotta	279	2.7
Cottage cheese	56–376	1.3–5.3
Cheddar	154	0.5
Emmental	110	0.4

Conclusion

The present paper describes the main groups of polar lipids found in milk. Polar lipids are not only constituents of milk fat globule membranes, but also major components of biomembranes. They serve as a barrier that isolates cell's interior from the environment – any change or damage to the structure of these lipids, caused by external factors, is the primary means of signaling a threat to the entire cell. From these basic functions, cells have evolved a variety of mechanisms of intercellular and intracellular

signaling, with specific functions activated based on the level of polar lipids and their metabolites.

There has been an increasing number of reports documenting the health benefits of milk lipid consumption. Although they are present in milk in small quantities, their unique properties may help prevent and alleviate numerous diseases. Due to their amphiphilic properties, they play a major role in lipid metabolism and may prove useful in the prophylaxis and treatment of various diseases of affluence.

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EFFECT OF METHIONINE ON THE LUNGS RESPIRATORY PART IN RATS OF DIFFERENT AGES*

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Key words: methionine, lungs, age, rat.

Abstract

The aim of this work was to study the lungs respiratory part in rats of different ages after methionine administration. The experiments were conducted on 48 male 3 and 15 months old Wistar rats. Animals received 250 mg/kg of methionine daily for 21 days. Lungs tissue samples were taken at the end of the experiment. We used histological, morphometric, biochemical and statistical research methods. It was found that in the lungs of 3-month experimental rats, the area of the alveolus (in 19%) and the relative area of air spaces (in 11%) decreased, and the relative area of the parenchyma and stroma – increased (in 16%) at the end of the experiment. Elevated levels of hydroxyproline in the lung tissue of these rats may indicate an increase in the number of connective tissue elements. In 15-month-old animals treated with methionine the morpho- and biochemical signs of improvement in the functional capabilities of the lungs were observed. Thus, the administration of methionine with young rats reduces the activity of the lungs, and the adult animals, on the contrary, increases.

Introduction

Amino acids are structural units of proteins, but also have their own biological roles as active substances, that are not fully discovered yet. The irreplaceable sulfur-containing amino acid methionine, which occupies a central place in amino acid metabolism, is of particular interest in this regard (BROSNAN et al. 2007).

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Methionine has a wide range of effects on metabolic processes in the body. It is essential for cell proliferation, in particular for protein and RNA synthesis (GELTINK and PEARCE 2019). The effect of methionine on the cardiovascular, digestive, endocrine and other systems is well studied (AISSA et al. 2017, LATIMER et al. 2018). But, at the same time, the effect of methionine on the morphofunctional state of the lungs remains poorly understood.

Most of the existing literature data are devoted to clinical and experimental studies of the methionine effect on the lung condition in a particular pathology and the effectiveness of its use for the correction of existing disorders (GNANADHAS et al. 2015, YOON et al. 2016). It was shown that the levels of collagen and elastin transcription, as well as the content of phosphatidylcholine, which is the main component of pulmonary surfactant, decreased in mice on a methionine restriction diet. A lack of methionine has been shown to alter significantly the lung response to cigarette smoke, increasing the risk of inflammation (JUBINVILLE et al. 2020). Model studies of chronic asthma have shown that S-adenosylmethionine (a methionine derivative) protects mouse lung tissue from damage and fibrosis by reducing oxidative stress (YOON et al. 2016). It was found that methionine reduces the risk of developing lung cancer (TAKATA et al. 2012), increases the effectiveness of antibiotic therapy for chronic infections of the respiratory system (GNANADHAS et al. 2015). At the same time, a number of studies have shown that prolonged exposure to high doses of methionine can lead to serious damage to elastic fibers and lung structure in general (STARCHER and HILL 2005).

Of particular interest is the question of effectiveness of the use of methionine to prevent the development of pathology or in healthy individuals, as a means of preadaptation and increasing the body's resistance to effects of various harmful environmental factors (YANKO et al. 2020). Until now, the question how the pronounced effect of using methionine to increase the functional activity of healthy lungs, remains open.

The results of the use of methionine in experimental practice are significantly influenced, on the one hand, by the dosage and duration of methionine administration, and, on the other, by the age and sex of the experimental animals. It is known that the lungs, like other organs, react differently to the same influences in the process of ontogenesis. This fully applies to age-related differences in the response of the lungs to the administration of methionine.

The aim of this work was to study the methionine effect on the lungs respiratory part of healthy rats of different ages.

Materials and Methods

Research object and experiment design

The study was conducted on 48 male Wistar rats at the age of 3 and 15 months. The rats were divided into 4 groups (12 animals each): I and III – control 3 and 15 month old animals, II and IV – experimental 3 and 15 month old rats, respectively. Control rats received 240–250 mg/kg methionine, which was included to the standard diet. The experimental rats received an additional oral 250 mg/kg of body weight dose of methionine (Sigma-Aldrich, Germany). Thus, the total amount of methionine that the experimental animals received was ≈500 mg/kg of body weight. Such a dose of methionine can be considered as a prophylactic one, since it does not lead to a significant increase in its content in the body and the occurrence of homocystenemia, but is sufficient to correct a possible deficiency of an amino acid in the body to the values of the physiological norm (ZHANG et al. 2004). The animals received methionine together with the cheese mass, with visual control of the complete consumption of the portion. The control rats received a similar portion of the cheese mass without methionine. Animals of all groups were kept in standardized conditions, on a standard diet. The total duration of the experiment was 21 days.

The rats were removed from the experiment by decapitation under ether narcosis. At the end of the experiment, the body and lung weights of the rats were measured and the lung index was determined. All research protocols corresponded to the provisions of the Council of Europe Convention on Bioethics (1997), the Helsinki Declaration of the World Medical Association (1996), the European Convention for the Protection of Vertebrates, which are used for experimental and other scientific purposes (Strasbourg, 1985), the general ethical principles of animal experiments, adopted by the First National Congress of Ukraine on Bioethics (2001), as well as a committee with biomedical ethics of the A. A. Bogomoletz Institute of Physiology, National Academy of Sciences of Ukraine.

Histological studies

Histological preparations of lungs tissue were prepared according to a standard procedure: fixed in Buen's liquid, dehydrated in spirits of increasing concentration and dioxane and poured into paraffin. The obtained preparations were used for morphological and morphometric studies. The sections were stained with Bemer's hematoxylin and eosin, and for the detection of connective tissue elements – by the Van Gyzon and Mason method (KIERNAN 2015). Microscopic preparations were photo-

graphed on a microscope "Nikon Eclipse E100" (Japan) using a digital camera. The morphometry of the preparations digital images was performed using the computer program "Image J".

The mean diameter of the alveolar lumen, the depth and area of the alveolus, the alveolus entrance width, the thickness of the interalveolar septum, the diameter of the respiratory bronchioles, the alveolar courses and sacs were measured on the lungs tissue histological sections. The relative area of parenchymal tissue, stroma, air spaces were measured and their ratio was determined. Morphometric measurements were performed on sections where the alveolar passages and alveolus are clearly visible (WEIBEL 1964, YANKO et al. 2021).

Biochemical studies

For biochemical studies, a sample of lung tissue was washed with saline from blood residues and dried to constant weight. The concentration of total hydroxyproline in the lung tissue was determined photometrically by oxidation of hydroxyproline with chloramine T (KLIMENT et al. 2011).

Data Analysis

Statistical processing was carried out using variation statistics methods using the computer program Statistica 6.0. The normal distribution of digital arrays was verified using the Pearson criterion. When the distribution was normal, the Student's *t*-test was used to estimate the difference in the reliability of the difference between the control and experimental groups. Differences were considered significant at $p < 0.05$.

Results and Discussion

Lung mass and lung index in 3-month-old rats, after methionine administration, were at the level of control values. Whereas in 15-month-old animals these indicators, compared with the control, were lower by 15% and 13% respectively. From this it follows that the lungs of adult rats are more sensitive to the effects of methionine.

The lungs respiratory part (LRP) was represented by respiratory bronchioles (RB), alveolar courses (AC), alveolar sacs (AS) and alveolus. It is difficult to identify structural differences between AC and AS in histological sections, as well as the differences between peripheral RB and AC, they is why they are generally considered to be the one group (Figure 1).

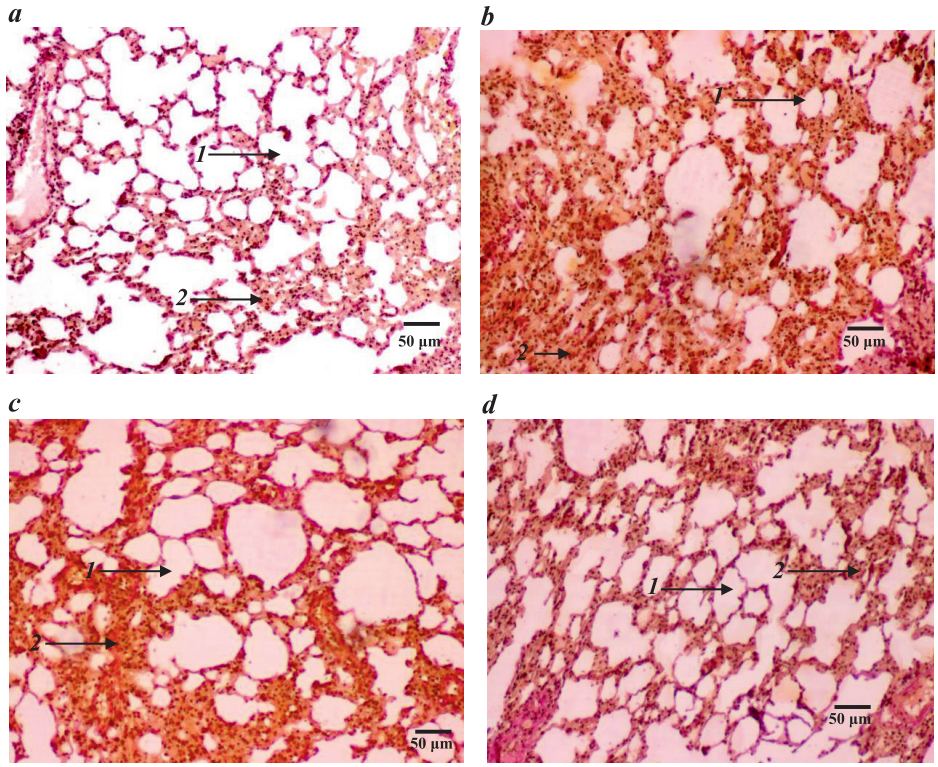

Fig. 1. Micrograph of the lungs respiratory part in control (*a* – 3 months old; *c* – 15 months old) and experimental rats (*b* – 3 months old; *d* – 15 months old). Van Gieson staining, > 200

Note. Micrographs show a decrease in the size of the alveolus and the relative area of air spaces (*1*), as well as in increase in the relative area of the parenchyma and stroma (*2*) in 3-month-old rats, after the administration of methionine (*b*). In 15-month-old experimental animals, on the contrary, the size of the alveolus, the relative area of air spaces (*1*) increased, and the relative area of connective tissue and parenchyma in the lungs (*2*) decreased (*d*).

Morphological differences in LRP structure of control rats of different ages were revealed. Thus, in the lungs of 15-month-old rats, compared to 3-month-old rats, the area of alveolus was smaller by 20%, their depth – by 13%. The thickness of the interalveolar septum was larger by 26%. The relative area of the parenchyma and stroma was 17% larger and airspace was 13% smaller. The concentration of hydroxyproline in the lungs tissue of adult rats was 88% higher than in young animals (Table 1). This nature of differences in the main morphometric parameters of LRP of young and adult rats corresponds to the general pattern of decrease in the functional activity of the lungs with age.

Morphometric parameters of LRP, after methionine administration, depended on the age of the animals and changed in different ways. Thus, in 3-month-old experimental rats, the average area of the alveolus was

smaller than in control animals of the same age by 19%, their depth – by 13% and the width of the entrance to the alveolus – by 14%. In 3-month-old rats, a significant increase in the relative area of the parenchyma and stroma (by 16%), a decrease in the relative area of air spaces (by 11%) and the ratio of the area of air spaces to the area of the parenchyma and stroma (by 23%) were observed comparing to the control. This indicated a decrease in the air content of the alveolus and deterioration in the conditions for the processes of intrapulmonary gas exchange (Table 1).

Table 1

Morphometric indicators of the lungs respiratory part (n = 12, M ± m)

Indicators	3 month old rats		15 month old rats	
	control	experience	control	experience
Mean diameter of alveolus lumen, μm	24.0±0.5	21.9±0.3	22.4±0.5	23.3±0.8
Cross-sectional area of alveolus [μm^2]	746±22	600±23*	600±15**	672±21*
Depth of alveolus [μm]	22.6±0.6	19.6±0.7*	19.7±0.9**	22.7±1.0*
Width of the entrance to the alveolus [μm]	13.5±0.4	11.6±0.4*	12.3±0.4	13.9±0.6*
Diameter of lumen of respiratory bronchioles, alveolar courses and alveolar sacs [μm]	63.1±2.9	58.1±1.5	67.8±2.1	68.8±2.7
Thickness of interalveolar septum [μm]	3.4±0.1	3.2±0.2	4.3±0.1**	3.2±0.1*
Relative area of parenchyma and stroma [%]	41.8±1.7	48.4±1.1*	49.1±1.7**	45.5±1.7
Relative area of air spaces [%]	58.2±1.3	51.6±1.1*	50.9±1.9**	54.5±3.6
The ratio of the area of air spaces / the area of parenchyma and stroma	1.39±0.12	1.07±0.08*	1.04±0.08**	1.20±0.07*

*- $p < 0.05$ – significance of differences in comparison with control
** - $p < 0.05$ – significance of differences in comparison with the control of 3-month-old rats

The changes in the LRP of 15-month-old rats treated with methionine were opposite. They were expressed as a significant increase in the area of the alveolus (by 12%), their depth (by 15%), the width of the entrance to the alveolus (by 13%); tendencies towards decrease in the relative area of the parenchyma and stroma, as well as increase in the relative area of air spaces in comparison to the control. This led to significant increase in the ratio of the area of air spaces to the area of the parenchyma and stroma of the lungs (by 15%). It should be noted that although the total area of the alveolar surface increased after the administration of methionine, it never went beyond the normal range and did not reach the size characteristic of emphysematous state. The thickness of the interalveolar septum in 15-month-old rats, after the administration of methionine, significantly decreased by 26% compared to the control animals (Table 1).

As it is known, the interalveolar septum consists of the epithelial layers of the alveolus, subepithelial basement membranes, a network of blood capillaries, as well as elastic, reticular and collagen fibers – its most pronounced structural component (KNUDSEN and OCHS 2018). It is obvious that a decrease in the thickness of the interalveolar septum, first of all, can be associated with a decrease in the content of connective tissue elements in it – elastic, reticular and collagen fibers. Such changes in the air-blood barrier lead to improved alveolar-capillary gas exchange. A similar nature of changes in the morphometric parameters of LRP in adult experimental rats, which were noted in our studies, may indicate improvement in the conditions of intrapulmonary gas exchange and functional activity of the lungs in general.

Staining by the Van Gieson method (KIERNAN 2015) revealed an increase in the number of collagen fiber bundles in the LRP of 3-month-old rats and decrease in adult animals. The majority of connective tissue elements was located around the RB, blood vessels, to a lesser extent, in the interalveolar septum (Figure 1).

Determination of concentration of hydroxyproline, an amino acid marker of collagen fibrillar protein, is often used to assess the condition of the connective tissue of various organs (LI and WU 2018). We revealed a significant increase in the concentration of total hydroxyproline (by 39%) in the lung tissue of young rats treated with methionine. Concentration of hydroxyproline in the lungs of 15-month-old rats, on the contrary, was by 35% lower ($p < 0.05$) than in control animals, that may indicate violation of the dynamic balance between the processes of collagen catabolism and biosynthesis (Figure 2). The multidirectional nature of changes in the

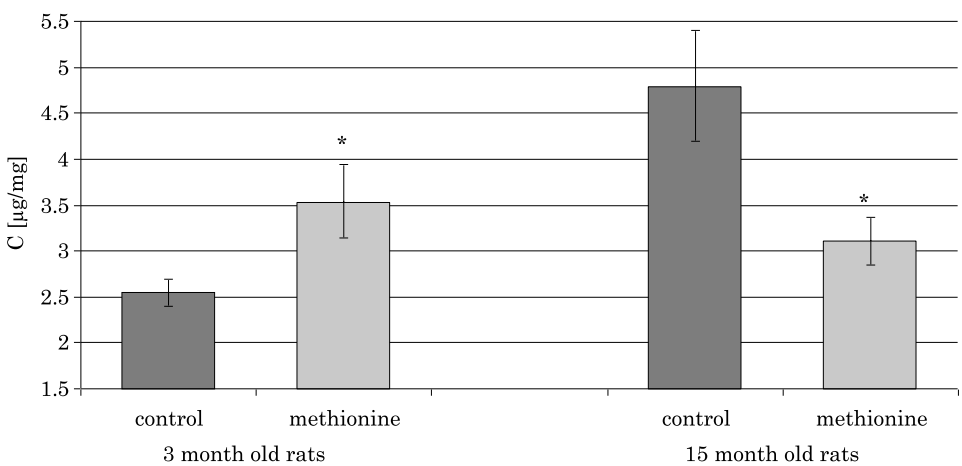

Fig. 2. Concentration of hydroxyproline in the lungs tissue in control and experimental rats
* $p < 0.05$ – reliability compared to control

concentration of total hydroxyproline in the lung tissue indicates the predominance of collagen catabolism processes and decrease in relative mass of connective tissue in the lungs of adult rats and, on the contrary, increase in its mass in young animals. These data may be important to explain the pathogenesis of disorders of the LRP.

The reasons of the identified age differences in the effect of methionine on the LRP state require further study and clarification. As you know, with age, a body's response to drugs can change. In old age, the effect of use of drug can both increase and decrease in comparison with young age. It is also known that age-related decrease in metabolic rate leads to slowdown in drug inactivation. The drugs can be contained in plasma in high concentrations and for a longer time. It is also necessary to take into account the fact that the ability to activate methionine-dependent enzymes in an aging organism may also decrease, and the number of receptors may decrease as well. Therefore, the amount of methionine supplied with a standard diet may not be sufficient. At a young age, with a balanced diet, methionine is quite enough, and its excessive administration leads to its accumulation in the body, and as a consequence to adverse consequences in the functioning of the lungs. Obviously, all this requires an age-dependent correction of the standard doses of the drug.

To date, there is no information about the features of the effect of methionine on the histomorphological and biochemical parameters of the lung state in animals of different ages. Therefore, further detailed study of the functions and mechanisms of the methionine effect on morphological changes in the lungs is urgent.

Conclusions

Thus, the administration of an additional amount of methionine (at a dose of 250 mg/kg) to standard diet of rats had different effects on the LRP of rats of different ages. The nature and severity of changes in the main histo- and biochemical parameters of LRP in 3-month-old rats indicated decrease in its activity. This was evidenced by decrease in the size of the alveolus and the relative area of air spaces, as well as in increase in the relative area of the parenchyma and stroma. Increase in the concentration of hydroxyproline in lung tissue of these rats may indicate increase in the number of connective tissue elements, which may reduce the efficiency of alveolar-capillary gas exchange. In 15-month-old animals treated with methionine, on the contrary, morpho- and biochemical signs of improvement in functional capabilities of the lungs were observed: the

size of the alveolus increased, their depth, the width of the entrance to the alveolus, the relative area of air spaces increased, the thickness of the interalveolar septum and the concentration of hydroxyproline in the lungs decreased. Such differences in the LRP response of young and old rats to the administration of methionine should be taken into account when prescribing methionine-containing drugs to people of different ages with impaired lung function.

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EXPRESSION OF AQUAPORIN 1, 4, 8 AND 9 IN THE LIVER OF RATS FED WITH THE STANDARD DIET AND SUPPLEMENTED WITH DRIED SEA-BUCKTHORN LEAVES (*HIPPOPHAE RHAMNOIDES* L.). A PILOT STUDY

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Key words: aquaporins, membrane transport, hepatocyte, bile, rats.

Abstract

The purpose of this study was to investigate the effect of a diet supplemented with *Hippophae rhamnoides* L. on the expression of AQP1, AQP4, AQP8 and AQP9 in the liver. The study was carried out on 8 male Wistar rats. Both control (n = 3) and experimental (n = 5) groups were fed ab libitum with a standard diet. The diet of the experimental animals was supplemented with dried sea buckthorn leaves. The rats were sacrificed after 14 days, which included a 7-day introductory period for acclimatization and habituation to diets, and 7 days of the actual experimental period. Western blot technique was used to analyze aquaporin expression in liver homogenates. It was found that the expression of AQP8 and AQP9 was higher than that of AQP1 and AQP4 in all animals tested. The present study found no statistically significant differences in the expression of individual AQPs between the control and experimental groups.

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Introduction

The discovery of water channels – aquaporins (AQPs), which was awarded the Nobel Prize in 2003, initiated a period of intensive research on the role of these proteins in the processes of membrane water transport and other small particles. Presently, it is known that these 'unusual' small proteins, with a molecular weight of about 30 kDa, located in practically all types of cells building the entire body, are involved in many key processes. AQPs play an important role among others in renal water reabsorption and urine concentration, in the regulation of water flow in brain, spinal cord and interstitial fluid surrounding the neurons, in airway hydration, salivary and gastric acid secretion, skin hydration and sweat secretion, cell differentiation and proliferation, milk synthesis during lactation and production of male semen (DAY et al. 2014, AGBANI and ALASTAIR 2021, AZAD et al. 2021, MICHAŁEK et al. 2021). To date, 13 of aquaporins (AQP0-AQP12) have been identified in mammals, 8 of which are located in the liver (AQP0, AQP1, AQP3, AQP4, AQP5, AQP8, AQP9 and AQP11) (MASYUK and LARUSSO 2006, GREGOIRE et al. 2015, CALAMITA et al. 2018, CHENG et al. 2021) In the light of recently published new data on aquaporins, these proteins are of particular interest in the context of bile production and secretion. It is well known that water makes up more than 98% of bile (GREGORIE et al. 2015). Although the transport and synthesis of many of its components is widely described in the literature, the molecular mechanism of water flow within hepatocytes and cholangiocytes is still not fully understood. Among all aquaporins localized in the liver, the best described in the literature are AQP1, AQP4, AQP8 and AQP9. AQP8 and AQP9 are located in hepatocytes, which make up about 80% of all cells that build the liver (TANI et al. 2001, HUEBERT et al. 2002). While AQP1 and AQP4 are present in the cholangiocytes, the epithelial cells of the bile duct (MARINELLI et al. 1997, MARINELLI et al. 2000, TIETZ et al. 2003). The available data show that these aquaporins play a key role in bile secretion, and changes in their expression may affect its canalicular and ductal formation (MARINELLI et al. 1997, HUEBERT et al. 2002, MARINELLI et al. 2004).

The development of civilization and the accompanying increased consumption of processed food, containing numerous preservatives, an increase in the consumption of drugs, as well as environmental pollution negatively affect the liver function. Therefore, people are looking for new, natural and safe factors, the use of which will neutralize the negative impact and will have a protective effect on this organ. The sea buckthorn (*Hippophae rhamnoides* L.), which has long been known in folk medicine as a medicinal plant, is of particular interest in this regard. The multidi-

rectional and wide health-promoting effect of oil, fruit, juices and dried *Hippophae rhamnoides* L. includes anti-inflammatory, anti-cancer, antioxidant and immunomodulating properties (HOU et al. 2017, SHI et al. 2018, GÂLTAN and GUTT 2021). The studies published to date show that the use of sea buckthorn in the diet also affects the liver and protects it against the histopathological changes (GAO et al. 2003, GEETHA et al. 2003, MAHESHWARI et al. 2011, SOLCAN et al. 2013, CZAPLICKI et al. 2017, ZHANG et al. 2018, RAN et al. 2021). It has been shown, inter alia, that liver histology after dosed of sea buckthorn oil showed a reduction in necrosis and fat formation. In addition, taking of oil from sea buckthorn berries decrease the toxic effects of aflatoxin B1, which leads to a reduction in total serum proteins and specifically reduced albumin (SOLCAN et al. 2013). It was also found that the dietary additive of compounds from sea buckthorn berries significant effects in inhibiting the activation of liver stellate cells and reduced the level of inflammatory factors (ZHANG et al. 2018). Particularly noteworthy are results of the study by GAO et al. (2003) on the effect of sea buckthorn in patients with cirrhosis of the liver. It has been shown that after the treatment with sea buckthorn, the levels of laminin, hyaluronic acid, type III and IV collagen, total bile acid in serum decreased significantly compared to before and after treatment in the control group. Sea buckthorn significantly shortened also the time of normalization of transaminases. These findings allowed the authors to suggest that sea buckthorn may be a promising drug for the prevention and treatment of liver cirrhosis. Despite many interesting data, the available literature lacks information regarding the effect of dietary application of *Hippophae rhamnoides* L. on aquaporins located in the liver. Among the many health-promoting properties of this plant, can a positive effect on the expression of AQP1, AQP4, AQP8 and AQP9, and thus on the production and secretion of bile be also observed? Preliminary studies were conducted in response to this question and wide possibilities associated with the use of sea buckthorn in order to identify and analyze the aforementioned aquaporins in the liver of rats fed a standard diet enriched with dried sea buckthorn leaves (*Hippophae rhamnoides* L.).

Materials and methods

Animals and experimental design

All experiments were performed in accordance with the principles and procedures of local Commission of Ethics for Care and use of Laboratory Animals (No. 43/2014). The study was carried out on 8 male Wistar rats.

During the experiment the animals, were remained unified and controlled environmental condition. The rats were kept individually in metabolic cages with 12 hours of light and 12 hours of darkness per day. The ambient temperature was 25°C ± 2°C and relative humidity was ~70%. From the 65 – 70th day of life (animals had a body weight 150 ± 5g) rats were divided into two nutrition groups. Rats from the control group (n = 3) were fed ab libitum with the standard diet (Table 1).

Table 1

Ingredient composition [g/kg] and analyzed nutrient content [%] of test diets fed in experiment (as fed basis)

Item	Control	Experimental
Corn starch	671.40	620.73
Sea buckthorn leaves	–	85.08
Soybean meal	303.17	271.07
Mineral mixture[1]	15.43	13.12
Vitamin mixture[2]	10	10
Analyzed nutrient content [%]		
Dry matter	90.11	89.99
Crude protein	15.86	15.40
Ash	3.73	3.58
Crude fat	0.54	0.82
Crude fibre	0.64	0.24
Total carbohydrates	79.23	79.96

[1] Provided per kg of diet: $CaHPO_4$, 735 g; K_2HPO_4, 81.8 g; K_2SO_4, 68 g; NaCl, 30.6 g; $CaCO_3$, 21.0 g; Na_2HPO_4, 21.4 g; MgO, 25 g; ferric citrate, 5.6 g, $ZnCO_3$, 8.1 g, $MnCO_3$, 4.2 g; $CuCO_3$, 0.33 g, KJ, 7.2 mg, citrid acid, 7.06 g.

[2] Provided per kg of diet: vitamin A, 20 000 IU; vitamin D, 2000 IU; vitamin E, 100 IU; vitamin K, 5 mg; choline, 2 g, para-aminobenzoic acid, 100 mg, inositol, 100 mg; niacin, 40 mg; calcium pantothenate, 40 mg; vitamin B_2, 8 mg; thiamine, 5 mg; vitamin B_6, 5 mg; folic acid, 2 mg; biotin, 0.4 mg; vitamin B_{12}, 0.03 mg

Experimental group (n = 5) were fed ab libitum with the standard diet supplemented with sea buckthorn leaves. After 14 days, which included a 7-day introductory period for the acclimatization of the animals and habituation to diets, and 7 days of the actual period, the rats were sacrificed.

SDS PAGE and Western blot

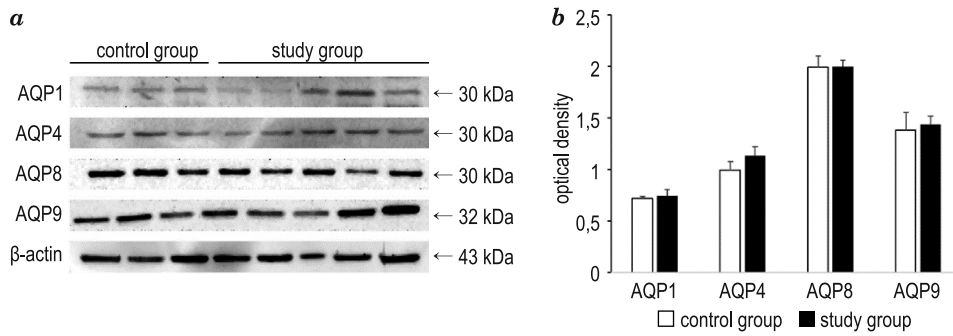
Immediately after slaughter, the livers were rapidly removed, washed with the 0.9% NaCl and cut into representative, small uniform pieces using dissecting tools. The liver samples were placed in lysis buffer (1% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.8, 2 mM PMSF, 1 mM EDTA) containing protease inhibitor cocktail at 1 : 200 (ab201111, Abcam).

Afterwards, the tissue samples were frozen in liquid nitrogen and were homogenized using a TissueLyser (QIAGEN). The homogenates were centrifuged at 20.800×g for 45 min at 4°C. The total protein in the obtained supernatants was determined by the modified Bradford method (Protein Assay Dye Reagent Concentrate, Bio-Rad). Subsequently liver homogenates were mixed with the Laemmli buffer (60 mM Tris HCl pH 6.8, 2% w/v SDS, 10% v/v glycerol, 4% betamercaptoethanol, 0.0005% bromophenol blue) in such proportions, so that after applying 10 µl of the sample to the wells, each of them contained 10 µg of total protein. The samples were warmed at 37°C for 15 min and loaded on 12% polyacrylamide gels and run for 120 min at 100 V. Subsequently, the proteins were then electrotransfered (12 V, 14 min) from the gels to PVDF membranes. The membranes were blocked with 5% non-fat-milk in PBS-T (80 mM Na_2HPO4, 20 mM NaH_2PO_2, 100 mM NaCl, and 0.1% Tween 20, pH 7.5) for 1 h and incubated overnight at 4°C with anti-AQPs primary antibodies diluted 1 : 500 – 1 : 2000 (depending on prior optimization experiments). In the current experiment, the following antibodies and dilutions were used: mouse monoclonal anti-AQP1 (Santa Cruz Biotechnology, sc-25287) 1 : 500, mouse monoclonal anti-AQP4 (Abcam, ab9512) 1 : 2000, rabbit polyclonal anti-AQP8 (Abcam, ab203682) 1 : 500, and anti-AQP9 (Santa Cruz Biotechnology, sc-74409) 1 : 500. The membranes were then incubated with a secondary rabbit anti-mouse or goats anti-rabbit horseradish peroxidase conjugated antibody (Santa Cruz Biotechnology, sc-516102; Dako, P 0448) diluted 1 : 100 or 1 : 200 respectively. The labeling was visualized by an enhanced chemiluminescence system (ECL Plus, Thermo Fisher Scientific) and exposure to a CCD camera (Versadoc 4000 MP, Bio-Rad). The obtained images were recorded in a digital form and modified (auto-scale was used, speckles were removed, and a representative band was cut out) using the Quantity One and PDQuest program (Bio-Rad). The expression of AQPs were normalized against β-actin (Santa Cruz Biotechnology, sc-47778) which was used as an internal control.

The results of the optical density were analyzed using STATISTICA 13 software (StatSoft, Kraków, Poland). The arithmetical means and standard deviations (X ± SD) were calculated. Due to the unequal number of variables between the groups and small number of variables in each group, nonparametric tests were used for further analysis. To assess the differences between the control and experimental groups, the nonparametric Mann-Whitney U-test were used. To evaluate the differences between the experimental groups, the nonparametric Kruskal–Wallis test with Dunn's multiple comparison test for post hoc analysis were used. The cut-off level for statistical significance was $p \leq 0.05$.

Results

The presence of four isoforms of aquaporins in rat livers was confirmed using Western blot technique. Figure 1 shows selective and representative results for each of them. In the present experiment all AQPs were detected as a single unglycolysated band with the molecular weight 30–32 kDa.



* Significance of differences ($p < 0.05$)

Fig. 1. Representative results of Western blot analysis of AQP1, AQP4, AQP8 and AQP9 in the rat's liver of control and experimental group

Weak expression of AQP1 and AQP4 were observed in all study animals. There were no significant changes in expression of these proteins between the control and experimental groups. Expression of AQP8 and AQP9 in rat's livers were higher compared to the remaining identified aquaporins. In comparison to AQP4 expression of AQP8 was statistically significant higher ($p < 0.05$). There were no statistically significant changes observed in the expression of AQP8 and AQP9 between the control and the experimental group. It is noteworthy that despite the lack of statistically significant differences observed between the studied groups, a detailed analysis of all band optical density seemed to indicate that the expression of AQP4 and AQP9 was slightly higher in rats fed a diet enriched with dried sea buckthorn leaves.

Discussion

There is no information in the literature regarding the effect of a diet supplemented with dried *Hippophae rhamnoides* L. leaves on the expression of AQPs in the liver. The results presented in this study are the first data in this field. It should be emphasized, however, that these were preliminary studies carried out on a small number of animals and the exper-

imental period was relatively short. The lack of recent data on liver AQPs in the context of bile production further complicates the interpretation of the results. It is widely known that bile formation is initiated by hepatocytes and is modified by secretory and absorptive processes in cholangiocytes within of bile duct. Final modification of bile composition occurs in gallbladder (BOYER 2013)). Synthesized in hepatocytes osmotically active substance such as bile salts and glutathione, are secreted into to canaliculus via the bile salt transporters Bsep and via the glutathione organic anion transporters Mrp2 (LEE et al. 2000, LEHMANN et al. 2008). Hydrocarbonates are also transposed to the light of the canalicus via the Cl^-/ HCO_3^- exchanger AE2, which together with the bile salts and glutathione constitute the major driving force for water movement from the sinusoidal blood to the bile canaliculus across hepatocytes (LEHMANN et al. 2008). This targeted water flow is mainly determined by the AQPs located in hepatocytes, among which AQP8 and AQP9 seem to play a particularly important role (Figure 2) (Marinelli et al 2004). In rat hepatocytes under basal conditions AQP8 is located mainly intracellularly to a lesser extent to the canalicular membrane, while AQP9 at the basolateral membrane (GREGOIRE et al. 2015, ELKJAER et al. 2001, NICCHIA et al. 2001). The study demonstrated that in response to stimulation by choleretic agonist such as dibutyryl cyclic adenosine monophosphate (cAMP) or glucagon, AQP8 is redistributed to the canalicular plasma membrane, thus increasing the permeability apical surface cell for water (GARCIA et al. 2001, HUEBERT et al. 2002, GRADILONE et al. 2003, MARINELLI et al. 2003). This mechanism seems to be similar to that seen in the kidney collecting duct (CD). In the absence of vasopressin (AVP) stimulation, present in the principal cells of CD AQP2 is mainly located in intracellular vesicles. Under AVP stimulation, the intracellular level of cAMP increases, as a result, the transport and fusion of this protein with apical plasma membrane and increased inflow of water to the renal cells of CD take place (MICHAŁEK et al. 2014). While AQP8 modulates the canalicular water flow, AQP9 localized in basolateral of hepatocytes enables its uptake (ELKJAER et al. 2000, NICCHIA et al. 2001, HUEBERT et al. 2002, LEHMANN et al. 2008). It is believed, that during the bile formation AQP9 facilitates the water transport from the sinusoidal blood to the liver hepatocytes (LEHMANN et al. 2008, MARINELLI et al. 2003). In the present experiment it was observed relative stable expression of total AQP8 expression in both control and experimental group, while in animals fed with dried *Hippophae rhammoides* L. leaves expression of AQP9 was slightly higher. This slight increase in a total amount of AQP9 may suggest that dietary use of dried sea buckthorn leaves will increase water transport via AQP9 from the sinusoidal blood to

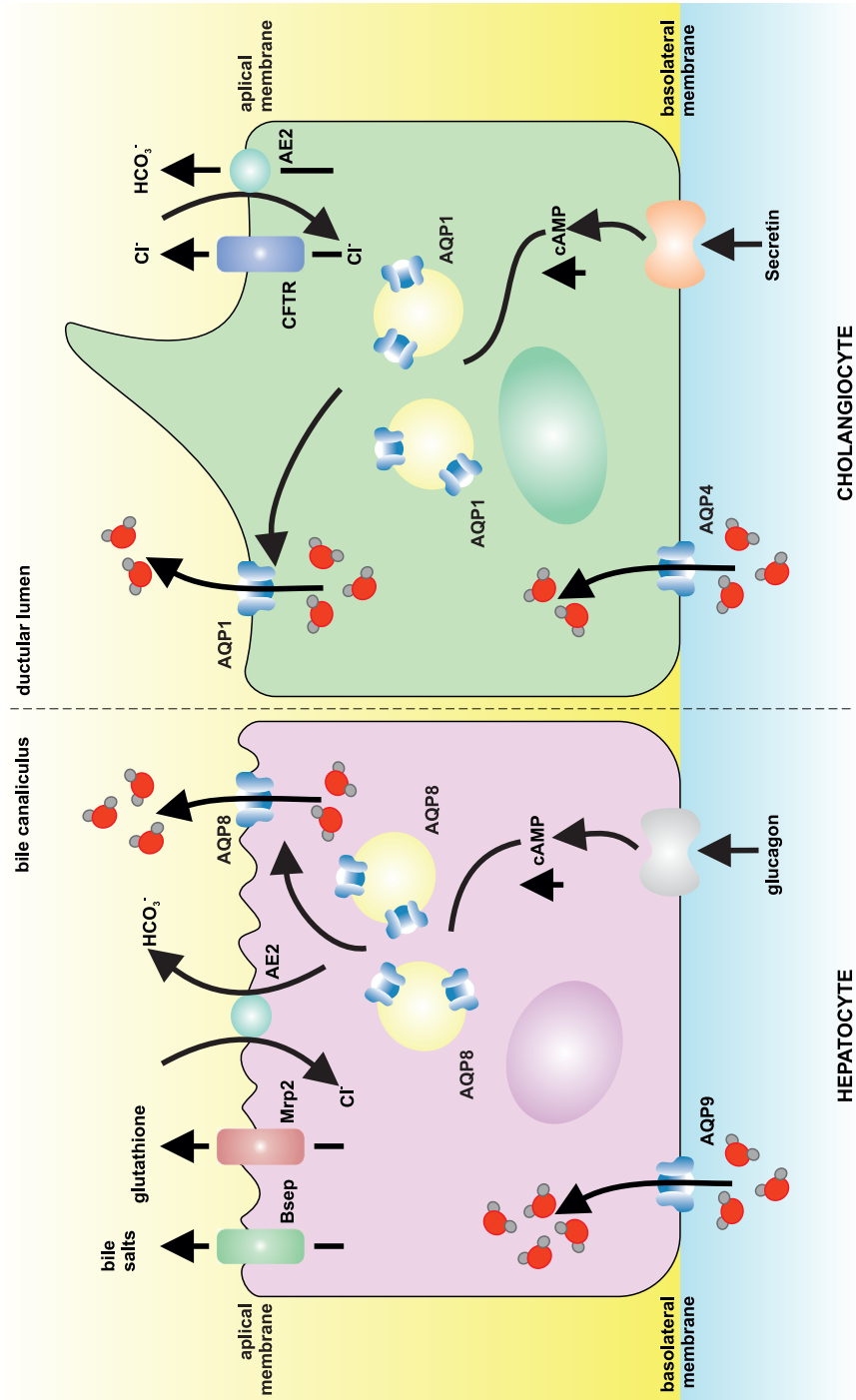


Fig. 2. Diagram showing the potential mechanism of water transport in hepatocytes and cholangiocytes

Source: own study based on MARINELLI et al. (2004)

the hepatocytes in rats from the experimental group. A consequence of the increase in water flow across the basolateral membrane should be its increased apical transport to the bile canaliculus. In the present study, no increase in the expression of AQP8 was found in animals fed a diet supplemented with dried sea buckthorn leaves, whatever the localization of this protein may have changed from intracellular compartments to the canalicular membrane.

AQP1 and AQP4 are mainly located in the cholangiocytes, that account only 3–5% the liver cell population (MARINELLI et al. 2004). Although cholangiocytes make up a small percentage of all cells that make up the hepatobiliary system, they play a significant role in bile formation, producing as much as 40% of total bile volume in some species (MASYUK and LARUSSO 2006). According to MARINENELLI and coworkers (2004) intensive processes taking place within bile duct related to bile formation, suggest that the amount of transcellular water movement across an individual cholangiocyte is potentially up to 10 times higher than across individual hepatocytes. Driving force for the water transport across cholangiocytes to the lumen of bile duct is osmotic gradients, created by secreted Cl^- via CFTR channels and HCO_3^- via AE2 transporter (MASYUK and LARUSSO 2006). Like AQP8 in hepatocytes, under basal conditions in cholangiocytes AQP1 is mainly located in intracellular vesicles (MARINELLI et al. 1997, MARINELLI et al. 1999). Under secretin stimulation and an increase intracellular cAMP synthesis, this protein undergoes translocation to the apical plasma membrane and thus promotes the water transport across the biliary epithelial cells (MARINELLI et al. 1997, MARINELLI et al. 1999). Unlike AQP1, whose subcellular distribution depends on physiological conditions, AQP4 is exclusively expressed in basolateral membrane of cholangiocytes (MARINELLI et al. 2000). During the bile formation, water enters the cholangiocytes via AQP4 located in the basolateral membrane and exits via AQP1 present in apical cell surface (Figure 2.) (MARINELLI et al. 2000). In the present experiment, the total expression of both AQP1 and AQP4 in liver homogenates were significantly lower than AQP8 and AQP9. The observed low expression of AQP1 and AQP4 is most likely associated with a significantly lower number of cholangiocytes compared to hepatocytes in liver. In the present preliminary study, only a slightly increase in the expression of total amount of AQP4 in the liver homogenates of animals fed with the diet supplemented with the dried sea buckthorn leaves was found, which may indicate a slight increased inflow of water to the cholangiocytes across the basolateral membrane. As in hepatocytes, increased transport of water inside the cell should be accompanied by an increase of water movement across the apical plasma membrane. It is possible that in

the tested animals, the slight increase of the total amount of AQP4 was accompanied by a change in AQP1 localization and an increase in its expression in apical plasma membrane of cholangiocytes. Despite the lack of significant changes in total amount of the studied AQPs, a slight increase in the expression of AQP4 and AQP9 suggests that perhaps long-term use of dried sea buckthorn leaves in the diet will have a positive effect on bile formation and secretion. Among the few data that can be used in the analysis of the presented results is information from research carried out by XING and coworkers (2012). According to these authors, one of the effects of using sea buckthorn pulp oils (SPBO) in the diet is the stimulation of the secretion of enterohormones, such as secretin. As mentioned earlier, this hormone accumulates translocation of the AQP1 from intracellular compartment to the apical plasma membrane and thus promotes the water transport across the biliary epithelial cells.

Conclusion

The results of the preliminary analysis of the dietary effect of the addition of dried sea buckthorn leaves on the expression of selected aquaporins in the livers of rats, obtained in the present study, seem to be quite promising and prompt to undertake further research in this direction. The observed slight increase in the total amount of AQP9 and AQP4 suggest that perhaps long-term dietary use of *Hippophae rhamnoides* L. may have a positive effect on liver function in terms of bile production and secretion. However, this issue requires further research, which will take into account a larger group of animals, the duration of the experiment and the use of the diet will be longer, and the WB analysis will be supplemented with studies enabling the detailed location of these proteins.

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