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Challenges of Ecology and open Questions

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INTERNATIONAL SEMINAR OF ECOLOGY – 2020
Challenges of Ecology and open Questions, Online SEMINAR

During last 13 years, the Seminar of Ecology has become well recognizable and expected scientific forum in the field of ecology. Annually, well known scientists from Bulgaria and abroad are invited to present plenary lectures and to make the auditorium familiar with the achievement and challenges of this branch of the science. Professors, scientists, PhD and Master Degree students take part to present and discuss their results. Very complicated epidemiological situation did not stop the Seminar of Ecology. Online "International Seminar of Ecology - 2020", organized by Section "Biology" – USB and the Institute of Biodiversity and Ecosystem Research (IBER-BAS) was held on April 23rd and 24th in Sofia. The scientific forum was dedicated to the 10th anniversary of IBER - BAS.

During the online sessions, a total of 44 presentations were reported as 4 plenary lectures, 18 oral presentations and 22 posters presentations in the six topics of the Seminar as follows:

- Biodiversity and conservation biology;
- Biotic and abiotic impact on the living nature and mechanisms of adaptation;
- Ecosystem research, service and ecological agriculture;
- Landscape ecology;
- Ecology and education;
- Other related topics.

Research teams from various universities, institutes, and organizations, ministries and departments, both from Bulgaria and abroad (Austria, Germany, Egypt, Romania, Russia, Turkey, Czech Republic and Switzerland) participated in the Seminar. Traditionally, six young scientists and students were nominated and awarded with a diploma and books for the "Best oral presentation" and "Best posters presentation".

The Organizing Committee of the "International Seminar on Ecology - 2020" would like to express its gratitude to the Pensoft Publishers Ltd., for providing free of charge on-line platform GoToMeeting, that has allowed to perform the Seminar. We also would like to thank the companies "BULGAP" Ltd. and "Pensoft Publishers" Ltd. for the financial support provided.

We make our thanks to the governing body of IBER-BAS for the assistance in conducting the Seminar and the books given to awarded young colleagues, to everyone who contributed, assisted and supported this edition of the Seminar, to USB for dissemination of this scientific event. The Organizing Committee was given many positive feedback from the participants - one of the biggest acknowledgments for well done work.

The Seminar was held with the financial support of the companies: "BULGAP" Ltd. and "Pensoft Publishers" Ltd.

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The Effect of Seasonality of Climate Conditions on the Structure and Functional Performance of Photosynthetic Apparatus of Medicinal Plant Petasites hybridus

Borislava V. Borisova[1*], Svetlana M. Momchilova[2], Dimitrina P. Koleva[3], Albena P. Ivanova[4], Albena B. Momchilova[1], Liliana T. Maslenkova[1]

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Abstract. The aim of this study was to relate annual variation in the structure and the functional performance of photosynthetic apparatus of a local population of medicinal plant *Petasites hybridus* (L.) to environmentally induced constraints in its natural habitat. The comparative analyses of chlorophyll thermoluminescence (TL) and photosynthetic oxygen evolution, used to assess functionality and recombination events of PSII reaction centres revealed specific changes in correlation to the climate conditions during the seasons. The analysis of lipid classes and fatty acids composition of monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyl diacylglycerol (SQDG) and phosphatidyldiacylglycerol (PG) show the existence of qualitative and quantitative differences that can contribute in this regard. The obtained results are discussed in terms of a possible relationship between seasonal variation of photosynthetic performance and modulation in the profile and accumulation of secondary metabolites with therapeutic activity in this medicinal plant.

Key words: *Petasites hybridus* L., chloroplasts, fatty acids, lipid composition, oxygen evolution, photosynthetic activity, thermoluminescence.

Introduction

The utilization of medicinal plants to produce natural compounds with important therapeutic properties has gained increasing attention over past decades due to growing tendency to replace synthetic drugs with natural ones (Jimenez-Garcia et al., 2013). The beneficial medicinal effects of plant materials typically result from the combinations of biologically active substances (BAS), products of secondary plant metabolism (Briskin, 2000). Much of the medical plants used in pharmacy and medicine are collected from their natural habitat. Fluctuations in environmental factors as well as various unfavorable situations known to influence

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general metabolism should also impact on secondary metabolism, i.e., on the biosynthesis and accumulation of natural products (Selmar & Kleinwaechter, 2013). Clarification of these relationships is essential to define their impact on the efficacy and therapeutic potential of phytochemical preparations of medicinal plants (Gobbo-Neto & Lopes, 2007). In order to answer these questions in the present study, comparative analyses of the structure and functional activity of the photosynthetic apparatus of *Petasites hybridus* are provided depending on the seasonal variations in the climatic factors of the environment.

The genus *Petasites* Mill. (Asteraceae) is widely vegetated in Europe, Northwest Asia and North America and has a long history of use in alternative medicine. Taxonomic survey of *Petasites* reveals the existence of 18 species of this genus. In Bulgaria, three *Petasites* taxa can be naturally found: *P. hybridus* (L.) P. Gaertn., B. Mey. & Scherb. (= *P. officinalis* Moench), *P. albus* (L.) Gaertn. and *P. kablikianus* Tausch ex Bercht. Representatives of genus *Petasites* are shown to be potential sources of high levels of bioactive substances with very promising aspects of therapeutic utility (Aydın et al., 2012). Main biologically active compounds are secondary metabolites classified as sesquiterpene esters of petasin and isopetasin. Purified preparations of *P. hybridus* roots, free of pyrrolizidine alkaloids are finding an increasingly widespread pharmacological application for the prophylaxis and treatment of migraine, bronchial asthma and allergic rhinitis - diseases affecting many people (Lipton et al., 2004). Extracts are also successfully used for the prevention of gastric ulcer, urinary tract irritation and respiratory problems (Ziolo & Samochowiec, 1998). Comparing the results of the structural-functional peculiarities of photosynthetic apparatus during the seasons with planned detailed phytochemical analyses of the composition, amount and antioxidant capacity of secondary metabolites will contribute to elucidating the physiological basis of variation in the metabolic profile and the accumulation of biologically active substances of this medicinal plant.

Materials and Methods

The study was conducted with plants growing at the region of Kokalyane, Devil's bridge (lat. 42°33′′37′ N, long. 23°25′′18′ E; altitude 650 m; Vitosha Mountains region, humid continental climate). The measurements were done with isolated thylakoids from leaves collected on three occasions during 2019 (April, August and November), chosen to represent different temperature and water supply during the seasons.

Averaged samples of leaves of 3-4 plants collected during the respective seasons were homogenised in 50 mM Na-tricine (pH 7.8), containing 3 mM Na-ascorbate, 10 mM NaCl, 5 mM $MgCl_2$, 0.4 M sucrose and 5% PEG-6000. The resulting slurries were passed through 8 layers of cheesecloth and the broken chloroplasts (thylakoids) were collected by centrifugation at 1000 × g for 10 min. The pellets were washed twice in 10 mM Na-tricine (pH 7.8) containing 0.4 M sucrose, 10 mM NaCl and 5 mM MgCl and then resuspended to concentration of 1 mg Chl/ml in 50 mM Na-Mes (pH 6.5) instead of Tricine buffer and stored on ice for 1 h in the dark before measurements. The pigment content was determined spectrophotometrically (Lichtenthaler, 1987).

Thermoluminescence (TL) measurements were carried out in darkness using computerized equipment, described in detail in (Zeinalov & Maslenkova, 1996). Samples of isolated thylakoid membranes were illuminated at 2-5°C to generate charge pairs within the PSII reaction centres and then rapidly cooled down in liquid nitrogen to trap those charge-separated states. Subsequent warming of the samples reveals thermoluminescence emission with characteristic peaks (Sane & Rutherford, 1986). Decomposition an alysis of TL glow curves was carried out using Origin Pro 8.

Oxygen-evolving reactions were measured using polarographic oxygen rate electrode (Joliot-type) and thylakoid membranes (100 µl sample volume, 300 µgChl/ml) without any artificial electron acceptors, as described in (Zeinalov, 2002). Oxygen flash yields were induced by saturating (4 J) and short ($t_{1/2}$ = 10 µs) periodic flash sequences with 650 ms dark spacing between the flashes. The initial oxygen burst was recorded after irradiation with continuous white light (450 µmol photons m^{-2} s^{-1}). The kinetic parameters of oxygen evolution (initial S_o and S_1 state distribution, misses and double hits) were determined by the least square deviations fitting of the experimentally obtained oxygen flash yields with the theoretically calculated yields, according to the model of Kok et al. (1970). Deconvolution of the oxygen burst decay was performed by fitting of the function with two exponential components: $A_1e(tk_1) + A_2e(tk_2)$, where A_1 and A_2, and k_1 and k_2 were the rate constants of the fast and slow components of the oxygen burst decay, respectively.

For TEM analysis small segments (1-2 mm^2) from the middle part of fully expanded leaves were taken and fixed in 3% (m/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 12 h at 4 °C The leaf segments were post fixed in 1% (m/v) $KMnO_4$ in the same buffer for 2 h at room temperature. After dehydration by increasing concentrations of ethyl alcohol (from 25 to 100%), the samples were embedded in Durcupan (Fluka, Buchs, Switzerland) and cross-sectioned with Reichert-Jung (Wien, Austria) ultramicrotome. Observation and documentation were performed by JEOL 1200 EX (Tokyo, Japan) electron microscope.

The lipids were extracted with chloroform/methanol/water as described by Bligh & Dyer (1959). The fresh aerial part of plant (25-30 g) was homogenized with 20 ml methanol and refluxed for 5 minutes in order to inactivate the lipases. An equal volume of chloroform was added and after 24h the mixture was filtered, and an equal amount of water was added. The lower layer (total lipophilic extract) was evaporated under vacuum and kept at –30°C. The amounts of lipophilic components were determined gravimetrically.

For lipid classes and fatty acids analyses part of the total lipophilic extract (50 mg) was applied on 20 × 20 cm silica gel G (Merck) plate (layer thickness 0.5 mm) and then the plate was developed with chloroform-methanol-acetone-acetic acid (70:14:24:0.4 v/v/v/v) as a mobile phase. The spots of the main lipid classes were visualized under UV-light, scrapped off with the silica gel layer and transferred in small vials with Teflon screw caps. Five ml of 15% acetyl chloride in absolute methanol were added and the vials were heated for 4 hours at 55°C (Christie, 1989). After cooling, the samples were diluted with water and the obtained fatty acids methyl esters (FAME) were extracted twice with hexane (2 × 5 ml). The FAME in combined hexane extracts were purified by preparative thin–layer chromatography (TLC) on 20 × 20 cm silica gel G (Merck) plates (layer thickness 0.5 mm) developed with hexane–acetone (95:5 v/v). The spots of the FAME were visualized under UV light, scrapped off with the silica-gel layer and eluted with diethyl ether. The amount of each sample was determined gravimetrically.

Fatty acid analysis was performed using Gas Chromatograph with Flame Ionization Detector Agilent 7890B, equipped with Agilent7693 Autosampler with 10 µl syringe and with capillary column SGE BPX70 (60 m x 0.25 mm x 0.25 µm). Nitrogen was the carrier gas at flow rate of 1.2 ml/min. The column temperature was programmed from 80°C (hold for 1 min) to 130°C by step of 8°C/min and then to 250°C by step of 5°C/min. The injector and detector temperatures were 245°C and 255°C, respectively; split 15:1. Instrument control, data acquisition and data processing were performed by GC software Clarity v.8.0.0.125. The fatty acids were identified by comparison of the retention times with that of reference mixture F.A.M.E. Mix C8-C24

(Sigma-Aldrich). The relative amounts of the fatty acids were determined from peak areas of the respective methyl esters.

The fluidity of the membrane lipids was expressed by the level of unsaturation, calculated as unsaturated/saturated ratio (16:1+18:1+18:2+18:3)/ (16:0+18:0).

Results and Discussion

The production of secondary metabolites by plants growing in natural populations is conditioned by environmental factors (Gobbo-Neto & Lopes, 2007). Constantly changing environmental conditions (temperature, water supply, light intensity, UV radiation, etc.) induce physiological adaptation by influencing the activity of primary metabolic reactions such as photosynthesis, which is a major physiological process that determines plant growth and productivity. Properties of photosynthetic apparatus may contribute to a great extent to plant habitat separation and adaptation to environmental factors, but currently there are no comprehensive studies on the effects of abiotic and phenological factors on growth, photosynthetic activity and accumulation of sesquiterpenes in *Petasites.* The most common species and the main medicinal plant in *Petasites* genus, used in European phototherapy, is *P. hybridus* (common butterbur). The plant generally inhabits humid areas preferably in partial shade, but if there is appropriate amount of water, it can grow in full sun. Plants are considered drought intolerant compared with many other plant species and they can encounter temporal increases in drought stress during summer due to increasing temperatures, decreasing precipitation, or both. *Petasites* blooms in the spring, and later develops leaves, thus early flowering may be considered as an advantageous defense strategy of the plant. The environmental conditions during the measurement periods were characterized by average spring (in April) and autumn (in November) minimum and maximum temperatures of 5 and 16.7°C, and 6.2 and 13.7°C, respectively, and there was non-limiting water supply (75.7 mm and 69.7 mm average monthly amount of precipitation). For the summer-grown plants (in August) the corresponding temperature values were 15.4 and 30.1°C and there was a progressive increase in drought from spring to summer (34 mm average monthly amount of precipitation). The analysis of plant water status of investigated samples determined by measuring the hydration of leaves (H[gH_2O(gDW) show lower values of summer (3.66) and autumn (4.5) in comparison to spring samples (5.75). Similar values were obtained in preliminary studies in two-year period (2015-2016, unpublished data). Drought stress (i.e. the combination of water deficit, high light and high temperatures) can reduce stomatal conductance, making the photosynthetic apparatus susceptible to photodamage. Perturbations by stressful environments are first manifested in alterations in structure of thylakoid membranes and the photochemical efficiency of photosystems, especially photosystem II (PSII). Moreover, PSII oxygen-evolving enzyme complex of thylakoid membranes appears main stress sensitive site in plants. The possibility of fast and reliable monitoring of the effectiveness of the operation of PSII centers is the first prerequisite to solve site and mechanisms of stress injury and adaptation to biotic and abiotic environmental factors. In this regard, the kinetic analysis of oxygen-evolving reactions in continuous and pulsed lighting and thermoluminescence (TL) emission as indicators of energy balance and functioning of PSII reaction centers, have important application in plant stress physiology.

Upon illumination by a single turn-over flash the isolated chloroplasts showed a TL emission curve which could be well fitted with only one component representing the main TL B-band ($S_{2(3)}Q_B^-$ charge recombination), related to dark distribution of the S_2 and S_3-states of oxygen evolving complex and Q_B/Q_B^- ratio. The analysis of the obtained data (Table 1) revealed distinct differences in TL parameters. In summer samples the relative contributions of B-band to the overall TL emission were significantly lower (29.6%) in comparison to spring samples (44.7%) and a shift of the maximal emission temperature of B band from 27.5°C to lover values (23.5) was also observed. During autumn, the relative contribution of B-band increases to 35.7% accompanied by T_{max}, centered at 25°C. It is reasonable to suggest that the TL data reflects seasonal dynamics in the relative number and stability of PSII reaction centers.

Another reliable approach used to study the properties of PSII complex in *Petasites* thylakoids in correlation to the climate conditions during the seasons was analysis of the kinetics of oxygen-evolving reactions under continuous and flash excitation (Maslenkova et al., 1993). The amplitudes of the oxygen burst A (%) under continuous irradiation, which is a measure of the oxygen volume evolved, differ in thylakoids from the investigated samples, reaching the lowest values in summer thylakoids (Table 1). The induction curves after oxygen burst exhibit biphasic exponential decay. The ratio between the amplitudes of the fast (A_1) and the slow (A_2) components after decomposition of oxygen induction curves and the respective time constants t_1 and t_2 (Table 1) suggest a decrease in the proportion of functionally active PSIIα centers in thylakoids from summer plants which could be attributed to the reduced grana formation and dominant operation of the cooperative mechanism of oxygen evolution in stroma situated PSIIβ centers. It is supposed that the cooperative mechanism is realized by diffusion of oxygen precursors mainly within PSIIβ centers and is characterized by a time constant lower than that of the non-cooperative Kok's mechanism, realized by PSIIα centers (Zeinalov & Maslenkova, 1996). Analysis of the flash-induced oxygen yield patterns reveals significant inhibition of the active PSII centers (S_0+$S_{1)}$ that evolve oxygen by non-cooperative mechanism (PSIIα centers) for summer samples without changes in miss parameter, suggesting that the resting PSII centers functioned nearly as in the spring plants, accepted as control. A gradual increase in the number of the active PSII centers (to 51.7%) during autumn is observed.

T(ºC) and B (%) are B band emission temperature, recorded after one turn-over flash and the contribution of B-band to overall TL emission; A is the amplitude of the initial oxygen burst; A_1 and A_2, and t_1 and t_2 represent amplitudes and time constants of fast and slow components of initial oxygen burst; S_0+S_1 and α and β are the sum of active oxygen evolving centers working through non-cooperative mechanism; α and β are the values of misses and double hits, according to the Kok's model.

The presented comparative analysis of the functional properties of the PSII complex during the seasons show an increased proportion of inactive (photoinhibited) PSII centers during summer as precipitation decreases and temperature rises, i.e. when drought increases and gradually recovered as temperatures fall and appropriate water supply are established during the autumn (Table 1). The number and the stability of PSII centers were maximal in spring growth conditions when water was abundant and temperature was moderately high. The observed lover values in PSII activity of autumn are most likely due to aging processes of chloroplasts (Fig. 1c).

To the best of our knowledge there are no microscopic investigations on chloroplast structure in *Petasites* leaves. The data obtained by TEM-analysis revealed that the structure of the chloroplasts in leaves collected in spring were characterized by an elliptical shape and well-developed inner membrane system (Fig. 1a). The grana represented different height. The number of thylakoids in them varies from 8-10 to 30, connected by well-developed and evenly spaced stromal thylakoids.

The inner membrane system of summer chloroplasts was characterized by an increase in the number of lower grana with a smaller number of thylakoids (8-15) and stromal thylakoids of greater length. A few small plastoglobulus in the stroma were noted. The chloroplasts of autumn collected leaves differed from the spring chloroplasts by the great amount of plastoglobulus and relatively small proportion of the internal membrane system, which were related to the natural aging processes.

Table 1. Seasonal changes in the kinetic parameters of thermoluminescence (TL) and oxygen evolution in isolated thylakoids.

Sample	T (°C)	B (%)	A (%)	A_1/A_2	t_1 [s]	t_2 [s]	S_0+S_1 (%)	α (%)	β (%)
Spring	27.5	44.7	100.0	2.09	0.32	2.78	100.0	15.6	4.9
Summer	23.5	29.6	28.6	0.56	0.57	4.1	20.3	16.1	4.5
Autumn	25.0	35.7	61.4	1.38	0.44	3.6	51.7	15.2	5.1

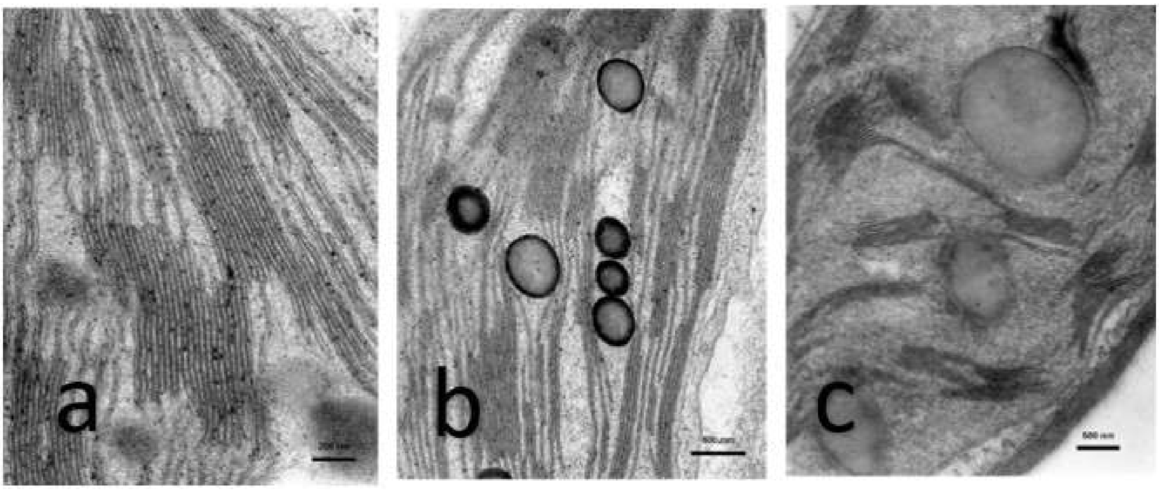

Fig. 1. Chloroplast ultrastructure in **a**: spring, **b**: summer, **c**: autumn.

It is well known that lipids play an essential role in maintaining the integrity and functional activity of chloroplast membrane. In this respect the elucidation the impact of varying environmental conditions during seasons and phenological factors on changes in lipid classes and fatty acid composition of *Petasites* membranes could bring important information in the interpretation of the obtained results. The lipid classes of thylakoid membranes were quantified at three different months during spring, summer and autumn (Table 2).

Photosynthetic membranes of plants are characterized by a high content of glycolipids dominated by galactolipids MGDG and DGDG. The fraction of MGDG slightly increase in summer, but the MGDG/DGDG ratio (1.1) is equal to the spring samples (Table 2). During the autumn fractions of MGDG in the membrane decreased and the MGDG/DGDG ratio also decreased (to 0.69) at the expense of the increased amount of DGDG. The fractions of the minor lipid constituents of thylakoid membrane show a gradual decrease of the amount of SQDG from spring to autumn while the amount of PG was found nearly constant during the seasons.

Six common fatty acids were detected in the lipid classes of the respective thylakoid preparations, including palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) and the seasonal variations in their relative content are presented in Table 3. Small quantities of some unusual fatty acids, early reported for *Petasites* thylakoid preparations (Yordanova et al., 2017) are not included in this analysis. The most abundant fatty acid in all the lipid classes was linolenic acid followed by palmitic and linoleic acids. The results revealed changes in fatty acids profiles of MGDG and DGDG in summer chloroplast membranes including decreased level in trienoic fatty acids and consistent increases in saturated 16:0 and 18:0 and in dienoic 18:2 levels. The changes in the level of unsaturation (Table 3), calculated as unsaturated/saturated ratio (16:1+18:1+18:2+18:3)/(16:0+18:0) were most obvious for MGDG reaching lower values during the summer and increasing during spring and autumn. The observed changes are consistent with the data for PSII activity of studied samples (Table 1). The content of polyunsaturated fatty acids in the lipid matrix is one of the major factors determining membrane fluidity. The decreased level of lipids unsaturation reduces the mobility of lateral separated pigment-protein complexes and electron carriers in the electron transport chain thus affecting the effectiveness of photosynthetic machinery. It has been proven that polyunsaturated fatty acids in thylakoid lipids play an important role in the stability of oxygen evolving machinery and increases in unsaturated fatty acids in membrane lipids protects PSII against photoinhibition (Sui & Han, 2014).

Table 2. Lipid classes in thylakoid membranes. The values obtained are means ± s.e. from three parallel measurements.

Seasons	Lipid classes (% of total)			
	MGDG	DGDG	SQDG	PG
Spring	25.92±1.1	25.92±1.0	27.77±0.7	20.37±1.0
Summer	30.76±1.0	27.70±0.4	23.07±0.5	18.46±0.9
Autumn	22.92±0.8	33.33±0.8	20.83±1.2	22.92±0.5

Table 3. Seasonal variations in relative fatty acids content of lipid classes. The values obtained are means ± s.e. from three parallel measurements; U/S, unsaturated to saturated fatty acids ratio; n.d. – not detected.

Membrane lipids	Sample	16:0	16:1	18:0	18:1	18:2	18:3	U/S
MGDG	spring	5.6±0.3	n.d.	1.4±0.2	1.7±0.7	1.6±0.2	88.2±0.1	12.96
	summer	7.9±1.2	n.d.	1.5±0.3	3.3±0.8	3.5±1.3	79.9±0.2	9.24
	autumn	2.5±0.6	n.d.	0.8±0.9	1.8±0.4	1.3±0.3	87.4±2.2	22.07
DGDG	spring	17.9±0.3	n.d.	2.8±0.3	n.d.	2.6±0.4	74.4±0.8	3.72
	summer	17.4±0.5	0.6±01	3.4±0.6	2.0±0.2	3.1±0.7	68.5±0.1	3.54
	autumn	12.4±1.1	0.5±0.1	2.7±0.1	1.9±0.4	2.0±0.2	75.3±1.4	5.05
SQDG	spring	27.6±2.3	9.9±0.7	4.2±0.8	1.9±1.3	9.6±1.8	38.5±0.1	1.98
	summer	27.7±0.6	13.0±0.4	2.3±0.3	1.9±1.1	11.1±0.1	38.9±1.7	2.03
	autumn	24.9±0.7	1.2±0.7	3.3±0.3	2.6±0.7	5.4±0.8	48.1±1.3	2.03
PG	spring	27.2±0.3	6.6±1.2	5.0±0.1	1.1±0.4	6.6±0.1	30.9±0.1	1.38
	summer	24.2±1.0	4.4±0.7	3.1±0.4	6.6±0.5	11.7±0.1	31.2±0.5	1.59
	autumn	28.6±1.1	10.9±0.2	3.0±0.6	2.9±1.2	6.5±0.1	37.2±0.2	1.82

Conclusion

The results of the present study on a local populatiobn of *Petasites hybridus* plants show seasonal variations in the activity of the photosynthetic apparatus expressed in strong inhibition of PSII activity during summer followed by a process of recovery during autumn and reacing maximal values in spring. These changes in photosynthetic performance are accompanied by modifications in the structute and composition of chloroplast membranes, connected with changes in membrane lipids and their fatty acid composition. The amount of unsaturated fatty acids is consistent with the data for PSII activity of studied species. A low level of unsaturation in summer thylakoid membranes makes PSII of drought intolerant *Petasite* extremely susceptible to photoinhibition and causes a significant reduction in photosynthetic activity. Unfavourable environmental conditions during summer induces stress-related metabolic responses as a result of which metabolic processes are shifted towards biosynthetic activities that consume reduction equivalents, especially the synthesis of secondary compounds such as terpenoids, phenols or alkaloids. Medicinal plants grown under water deficiency conditions reveal much higher concentrations of relevant natural products.

Acknowledgments

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Integrative Investigation on the Ecology of the Black Sea Mussel Mytilus galloprovincialis Lam. and its Habitat

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Abstract. The present work is reporting a preliminary investigation on the ecology of the Black sea mussel (*Mytilus galloprovincialis* Lam.) and its habitat by monitoring of the physicochemical indicators of the water, the microbiology and the radionucleids in the region of Shkorpilovci and Kavarna (NE Bulgaria). The samples were collected in the period of August 2018 to October 2019. The microbiological determination was performed by the use of the system "BIOLOG". *Enterococcus durans* and *E. haere* were isolated from the Shkorpilovci region and *Streptococcus gallolyticus ss gallolyticus*, *E. haere, Lactobacillus brevis* and *L. sakei ss sakei* were found from Kavarna. We isolated the lactic acid bacterium *Streptococcus gallolyticus ss gallolyticus*, a species associated with colon-rectal tumors on the one hand and as a producer of bacteriocins on the other. Additionally, the study includes information on the naturall and tehnogenic radionuclides in the marine environment. No technogenic radionuclides were found in the mussel samples. The calculated indicators for radiation hazard were in accordance with the norms quoted fixed in the local an interntional legislation.

Key words: Bivalvia, *Mytilus galloprovincialis* Lam, radionucleids, Black Sea, monitoring of the water, radiology, marine habitats.

Introduction

Marine mussels of the genus *Mytilus* are grown in aquaculture farms for human consumption worldwide. The economic value of these bivalve molluscs has resulted in considerable efforts to improve production by increasing survival and growth rates. Mussels, as filter feeders, are important for the marine ecology, but they also have an exceptional nutritional value, making them important foof in the human diet. Consumption of bivalve molluscs provide highly valuable proteins, essential vitamins, and minerals, as well as polyunsaturated fatty acids with health beneficial effects (Bongiorno et al., 2015).

The implementation of the Marine Strategy Framework Directive 79/923/EEC (CEC, 1979) requires adaptive management to improve the status of the marine

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environment. This adaptive management implies monitoring of the ecosystems using a set of appropriate indicators for their functional state (Guerry, 2005). Due to their sessile lifestyle, the filter-feeding apparatus and the lack of excretion mechanisms, the sea mussels have a significant capacity to bioaccumulate different xenobiotics from the environment and bioconcentrate them in their tissues to concentrations, that may significantly exceed the values in the environment. Therefore, a number of researchers share the view, that concentrations of pollutants in the tissues of organisms recognized as bio-indicators reflect most accurately the degree of environmental pollution and their analysis gain more valuable information than the analysis of the sea water (Baumard et al., 1999; Maanan, 2007). Such data can be used to quantify, evaluate, map and track the distribution of bioavailable components of different pollutants in marine and estuarine ecosystems (Phillips, 1994; Farrington et al., 1983).

The Black Sea mussel (*Mytilus galloprovincialis* Lamarck, 1819) is one of the few species that are considered to be a reliable species for the monitoring of the environment conditions in the marine ecosystems (Danellakis et al., 2011). The concept of using mussels as bioindicators for monitoring of coastal marine waters was termed "mussel-watch" (Goldberg, 1975). The mussels *M. galloprovincialis* are globally recognized as biomonitors and serve as control organisms in national and international programs (BIOMAR, BEEP, IOC-IMO, UNEP-GIPME). The Bulgarian Black Sea aquatory is seriously affected by chemical pollutants entering the sea with the waste waters of urban agglomerations, industry, agriculture and river runoffs. Molluscs, especially shellfish, live in the ever-changing environment of marine ecosystems concerning the increase of pollutions, climate change, eutrophication, changes in the water salinity, etc. A number of studies reported, that shellfish can be used as bioindicators to assess the toxic effects of chemical pollutants, heavy metals and other types of pressure. Typically, the effect of such pressure is related to changes in the response of the biological systems, as the effects were mediated at the cellular level. The estimation of the biological response revealing the effects of anthropogenic pressure even at the cell/molecular level may allow indirect integral assessment of the status of the ecosystem (Rodriguez-Ortega et al., 2009; Hering et al., 2003). The Black Sea mussel is in fact the only species of mussels grown as aquaculture in the Bulgarian Black sea aquatory. In Bulgaria its production in 2005 amounted to 170 tons, which accounted for 5.4% of the total aquaculture production in the country. In recent years, there is a steady trend towards the development of marine aquaculture on the Bulgarian Black Sea coast. The Black Sea mussel is used mainly for food in Bulgaria. The mussel tissue and beef are almost equal concerning the protein content, however the mussels contain 11 times more calcium and 2 times more phosphorus. Mussels can effectively purify seawater, so the cultivated production of Black Sea mussel may support the natural process of self-purification of the marine environment.

The present work is reporting a preliminary investigation on the ecology of the Black sea mussel and its habitat by monitoring of the physicochemical indicators of the water, the microbiology and the radionucleids in the region of Shkorpilovci and Kavarna.

Material and Methods

The study was conducted at the Department of Biology, University of Shumen, Bulgaria. The samples were collected from the regions of Kavarna (43.411059 N; 28.356250 E) and Shkorpilovci (42.957889 N; 27.89899 E) in the period of August 2018 to October 2019 (Fig. 1).

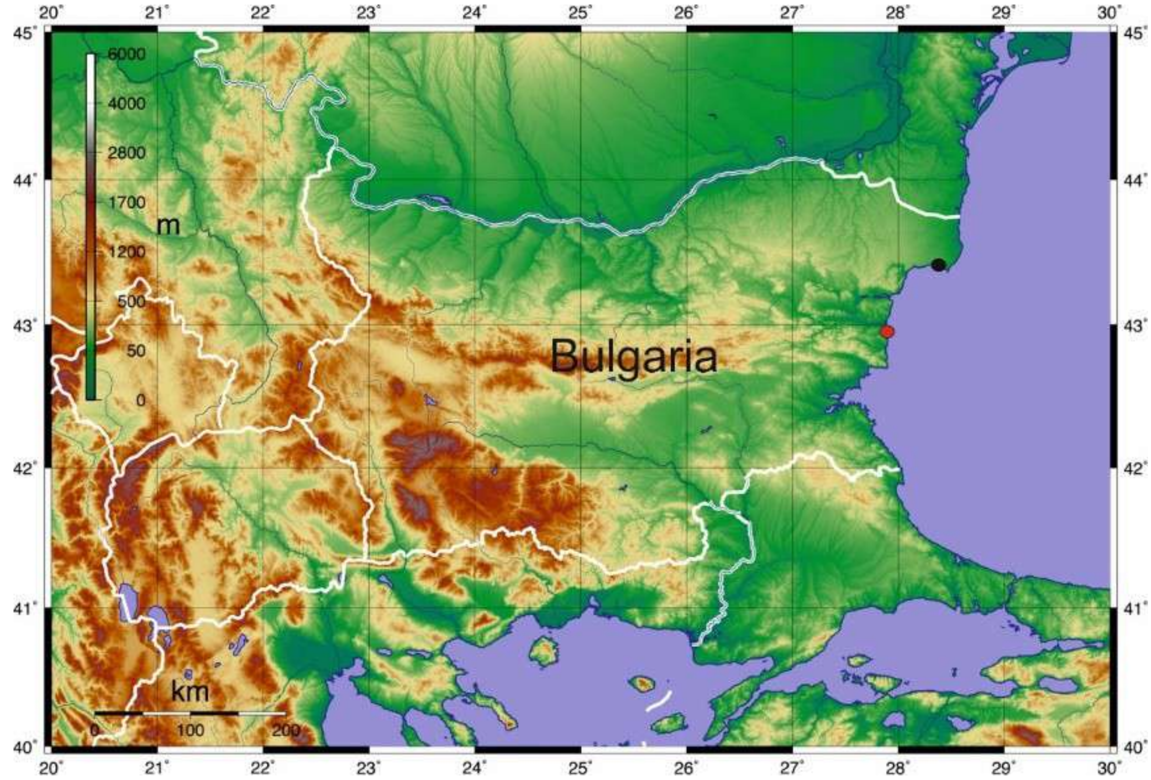

Fig. 1. Sampling locations at the Black sea coast. The red circle represent the sampling site at Shkorpilovci and the black one the sample site at Kavarna.

Collection of samples

The mussels were harvested from the northern part of the Bulgarian Black Sea aquatory. After collection, the samples (about 10 kg) were immediately refrigerated (4 °C) and transported to the laboratory for the further analyses.

In this study, we examined *Mytilus* mussels of similar size, weight, and shape to ensure maximal uniformity in the applied methods (Duquesne et al., 2004). The average length of mussels used in the study was 4.676 ± 0.47 cm.

Physico-chemical analysis of water

During mussel sampling we measured in situ the temperature, total salinity (by using YSI Model 33 salinity meter), and pH (by using ATC Piccolo HI 1280 pH-meter).

ɣ-*Spectrometric measurements*

The study included both determination of the concentration of natural radionuclides such as ^{40}K, ^{232}Th, ^{226}Ra, ^{214}Pb and others, as well as monitoring of the accumulation of the technogenic radionuclides.

Microbiological analysis

Three subsamples (each of about 1 kg of mussels) were used for the microbiological analyses. The mussels were scrubbed free of dirt, washed in hypochlorite solution (20 mg l^{-1}), rinsed with sterile distilled water, and shucked with a sterile knife. Tissue liquor samples (about 100 g) were homogenized.

Microbial Identification Databases for the "Biolog" Systems

The microbial identification was performed by a manual microbial identification system Biologist VIO45101AM (Diahim LTD, Sofia, Bulgaria). The isolated strains were screened on BL4021502 Tryptic Soy Agar (TCA), cultured for 24 hours at 37°C and then subjected to Gen III plaque identification to identify Gram positive and Gram negative aerobic bacteria. The microscopic pictures were performed using stereomicroscope OPTIKA (Italy) with a DinoEye, Eyepiece camera with 5 megapixels. Photographs were performed by using a with a Canon EOS 60D camera.

Results

The microorganisms were isolated from *M. galloprovincialis* Lam. collected from both sample sites at north Bulgarian Black Sea aquatory.

After 24 h of cultivation on different media, various microbial colonies were obtained. Data are represented on Table 1, Fig. 2 and 3.

Parallel to the performance of the microbiological experiments we conducted a physicochemical analysis of the sea waters. The results are summarized in Table 2.

Gamma spectrometry analysis of the samples was performed with Ge(Li) detector for a period of 24 h. Gamma spectra were analyzed with the ANGES program (Mishev & Vidolov, 2020, Program ANGES, Research Contact 9493/RO, Vienna, Austria, IAEA). The analysis of the gamma spectrums include: detecting of the peaks and determination of their energy and area; identification of the nuclides; calculation of the specific activities of the nuclides. Specific activities of radionuclides identified in the samples are represented on Fig. 4 and 5.

In the sand samples, collected from Kavarna and Shkorpilovci were found natural radionuclides ^{212}Pb, ^{214}Pb, ^{214}Bi, ^{208}Tl, ^{228}Ac, ^{226}Ra and ^{40}K. The values obtained for the specific activities of radionuclides in the samples are close. The values of the specific activities of ^{40}K in both samples were about 500 $Bq.kg^{-1}$.

The calculated indicators for radiation hazard (I_r), external hazard (H_{ex}) (Beretka & Mathew, 1985), absorbed dose (D) and annual effective dose (E) (UNSCEAR 2000; Veiga et al., 2006) were in accordance with the norms termed in the local and international legislation. The obtained values are presented in Table 3.

Table 1. Number of obtained colonies on the different media.

Media/ region	MRS agar	Cetrimid agar	Hromokult agar	MacConkey agar	MPA agar	M17	Strain BIOLOG
Kavarna (08.2018)	200.10^{-2}	76.10^{-2}	58.10^{-3}		246.10^{-3}		*Lactobacillus brevis; L. sakei sakei; Streptococcus gallolyticus gallolyticus; Enterobacter aerogenes*
Shkorpilovci region (08.2018)			78.10^{-3}	10.10^{-3}	90.10^{-3}		*Enterococcus hirae; Enterococus durans*
Kavarna (10.2018)	58.10^{-2}		68.10^{-3}	84.10^{-5}	128.10^{-3}		*Streptococcus gallolyticus gallolyticus*
Shkorpilovci region (10.2018)				56.10^{-3}	12.10^{-5}	101.10^{-3}	*Enterococcus hirae*
Shkorpilovci region (02.2019)			71.10^{-3}	94.10^{-1}	130.10^{-3}		*Enterococus durans*
Kavarna (02.2019)	28.10^{-2}		3.10^{-3}	2.10^{-3}	1.10^{-1}		*Streptococcus gallolyticus gallolyticus*
Shkorpilovci region			28.10^{-2}		150.10^{-3}	200.10^{-3}	*Enterococcus hirae;*

(06.2019)							*Enterococus durans*
Kavarna (06.2019)	67.10^{-3}	46.10^{-3}			132.10^{-3}		*Streptococcus gallolyticus gallolyticus*
Kavarna (10.2019)	70.10^{-2}		55.10^{-5}		14.10^{-5}	79.10^{-3}	*Streptococcus gallolyticus gallolyticus*
Shkorpilovci region (10.2019)				46.10^{-3}	2.10^{-5}	88.10^{-3}	*Enterococcus hirae*

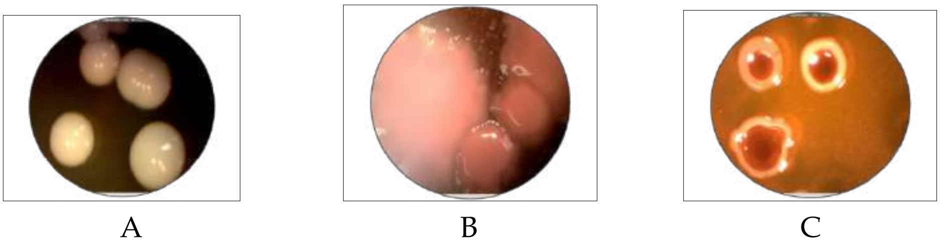

Fig. 2. Photographs of the colonies of the isolated species: A) colonies of Lactic acid bacteria on media MRS agar B) colonies of *Enterocucus durans* on media MacConkey agar; C) colonies of *E. hirae* on media Hromokult.

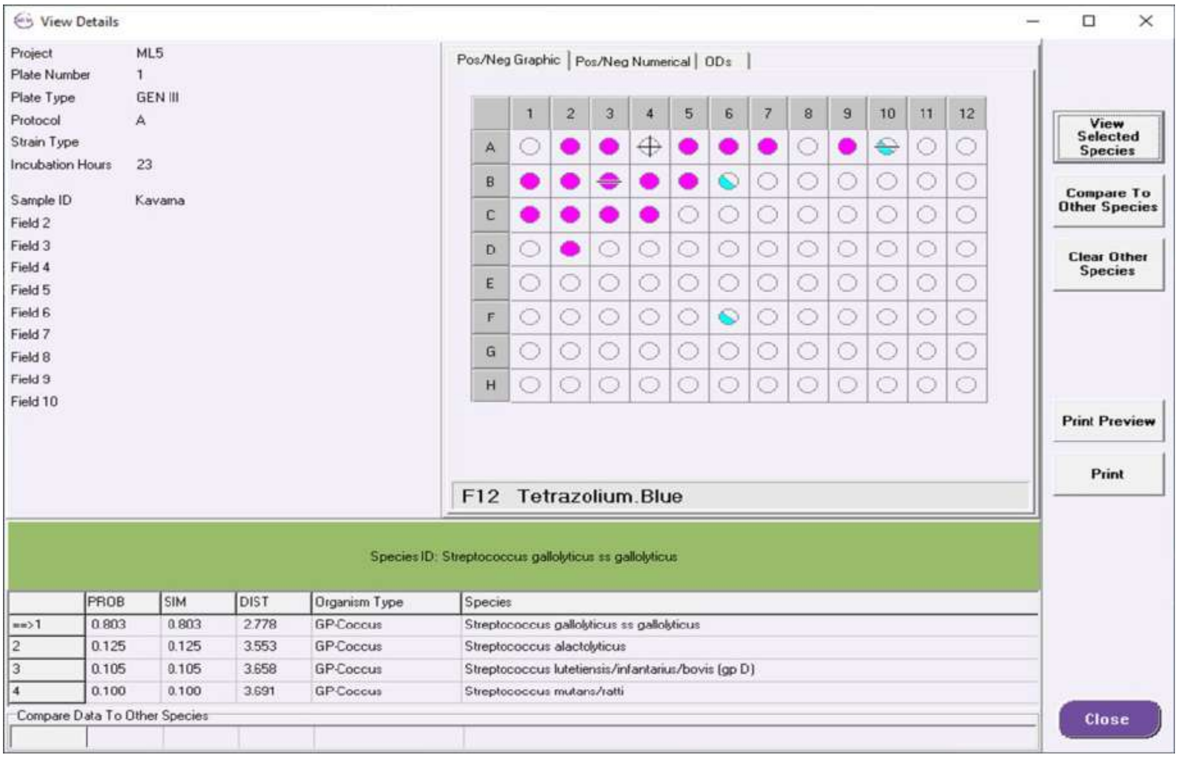

Fig. 3. Diagram from a test with the Microbial identification system Biologist VIO45101AM, demonstrating the presence of *Streptococcus gallolyticus ss gallolyticus.*

Table 2. The physicochemical parameters of the waters.

Region	Date	Depth m	temperature [° C]	pH [pH]	salinity [ppt]	dissolved O2 [mg/l]
Kavarna	08.2018	2 to 4	27.9	8.30	13.7	7.3
Shkorpilovci	08.2018	2 to 4	27.2	8.27	13.6	7.1
Kavarna	10.2018	2 to 4	26.7	7.26	11.5	8.5
Shkorpilovci	10.2018	2 to 4	24.6	7.35	10.5	8.2
Kavarna	02.2019	2 to 4	5.1	7.61	11.1	8.8
Shkorpilovci	02.2019	2 to 4	7.4	7.78	9.2	8.5
Shkorpilovci	06.2019	2 to 4	27.7	8.26	13.5	7.5
Kavarna	06.2019	2 to 4	26.2	8.25	13.5	7.2
Shkorpilovci	10.2019	2 to 4	25.7	7.36	11.2	8.8
Kavarna	10.2019	2 to 4	23.2	7.25	11.2	8.4

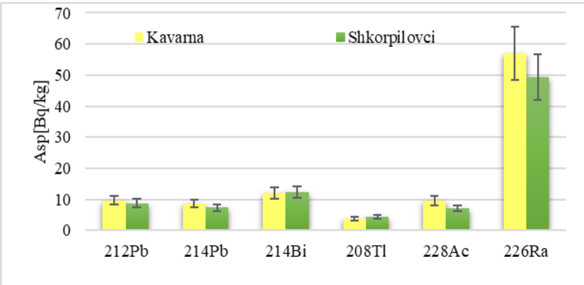

Fig. 4. Activity concentration of samples of sand collected from Kavarna and Shkorpilovci.

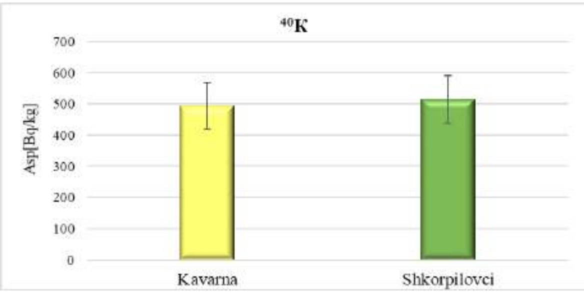

Fig. 5. Specific activity of 40K in sand samples collected from Kavarna and Shkorpilovtsi.

Table 3. Value for the indicators of radiation hazard, external hazard, absorbed dose and annual effective dose obtained for the sand samples collected from Kavarna and Shkorpilovci, and their referent norms.

	I_r [Bq.kg^{-1}]	H_{ex} [Bq.kg^{-1}]	D [nGy.h^{-1}]	E [mSv.y^{-1}]
Kavarna	0.60	0.21	38.96	0.048
Shkorpilovci	0.59	0.20	38.08	0.047
Norms	1	1	55	1

Discussion

The values observed for Fecal coliforms (FC) were conformed also for *E. durans* and *E. hirae* counts (Table 1 and Figures 3 A). In particular, *Enterococcus* was responsible for the FC peak in August (the two counts coincided), suggesting the presence of particular environmental conditions, which influenced the quality of the mussels harvested in that month. These bacteria were found in the region of Shkorpilovci. The lactic acid bacteria, which were isolated, belonged to *Streptococcus gallolyticus gallolyticus*, *Lactobacillus brevis* and *L. sakei sakei* from the region of Kavarna, as the peak in the concentration was detected in August (Table 1 and Figures 3 A, 4). The high concentrations of FC, detected in the warmer months may be related to the increased metabolic activity of the moluscs. The high metabolic activity of the mussels is directly related to the increase of the temperature of the sea waters from one side (Table 2) and their biological cycle from the other. In previous investigations of our working group we demonstrated experimentally, that from the black mussels can be isolated two stems of lactic acid bacteria belonging to *L. plantarum*, which show antifungal activities (Ibryamova et al., 2020). For the north section of the Bulgarian Black sea aquatory, for June 2018 were reported high levels of *E. coli* and *Pseudomonas aeroginosa* (Ignatova-

Ivanova et al., 2018). The temperature of the sea waters may affect the number of the microbs by concentration of the nutients. Simmilar results were reported previously (Grimes et al., 1986; Ramon & Richardson, 1992). The peak of *Enterobacter* observed in August can be related to some particular environmental conditions. In fact, the characteristics of the mussels can be affected by a variety of extrinsic and intrinsic factors, such as water temperature and salinity, food availability and gametogenic cycle of the animals (Okumus & Stirling, 1998). According to Ordinance No. 4 from 20.10.2000 for the quality of fisheries water and the breeding of shellfish, the amount of fecal coliforms in the inter-shell content should be less than 300 NVB - in our samples from October 2018, the values were exceeded by 40 times. The probable cause for the increased quantities of fecal coliforms is the pollution of the seawater at the end of the summer season, which remains relatively high until the beginning of October. No pollution was reported at the same site in June.

The gamma-spectrometric analysis revealed, that technogenic radionuclides can be detected in the sand samples, collected from the Kavarna and Shkorpilovci region. Tehnogenic radionuclides were not detected in the shells and the soft tissues of the mussels colected from theses regions. The indexes for radiation hazard, external hazard, absorbed dose and annual effective dose obtained from the sand samples are lower than the referent values set in the UNSCEAR.

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How Chlorella Species Isolated from Contrasting Habitats Respond to UV-B Induced Stress?

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Abstract. We hypothesize that algal species isolated from habitats with extreme environmental conditions would differ in their susceptibility to UV-B irradiation having better survival strategies. The aim of this study was to evaluate UV-B induced stress response of *Chlorella* species, isolated from different habitats: *Chlorella vulgaris* (Antarctic) - from soil in island Livingston, Antarctic, *Chlorella vulgaris 8/1* (Thermophilic) - from thermal springs in region of Rupite, Bulgaria and *Chlorella kessleri* (Mesophilic) - from the Trebon collection. Unicellular green algae were chosen as a model organism because they are a robust model in genotoxicology due to the following reasons: photosynthetic eukaryotic organisms with typical for plants cell structure and genome organization; cell - organism with short life cycle - the response of a single cell is equivalent to the response of an individual organism; not expensive microbiological and molecular methods. *Chlorella* species were cultivated on TAP medium under standard conditions 23 °C ± 0.1° and 60 µmol m−2 s^{-1} in a growth chamber Phytotron GC 40. Cell suspensions in the end of the exponential/ beginning of the stationary phase were used. Cells were irradiated in BLX-254 (Life Technology, UV crosslinker, λ= 312nm). Cell response of *Chlorella* species was examined based on spot-test, micro-colonies assay, growth rate and DSB induction. The results demonstrated strong bioactivity of UV-B doses equal to or higher than 250 J/m^2. The magnitude of photoreactivation sectors revealed that *Chlorella* species are photoreactivation and dark-repair proficient but differ in their capacity to overcome damages induced by UV-B light. New data were provided concerning UV-B capacity to induce DNA double-strand breaks in *Chlorella* species. Based on the complex of methods used, it was established that according to their resistance to UV-B induced stress, the different species can be arranged in the following order: *Chlorella vulgaris* Antarctic > *Chlorella vulgaris 8/1* > *Chlorella kesseri*.

Key words: UV-B irradiation, *Chlorella*, DSBs, photo-reactivation.

Introduction

In recent decades, the sun's ultraviolet-B (280-315 nm) reaching the earth's surface has increased due to ozone depletion (Kshama & Agrawal, 2017). Ultraviolet-B (UV-B), being high energy can impact the biota at different levels causing various biological damage including sunburn, skin cancer, inhibition of immune responses etc (Gill et al., 2015; Rigo et al., 2015).

The bioactivity of UV-B irradiation results from both direct and indirect mechanisms involving endogenous sensitizers and the generation of active oxygen species. Primary radicals formed as a result of UV irradiation lead to the formation of lipid radicals, which react with oxygen to produce lipid peroxyl radicals (Pouneva & Minkova, 2010; Medeiros et al., 2015).

Due to the potential impact of UV-B on human health, extensive research has been carried out in determining the mechanisms of UV-B induced damage in mammalian systems (Rigo et al., 2015). DNA is one of the key targets for UV-induced damage in a variety of organisms ranging from bacteria to humans (Sinha & Hader, 2002).

Although plants, as photosynthesizing organisms, have an absolute need for sunlight and are particularly sensitive to occurring changes, modern knowledge of the mechanisms of UV-B effects on plants is not as satisfactory as in mammals. At present state of knowledge, UV-B can affect many processes in plants - inhibition of photosynthesis, inactivation of enzymes, damaging DNA etc. (Wong et al., 2015).

Organisms respond to environmental impact by developing a series of physiological, biochemical and molecular strategies.

Previously, we have described that strains more resistant to various inducers of oxidative stress or isolated from contrasting habitats differ in their DSBs repair capacity, cells membrane stability, activation of antioxidant and/or chaperone systems (Chankova et al., 2002; 2013; 2014; Chankova & Yurina, 2012; 2016).

Following these findings here we hypothesize that algal species isolated from habitats with extreme environmental conditions would differ in their susceptibility to UV-B irradiation having better survival strategies. *Chlorella* species have been chosen because they are widely spread photosynthesizing unicellular eukaryotes with a short life cycle, cell structure and genome organization typical for plants so the results could be extrapolated to higher plants, with haploid genome - recessive mutations could be revealed at the next generation, routine inexpensive laboratory cultivation techniques could be applied, very suitable organism for molecular studies.

The aim of this study was to compare UV-B susceptibility of *Chlorella* species, isolated from different contrasting habitats at different levels: cellular and molecular.

Materials and Methods

Species and cultivation: Chlorella vulgaris Antarctic – isolated from soil in island Livingston, Antarctic, *Chlorella vulgaris 8/*1 - from thermal springs in the region of Rupite, Bulgaria, cultivated since 1975 in our lab, and *Chlorella kessleri* Mesophilic - from the Trebon collection.

Cell suspensions were cultivated on TAP (Tris Acetate Phosphate) medium (Harris, 1989) under continuous light of 60 μmol m−2 s^{-1} and a temperature 23 °C ± 0.1° in a Phytotron GC 40 growth chamber. Cell suspensions in the end of exponential and the beginning of the stationary phase (5-days old) were used.

UV-B Irradiation: Five-days old cell suspensions with a density $1x10^6$ cell/ml were irradiated with UV-B (λ= 312 nm) in BLX-254, Life Technology, UV crosslinker. The irradiation was done with doses in the range 50, 100, 250, 500 and 1000 J/m^2 in a dark or yellow light to prevent photo-reactivation. After the irradiation, samples were split in two- samples: "with" photo-reactivation (kept at continuous light of 60 μmol m^{-2} s^{-1} for 24 h) and "without" photo-reactivation (kept in a dark for 24h). After that, Petri dishes were cultivated in a Phytotron GC 40 growth chamber at temperature 23 °C ± 0.1° at continuous light of 60 μmol m^{-2} s^{-1}.

Genotypes resistance of species to UV-B was assessed based on several endpoints:

Spot-test (Harris, 1989) - 10μl of cell suspension irradiated with appropriate UV-B doses were pipetted on solid TAP medium in Petri dishes to form drops. Every drop contained 1000 cells. The Petri dishes were kept in the growth chamber at "with" and "without" photo-reactivation conditions.

Micro-colonies survival assay (Vlcek et al., 1987) **-** very rapid method allowing to obtain information concerning strains survival in a next 72 hours after the UV-B irradiation. Observations were made under a microscope Amplival at a magnification of 16/40. The method is based on counting of the survived microcolonies and single none divided cells vs dead cells and microcolonies 72 hours after the irradiation with appropriate doses of UV-B.

Growth rate (Harris, 1989; Shevchenko, 1979) - this method provides useful information concerning population potential to restore its reproductivity and the rate of growth after provocative exposure (in our case UV-B irradiation. Observation was made microscopically using Amplival microscope at a magnification of 16/40. Cell density was counted microscopically on a hemocytometer (Burker) every 24 hr for 72 hours

Doses determining three levels of lethality - LD_{20}, LD_{50} and LD_{80} were calculated in order to compare species sensitivity to UV-B irradiation (Lidanski, 1988)

Photo-reactivation sectors (Harm, 1968, Serafin et al., 2003; Kiefer, 2012). The magnitude of photo-reactivation sectors (PRS) provides information about photo-reactivation proficiency or photo-reactivation deficiency of species.

DSBs induction and repair capacity by Constant field gel electrophoresis (CFGE)

Chlamydomonas reinhardtii protocol (Chankova and Bryant, 2002; Chankova et al., 2005) was optimized for *Chlorella* species. Additional step for cell wall disruption with sonicator BANDELIN Sonopuls HD 2070 was added due to the differences in the cell wall composition of *Chlamydomonas reinhardtii* and *Chlorella*.

Parameters for cell wall degradation were: *Chlorella vulgaris* (Antarctic species) - 3 minutes 4 cycles, 75% strength; *Chlorella kessleri* (mesophilic species) - 3 minutes, 2 cycles, 25% strength; *Chlorella vulgaris* 8/1 (thermophilic species) - 3 minutes 4 cycles, 25% strength.

Data analysis. The **e**xperiments were repeated at least three times using independently grown algal cultures. One-way ANOVA with Tukey multiple comparison test was performed to compare mean differences among genotypes (GraphPad Prism 6.04).

Results

Survival after UV-B irradiation

Spot-test was used for initial evaluation of the bioactivity of UV-B irradiation depending on the dose-range and the genotype. Reducing the intensity of spots was read at irradiation doses equal to or greater than $250J/m^2$ in photo-reactivation conditions for both species – *Chlorella vulgaris* 8/1 and *Chlorella kessleri.* Only irradiation with two-fold higher dose - 500 J/m^2 resulted in slight reduction of spots intensity in *Chlorella vulgaris* Antarctic that is informative for highly expressed capacity of Antarctic *Chlorella vulgaris* to repair at light conditions damages induced by UV-B.

More clear differences in species response were expressed in samples "without" photo-reactivation. The most sensitive was *Chlorella kessleri*>*Chlorella vulgaris* 8/1>*Chlorella vulgaris* Antarctic (data not shown).

As a next step, the micro-colonies survival assay was performed in order to obtain more detailed information concerning species sensitivity to UV-B irradiation.

Cell survival data of samples with photo-reactivation presented in Fig. 1A show that this parameter was significantly reduced after UV-B irradiation with doses equal to or higher than $500J/m^2$ in all three species. Comparing the slope of curves, it is evident that both species *Chlorella vulgaris* 8/1 and *Chlorella kessleri* follow the same trend. Spots test information was confirmed by those of micro-colonies survival assay – dose 1000 J/m^2 can induce around 100% lethality for *Chlorella vulgaris* 8/1 and *Chlorella kessleri*. Even at this high dose, *Chlorella vulgaris* Antarctic has survived, albeit with low frequency.

Further, we have evaluated species cell survival at conditions preventing photo-reactivation.

Cell survival of *Chlorella kessleri* was dramatically decreased comparing with those of *Chlorella vulgaris* Antarctic and *Chlorella vulgaris* 8/1 in samples "without" photo-reactivation after 250 J/m^2 UV-B irradiation (Fig. 1B). Our data illustrate that *Chlorella vulgaris* Antarctic and *Chlorella vulgaris* 8/1 probably have a better dark-repair potential depending on the dose comparing with *Chlorella kessleri*. This trend is the same at higher doses only for *Chlorella vulgaris* Antarctic. The most sensitive was *Chlorella kessleri*>*Chlorella vulgaris* 8/1>*Chlorella vulgaris* Antarctic.

Based on our cell survival data after UV-B irradiation, three levels of lethality (LD_{20}, LD_{50} and LD_{80}) were calculated. These criteria are good tools to compare genotypes resistance to different mutagenic factors, in our case UV-B irradiation. Looking at the Table 1 it is obvious that doses for Antarctic chlorella are significantly higher than those causing the same level of lethality, for *Chlorella vulgaris* 8/1 and *Chlorella kessleri,* following dose-dependent relationships. In samples "with" photo-reactivation, doses that can induce these three levels of lethality in both species *Chlorella vulgaris* 8/1 and *Chlorella kessleri* were approximately similar (Table 1).

Data presented in the same table for samples kept in a dark for 24 h ("without" photo-reactivation) demonstrate approximately similar doses determining LD_{20} and LD_{50} for both species *Chlorella vulgaris* 8/1 and *Chlorella kessleri*.

Based on our data we can speculate that *Chlorella vulgaris* Antarctic was less susceptible to UV-B radiation comparing with *Chlorella vulgaris* 8/1 and *Chlorella kessleri*.

Further, sectors of photo-reactivation (PRSs) calculated by three methods (Harm, 1968; Serafin et al., 2003; Kiefer, 2012) have revealed similar tendencies:

Comparison of the results following the procedure described by Kiefer (2012) reveals that *Chlorella vulgaris* Antarctic and *Chlorella vulgaris* 8/1 are characterized with dose-dependent decrease in the magnitude of PRSs. No such trend was found for *Chlorella kessleri*. As it is seen in a Table 1 the differences among average PRSs are not large but they could be a good reason to assume that *Chlorella kessleri* probably has a less pronounced dark repair and "relies" mainly on its photo-enzyme repair.

Similar trend (Table 2) was found using one more method (Harm, 1968). Results revealed dose-dependent decrease in the photo-enzymatic repair capacity of both species, isolated from habitats with extreme environment - *Chlorella vulgaris* Antarctic and Chlorella vulgaris 8/1 and approximately similar sectors for all the doses in *Chlorella kessleri* (Table 2).

Data obtained according to Serafin et al. (2003) are in a Fig. 2. The bars show the magnitude of area between curves "with" and "without" photo-reactivation.

We have calculated again slightly increased PRS for *Chlorella kessleri* and approximately similar PRSs for *Chlorella vulgaris* Antarctic and *Chlorella vulgaris* 8/1.

Growth rate after UV-B irradiation

Our results show that in both *Chlorella* species - *Chlorella vulgaris* 8/1 and *Chlorella kessleri*, doses of 500 and 1000 J/m^2 can induce damages with lethal effect or such leading to full blocking of cell division - no doubling of cells was scored (Fig. 3A). Statistically significant correlation between both cell survival and growth rate was obtained for *Chlorella vulgaris* 8/1 and *Chlorella kessleri* - 0.895 and 0.912, respectively. In *Chlorella vulgaris* Antarctic at photo-reactivation conditions, cell division at doses of 500 and 1000J/m^2 was severely delayed.

In samples "without" photo-reactivation, cell division was completely inhibited. Single dead cells as well as dead micro-colonies were found after the irradiation with doses equal to or higher than 500 J/m^2. Strong decrease of doubling capacity was read in samples irradiated with 250 J/m^2 for *Chlorella kessleri*. The curves at Fig. 3B demonstrate that both species *Chlorella vulgaris* Antarctic and *Chlorella vulgaris* 8/1 isolated from extreme habitats, can overcome the UV-B induced stress to some extent. Some doubling potential of cells in *Chlorella vulgaris* Antarctic population was obtained even after the irradiation with 500J/m^2.

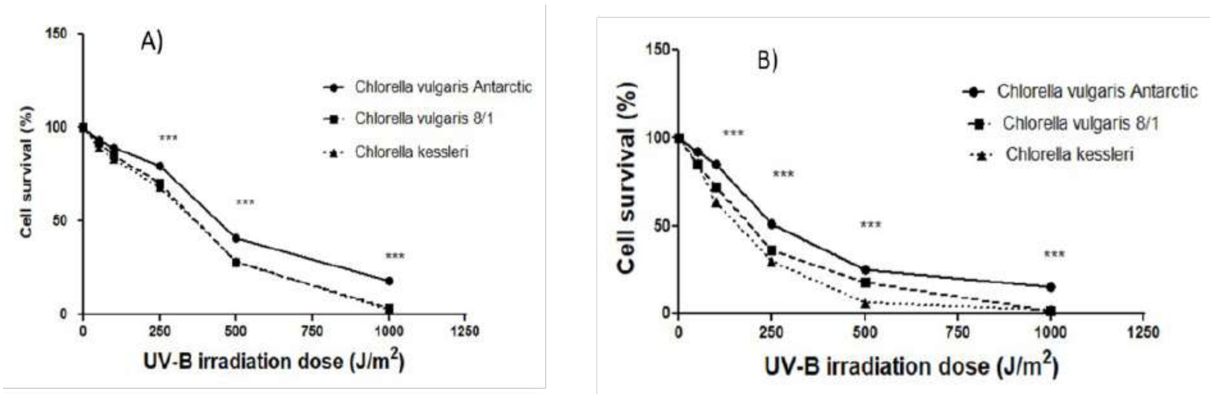

Fig. 1. Cell survival after UV-B irradiation at photo-reactivation conditions (A) and "without" photo-reactivation (B) of *Chlorella vulgaris* Antarctic, *Chlorella vulgaris* 8/1, *Chlorella kessleri* from 3 independently grown cell cultures. Where standard errors are not visible, they are equal to or less than the symbols on the graphs. The differences between *Chlorella vulgaris* Antarctic and the other two species are statistically significant *** p <0.001.

Table 1. UV-B doses determining three levels of lethality of Chlorella species, DMF and PRS. *Legend:* Data are averages from 3 independently grown cultures; PHR (+) represents samples grown at light - "with" photo-reactivation; PHR (-) - samples "without" photo-reactivation conditions; DMF – dose-modifying factor; PRS – photo-reactivation sector.

		Chlorella vulgaris Antarctic			*Chlorella vulgaris* 8/1			*Chlorella kessleri*		
		J/m$_2$	DMF	PRS	J/m$_2$	DMF	PRS	J/m$_2$	DMF	PRS
LD$_{20}$	(+) PR	239	2.07	0.52	138	2.12	0.53	120	2.03	0.51
	(-) PR	113			65			59		
LD$_{50}$	(+) PR	425	1.65	0.40	348	1.98	0.50	343	2.38	0.58
	(-) PR	257			175			144		
LD$_{80}$	(+) PR	940	1.32	0.24	627	1.37	0.27	626	1.88	0.47
	(-) PR	710			458			333		
∑ PRS		1.16			1.29			1.56		
Average PRS		0.39			0.43			0.52		

Table 2. Photo-reactivation sectors according to Harm (1968).

	LD_{20}	LD_{50}	LD_{80}
***Chlorella vulgaris* Antarctic**	0.590	0.393	0.252
***Chlorella vulgaris* 8/1**	0.540	0.493	0.275
Chlorella kessleri	0.525	0.575	0.472

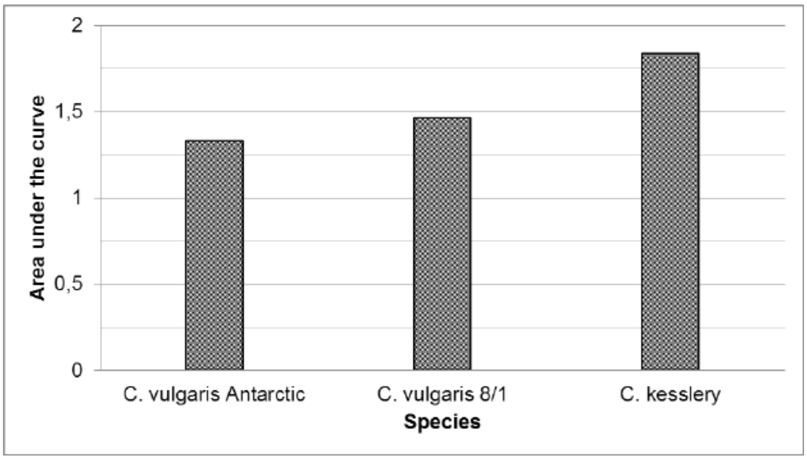

Fig. 2. Photo-reactivation sectors presented as Area under the curve based on Serafin et al. (2003).

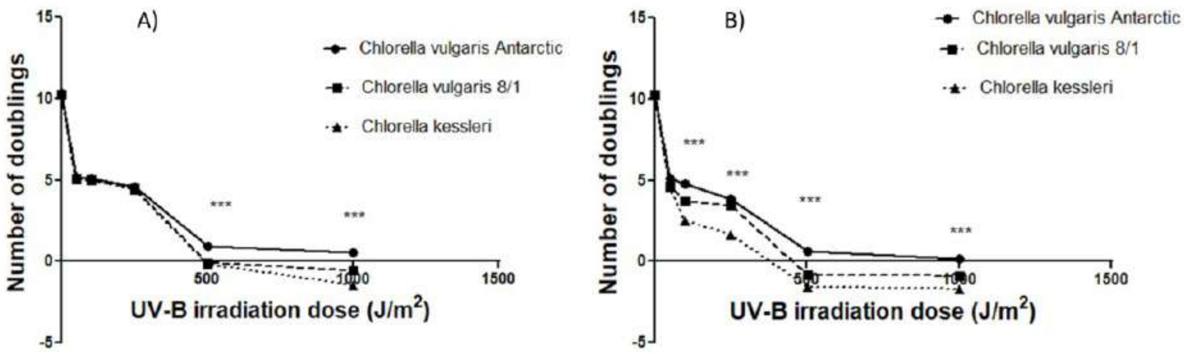

Fig. 3. Growth rate measured as number of doubled cells in samples "with" (A) and "without" (B) photo-reactivation. Data are averages from 3 independently grown cell cultures. Where standard errors are not visible, they are equal to or less than the symbols of the graphs. The differences between *Chlorella vulgaris* Antarctic and the other two species are statistically significant *** p <0.001.

Again, in conditions without photo-reactivation species UV-B resistance could be arranged as follows: *Chlorella vulgaris* Antarctic > *Chlorella vulgaris* 8/1> *Chlorella kessleri*.

Statistically significant correlation between the cell survival and the growth rate was obtained for *Chlorella kessleri* – 0.914.

The information presented above was also confirmed using another method (Shevchenko, 1979). Data presented in Table 3 illustrate the same tendency described above.

Up to 250J/m^2, the three algal species have a similar capacity to cope with the harmful action of UV-B. Over 500 J/m^2 the difference is noticeable. Some potential to recover cell population was found again for *Chlorella vulgaris* Antarctic.

In samples "without" photo-reactivation inhibition of cell division dependent on both the dose and genotype was revealed. The effect was most pronounced for *Chlorella kessleri*. Again some cell division was obtained for *Chlorella vulgaris* Antarctic.

These data have confirmed those obtained for cell survival. Again, *Chlorella vulgaris* Antarctic was shown as the most resistant and *Chlorella kessleri* as the most susceptible to UV-B irradiation.

Induction of DSB after UV-B irradiation

No statistically, significant differences were calculated among spontaneously occurred DSBs. DSBs, induced by UV-B in a dose range 50- 500 J/m^2 are presented in a Fig.4. All three *Chlorella* species respond to UV-B irradiation in a similar, dose dependent way up to a dose 250 J/m2. Doses higher 250 J/m^2 resulted to the formation of plateau for all three *Chlorella* species.

Chlorella vulgaris Antarctic and *Chlorella vulgaris* 8/1 isolated from habitats with extreme environment respond to UV-B irradiation in a similar way – approximately the same, lower DSBs levels from those measured in *Chlorella kessleri*.

Next, species DSBs repair capacity was evaluated when 24 h recovery time was given. Results revealed that when the recovery time is at optimal for cell growth conditions (Fig. 5A), the three species repair the UV-B induced DSBs in a similar manner.

Interestingly, in unfavorable conditions (Fig. 5B) while *Chlorella vulgaris* Antarctic and *Chlorella vulgaris* 8/1 follow the same trend of similar DSB repair capacity, *Chlorella kessleri* showed very low levels of DSB. Based on a correlation analysis, we can suppose that the low DSB levels measured after 24 h recovery time in unfavorable conditions are the result of huge DNA fragmentation rather than higher repair capacity.

Discussion

Climate changes and anthropogenesis contribute for increased levels of UV-B light that has an adverse impact on the biota, including microalgae. Microalgae are very important from ecological point of view as primary producers, as well as economically - as main sources of health supplements and pigments (Lai et al., 2019). Until now very reliable, information has been provided mainly on the negative effects of UV/UV-B on growth and development as well as on the photosynthetic apparatus of microalgae and plants (Pessoa, 2012; discussed in Apostolova et al., 2014; Ganapathy et al., 2017). A few studies report differences in the response of microalgae depending both on natural UV-irradiance of the environment (Pessoa, 2012) and specificity of habitats (discussed in Apostolova et al., 2014).

Table 3. Growth rate in samples "with" (+) and "without" (-) photo-reactivation. Legend: Data are averages from 3 independently cell cultures. Where standard errors are not visible, they are equal to or less than the symbols of the graphs. The differences are statistically significant *** p <0.001.

Doses	*Chlorella vulgaris* Antarctic	*Chlorella vulgaris* 8/1	*Chlorella kessleri*
	PHR (+) PHR(-)	**PHR (+) PHR(-)**	**PHR (+) PHR(-)**
control	2.38 2.37	2.30 2.29	2.28 2.20
50J/m^2	1.19 1.18	1.18 1.08	1.16 1.05
100J/m^2	1.18 1.10	1.16 0.85	1.14 0.58
250J/m^2	1.06 0.89	1.05 0.80	1.03 0.39
500J/m^2	0.22 0.15	-0.02 -0.19	-0.04 -0.36
1000J/m^2	0.13 0.03	-0.13 -0.21	-0.34 -0.39

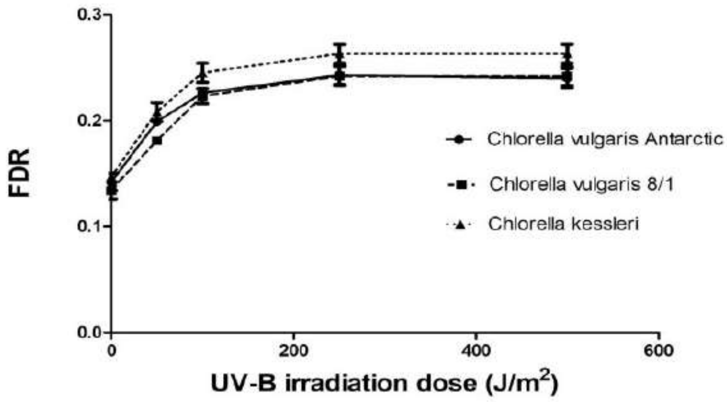

Fig. 4. DSB induction after UV-B irradiation with doses in the range 50 - 500 J/m^2. Data are averages from at least three independent experiments. Where standard errors are not visible, they are equal to or less than the symbols of the graphs.

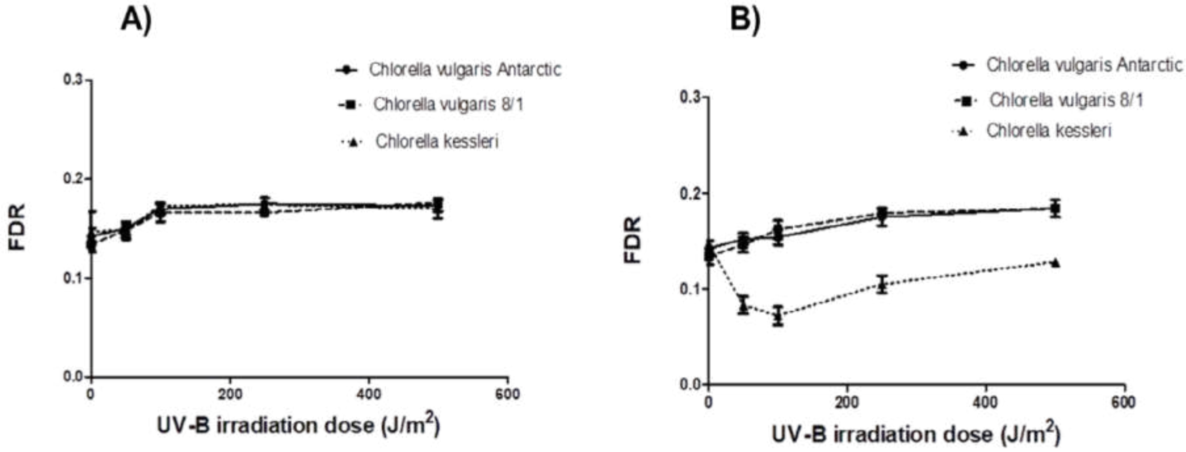

Fig. 5. DSB repair capacity after 24 h recovery time at: (A) optimal conditions (light, room temperature); (B) unfavorable conditions (dark, on ice). Data are averages from at least three independent experiments. Where standard errors are not visible, they are equal to or less than the symbols of the graphs.

UV-B irradiation directly or indirectly via generation of ROS may induce different types of DNA lesions - cyclobutane pyrimidine dimmers (CPD) and 6-4-photoproduct (6-4PP), DNA/DNA and DNA protein cross-links, double-strand breaks (DSB) and single-strand breaks (SSBs) leading to disruption both DNA structure and the processes of replication and transcription (Rastogi et al., 2010; He et al., 2002; Lesser, 2008; Rastogi et al., 2020). It has been supposed that DNA DSB and SSBs are formed not because of the direct absorption of UV radiation but rather as the consequence of the attempted repair of UV radiation-induced base damage in DNA – NER dependent manner (Wakasugi et al. 2014). Photolesions induced by UV-B irradiation could be overcome by a number of DNA repair mechanisms, including photo-reactivation, nucleotide excision repair (NER), base excision repair (BER), recombinational repair and post replication repair (Smith & Mpoloka, 2008; Jones & Baxter, 2017; Gill et al., 2015; Yin et al., 2017).

In the present work, we have attempted to broaden our understanding of the variety of types of damage induced by UV-B and capacity of *Chlorella* species, isolated from contrasting habitats to repair these damages.

The first step of our investigation was to compare cell survival and growth rate of species at photo-reactivation and none photo-reactivation conditions. Photo-reactivation (PHR), the so called "light repair," is a very old, evolutionary developed mechanism to overcome harmful effect of solar radiation using blue to near-UV light energy to repair UV-induced lesions - CPDs or (6-4) PPs, by directly rearranging bonds (Jones and Baxter, 2017). Comparing UV-B resistance based on doses determining three levels of lethality at conditions "with" and "without" photo-reactivation *Chlorella* species were arrange in the following way: *Chlorella vulgaris* Antarctic > *Chlorella vulgaris* 8/1~ *Chlorella kessleri. Chlorella vulgaris* Antarctic was more resistant to UV-B irradiation comparing with *Chlorella vulgaris* 8/1 and *Chlorella kessleri*. These results are in a good agreement with those of Pessoa (2012) where isolates of the marine microalga *Chattonella marina* (Raphidophyte) from Australia exhibits higher tolerance to high intensities of visible light than *C. marina* collected from Japan waters.

Looking at the slopes of survival curves and LD levels we can say that the three species are photo-reactivation and dark-repair proficient with the most pronounced capacity for *Chlorella vulgaris* Antarctic. Analysing the magnitude of photo-reactivation sectors (PRS) we have found the same trend using three methods good enough for such purpose. The magnitude of ∑PRS expressing the space between both survival curves - "with"and "without" photo-reactivation slightly increases from *Chlorella vulgaris* Antarctic to *Chlorella vulgaris* 8/1 and is higher for *Chlorella kessleri*. This finding show better expressed dark repair capacity of *Chlorella vulgaris* Antarctic and *Chlorella vulgaris* 8/1. Probably mesophilic *Chlorella kessleri* is mostly dependent on the photo-reactivation and probably with impaired dark repair.

The last step of our investigation was to evaluate DSBs repair capacity of species because it is known that the generation of SSBs and DSBs in UV-B irradiated cells, is observed extensively as a result of transcription/replication blockage (Rastogi et al, 2010; Marabini et al., 2020). We have measured similar quantities of spontaneously arisen DSBs for the species investigated by us. In the levels of DSBs induced by different UV-B doses, we did not find any differences that mean similar DNA susceptibility. Having in mind the present state of knowledge that genotypes resistance is rather related to repair capacity than to primary induced damages we were interested to compare the repair capacity of species. At conditions, not preventing DSBs repair no statistically significant differences among the species were found. In the case when post irradiation conditions prevent DSBs repair approximately similar levels of DSBs were measured for *Chlorella vulgaris* Antarctic and *Chlorella vulgaris* 8/1 and strong DNA degradation for *Chlorella kessleri*.

Conclusion

Our finding provides additional information concerning cellular and molecular differences of *Chlorella* species, isolated from contrasting habitats. Species investigated by us differ in their cell survival, growth rate, photoreactivation, dark and DNA double-strand breaks repair capacity. Both species isolated from extreme habitats are photo and dark repair proficient, while *Chlorella kessleri* is probably with impaired dark repair. UV-B induction of DSBs was confirmed.

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Relationships between Soil Microbial Activity, Bacterial Diversity and Abiotic Factors Along the Heavy Metal Contamination Gradient

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Abstract. In this study, the relationships between soil abiotic factors, heavy metals content and soil microbial activity, bacterial abundance, bacterial genotype richness and diversity were analysed in three sites along a Cu gradient (from 53 to 860 mg kg -1) and co-contaminants Zn and Pb, located in the region of Zlatitsa-Pirdop valley, Western Bulgaria. Long-term heavy metal contamination had a significant negative effect on soil microbial activity and our results showed that the dehydrogenase activity (DHA) decreased along the contamination gradient with up to 79% compared to the uncontaminated sample. The principal component analysis (PCA) showed that DHA correlated significantly and positively with total bacterial abundance (16S rRNA gene copies) and nitrate ions (NO_3-N), and negatively with soil pH, heavy metals and their bioavailable forms. Bacterial genotype diversity was mainly influenced by abiotic factors such as soil organic matter and sand fraction of the studied sites.

Key words: heavy metals, soil contamination, dehydrogenase activity, bacterial abundance and diversity, 16S rRNA gene.

Introduction

Soil contamination by heavy metals has been of a great concern on a global scale, because of their threats to the environment, food safety and human health. Heavy metals (HMs) are the main contaminants of Bulgarian soils, being widely distributed as a result of agricultural and industrial activities. Many studies underlined that heavy metals and soil abiotic factors altered the microbial communities in different manner concerning their activity, abundance, diversity, and structure (Wang et al., 2007; Zhang et al., 2013; Park et al., 2018, Wiatrowska et al., 2015; Zhao et al., 2019). In general, long-term contamination with heavy metals causes a

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reduction in the diversity, richness and activity of soil microbiome (Wang et al., 2007; Park et al., 2018). Since the microbial communities are more sensitive than soil physicochemical properties to human impact, changes in their microbial activity are often considered to be a good and adequate measuring of their response to environmental stress. One of the most commonly used sensitive indicators of microbial functioning in soil is the activity of dehydrogenase complex. Many authors reported a relationship between reduction of dehydrogenase activity and high concentrations of pollutants in soil, which in turn correlates with a decrease in microbial community diversity and biomass (Baćmaga et al., 2015; Wang et al., 2018; Wolińska & Stępniewska, 2012). By searching links between microbial communities and soil functions, many reports assessed the influence of different edaphic factors on the composition and functional diversity of soil microbiome (Chau et al., 2011; Furtak et al., 2019). Recent research works are focused on the combined effects of multiple factors on multiple processes in soil environment - microbial community-function coupling (Zheng et al., 2019).

This study aimed to analyse the relationships between soil abiotic ((pH, TOC, texture, NO3-N, NH4-N, SM, HMs (total and bioavailable forms)) and biotic (DHA, 16S rRNA gene copies, bacterial genotype richness and diversity) properties along a heavy metal contamination gradient. The object of our study was the long-term contaminated soils with Cu, Zn and Pb in the region of Zlatitsa-Pirdop Valley, Western Bulgaria. The Zlatitsa-Pirdop Valley is situated in one of the largest and the richest in copper-gold-pyrite deposit area of Europe, currently being developed by the company of "Chelopech Mining" EAD.

Material and Methods

Study area and soil sampling. The studied area is located in the region of Zlatitsa-Pirdop Valley, Western Bulgaria (Fig. 1). Topsoil samples (0-20 cm) were collected in May 2018 along a gradient of Cu contamination (from 53 mg kg^{-1} to 860 mg kg^{-1}) and co-contaminants Zn and Pb in the vicinity of Chelopech (42.6995°N, 24.0847°E) (Chel_1, Chel_4), Chavdar (42.6599° N, 24.0561° E) (Chav_3) and Karlievo (42.6852° N, 24.1059° E) (Karl_5) villages. Chel_1 was chosen as a control (uncontaminated) site, whereas Chel_4 and Karl_5 were high (Cu - 210 mg kg^{-1}) and very high (Cu - 860 mg kg^{-1}) contaminated sites, respectively (Table 1). Five subsamples per site were pooled randomly and used for further analyses.

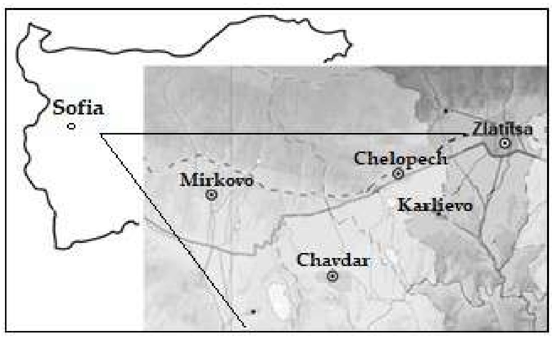

Fig. 1. Region of sampling sites' location (Chavdar, Chelopech and Karlievo villages) in the Zlatitsa – Pirdop valley, Western Bulgaria.

Soil physicochemical properties and heavy metal content. Soil pH was measured in 0.1 M $CaCl_2$ according to ISO 10390:2005(E). Soil texture was determined by the Kachinsky method (1958). The total organic carbon (TOC) was determined according to Chen et al. (2014). Soil nitrate (NO_3-N) and ammonium (NH_4-N) nitrogen, and inorganic phosphates were determined according to the methods of Keeney & Nelson (1982) and Olsen (1982), respectively. The soil moisture (SM) was calculated by oven dry method (105 °C). The total content of HMs was estimated after decomposition by *aqua regia*, whereas that of their bioavailable forms was determined after soil extraction with 0.01 M $CaCl_2$. The average pollution index (API) was calculated to evaluate heavy metal contamination at each site (Hakanson, 1980).

Dehydrogenase activity. We chose dehydrogenase activity (DHA) as a subject of our investigation, because the enzyme is closely related to the soil metabolic activity (Wolińska & Stępniewska, 2012). DHA was assayed by the method of Friedel et al. (1994) based on the reduction of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) to INT-formazan (INT-F). The enzyme activity was calculated in micrograms per gram dry soil per hour.

Bacterial abundance. Bacterial abundance (16S rRNA gene copies) was quantified by real-time quantitative PCR (qPCR) with bacterial domain-specific primer pairs Eub338f (5′-ACTCCTACGGGAGGCAGCAG-3′)/Eub518r (5′-ATTACCGCGGCTGCTGG-3′). Q-PCR reactions were set up using iTaqTM Universal SYBR®Green Supermix (BioRad) and the real time qPCR conditions were described in Aleksova et al. (2020).

Bacterial genotype richness and diversity. The values of bacterial richness (Chao1) and diversity (Shannon index and Simpson index of dominance) were calculated (Magguran, 2004) based on the restriction fragment length polymorphism (RFLP) data. Metagenomic DNA was extracted using E.Z.N.A soil DNA kit (Omega Bio-tek, USA) according to the manufacturer's instructions. The 16S rDNA clone libraries were constructed for each sample as described in Radeva et al. (2013) to estimate the bacterial genotype diversity. The obtained clones were subjected by restriction fragment length polymorphism (RFLP) analysis. Briefly, the clones were digested with the restriction enzyme *MspI* (FastDigest MspI, Thermo Fisher Scientific) following the manufacturer's instructions, subsequently were separated on a 3% agarose gel electrophoresis and the obtained patterns were grouped. The dominant 16S rDNA sequences were Sanger sequenced (Macrogen, Europe B.V., Amsterdam, the Netherlands).

Data analysis. One-way ANOVA followed by Tukey's test were performed to examine the differences in the values of soil properties (pH, TOC, SM, NO_3-N, NH_4-N, HPO_4), heavy metals, and microbial variables (DHA, 16S rRNA gene copies, Chao1, Shannon index and Simpson index of dominance). Cluster analysis (Algorithm: UPGMA, Similarity index: Bray-Curtis) and Principal component analysis (PCA) were applied to visualize the relationships between soil abiotic and biotic metrics. Statistical analyses were performed using SPSS for Windows, version 18.0.

Results and Discussion

Soil physicochemical properties, total content and availability of heavy metals

The values of studied soil variables are shown in Table 1. The soils from all sites were determined as loamy sand textured. Soils were acidic, well abundant of organic carbon, inorganic nitrogen (especially Chel_1 and Chav_3) and inorganic phosphates. The high NO_3-N content in soils of Chel_1 and Chav_3 was associated with their land-use manner for annual crop production, where the soil fertilization is a common practice. The concentration of Cu and Pb were under and close to the maximum permissible concentrations (MPC) according to Bulgarian Regulation 3/2008, whereas in Chel_4 and Karl_5, their concentrations exceeded the guideline limit (Table 1). The concentrations of Zn did not exceed the background values in all samples (Table 1). According to the API, Chel_1 (1.3) showed close to the background heavy metal pollution, and was considered as a control in our study. Chav_3 (1.41) showed medium heavy metal pollution, while Chel-4 (3.82) and Karl_5. (11.36) showed high and very high heavy metal pollution (Hakanson, 1980). Bioavailable forms of each HM were below 1.0% of the respective total concentration, except Zn in Karl_5, where its bioavailable forms were calculated to be 1.83% of the total Zn concentration.

Table 1. Soil physicochemical and microbial parameters. *Legend:* According to Regulation 3/2008, the maximum permissible concentrations of HMs (mg kg^{-1}) in arable soils at < 6.00 pH are: Cu ≤ 80, Zn ≤ 200, and Pb ≤ 60. Cu_b, Zn_b, Pb_b – Bioavailable form of the heavy metal; ND – No data; Different letters per row indicate significant differences between the mean values of the respective soil variable ($p<0.05$).

Parameters	**Sampling sites**			
	Chel_1	**Chav_3**	**Chel_4**	**Karl_5**
Physicochemical				
pH	4.60^{a}	5.30^{b}	4.80^{a}	5.20^{b}
Sand (%)	45.60^{a}	45.00^{a}	50.90^{b}	31.20^{c}
Clay (%)	17.00^{a}	19.20^{a}	18.30^{a}	18.90^{a}
Silt (%)	37.50^{a}	35.80^{a}	30.90^{b}	49.90^{c}
Soil moisture (SM; %)	5.00^{a}	8.60^{b}	4.30^{a}	7.30^{ab}
Total organic carbon (TOC; g kg^{-1})	13.95^{a}	17.44^{a}	24.42^{b}	16.86^{a}
NO_3-N (mg g^{-1})	38.67^{a}	62.41^{b}	2.36^{ce}	8.32^{de}
NH_4-N (mg g^{-1})	1.92^{a}	4.77^{b}	6.13^{bc}	2.60^{ab}
HPO_4 (mg g^{-1})	15.47^{a}	8.91^{b}	13.7^{2ab}	10.90^{ab}
Cu (mg kg^{-1})	53.00^{a}	86.50^{b}	270.00^{c}	860.00^{d}
Zn (mg kg^{-1})	86.50^{a}	42.00^{b}	80.00^{a}	180.00^{c}
Pb (mg kg^{-1})	35.00^{a}	31.56^{a}	67.50^{b}	175.50^{c}
Cu_b (mg kg^{-1})	0.20^{a}	0.20^{a}	0.25^{a}	0.65^{b}
Zn_b (mg kg^{-1})	0.23^{a}	0.25^{a}	0.65^{b}	3.30^{c}
Pb_b (mg kg^{-1})	0.05^{a}	ND	0.25^{b}	0.08^{a}
API	1.30^{a}	1.41^{a}	3.82^{b}	11.36^{c}
Microbial				
Dehydrogenase activity (DHA; μg g^{-1} h^{-1})	83.13^{a}	15.00^{b}	19.11^{c}	17.80^{bc}
Number of 16S rRNA gene copy (x10^{10})	5.38^{a}	2.74^{b}	2.00^{b}	1.82^{b}
Chao1 index of genotype richness (Chao1)	106.50^{a}	87.14^{ab}	75.36^{b}	63.63^{b}
Shannon index of genotype diversity (H')	3.74^{a}	3.43^{b}	3.63^{a}	3.34^{b}
Simpson index of genotype dominance (D)	36.58^{a}	24.50^{b}	34.20^{abc}	24.40^{bc}

Soil microbial properties

The activity of soil dehydrogenase enzymes varied from 83.13 μg g^{-1} h^{-1} in the uncontaminated soil (Chel_1) to 17.8 μg g^{-1} h^{-1} in the most polluted one (Karl_5), indicating a 79% reduction compared to the control (Table 1). Consequently, the dehydrogenase activity tended to decrease along the heavy metal contamination gradient. Many studies reported high sensitivity of DHA to heavy metals and their bioavailable forms. In most of the reported cases, the DHA is very sensitive to high soil concentrations of Cu and Zn (Murata et al., 2005; Wolińska & Stępniewska, 2012; Wiatrowska et al., 2015; Zhang et al., 2008). Resent results were in agreement with our earlier studies, where the rate of inhibition of dehydrogenase activity was up to 88%, and increase with increasing the soil Cu and Zn pollution (Kenarova & Radeva, 2010a; b).

The soils were well abundant of bacteria, whose number varied from 1.82 x 10^{10} (Karl_5) to 5.38 x 10^{10} (Chel_1) 16S rRNA gene copies (Table 1). The bacterial abundance in Karl_5's soil was not dramatically affected by the high HM concentrations, supposing already completed selection followed by a proliferation of HM resistant bacterial species.

The current uncertainties associated with microbial community assessment have, however, given rise to sometimes contrasting observations as to the impact of HM pollution to soil microbial communities. For example, several studies have outlined a lack of microbial community resistance to metal contamination (Gans et al., 2005), while others have demonstrated the opposite trend of extensive HM community resistance (Azarbad et al., 2016). There are also studies reported that HMs affected slightly bacterial abundance, but strongly their diversity (Stan et al., 2011). Our results confirmed the findings of Stan et al. (2011), clearly demonstrating a high bacterial genotype richness (Chao1) and diversity (Shannon and Simpson) in Chel_1, which decreased (significant or insignificant) with increasing the levels of HM contamination in the other sites of interest (Table 1).

Relationships between soil abiotic and microbial properties

Based on the data from Table 1, cluster and principal component analysis (PCA) were conducted to determine the relationships between soil abiotic and microbial properties. The cluster analysis demonstrated the high level of similarity between Chel_1 and Chav_3 (around 78%), both of them forming a cluster of non- to low- polluted arable lands (Fig. 2). The outliers Chel_4 and Karl_5 showed different levels of similarities with the cluster of Chel_1 and Chav_3, expressed by relatively high similarity of Chel_4 (68%) and low similarity of Karl_5 (47%) to the arable lands' environments.

Principal coordination analysis (PCA) was conducted to locate the sites of interest in the ordination space according to their soil physicochemical and microbial properties (Fig. 3). After determining the initial eigenvalues, two principal components were considered, and these components accounted for more than 77.93% of the total variance.

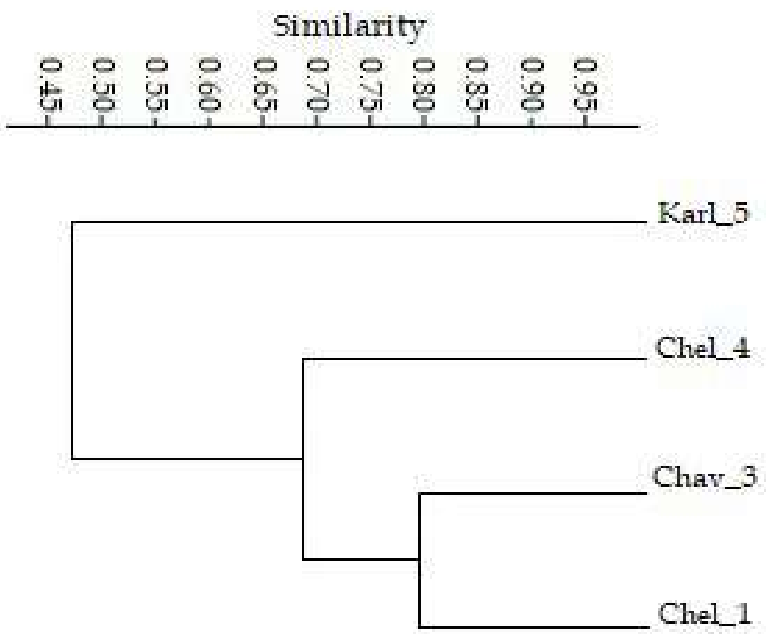

Fig. 2. Cluster dendrogram, representing the similarity among soils based on their physicochemical and microbial properties displayed in Table 1.

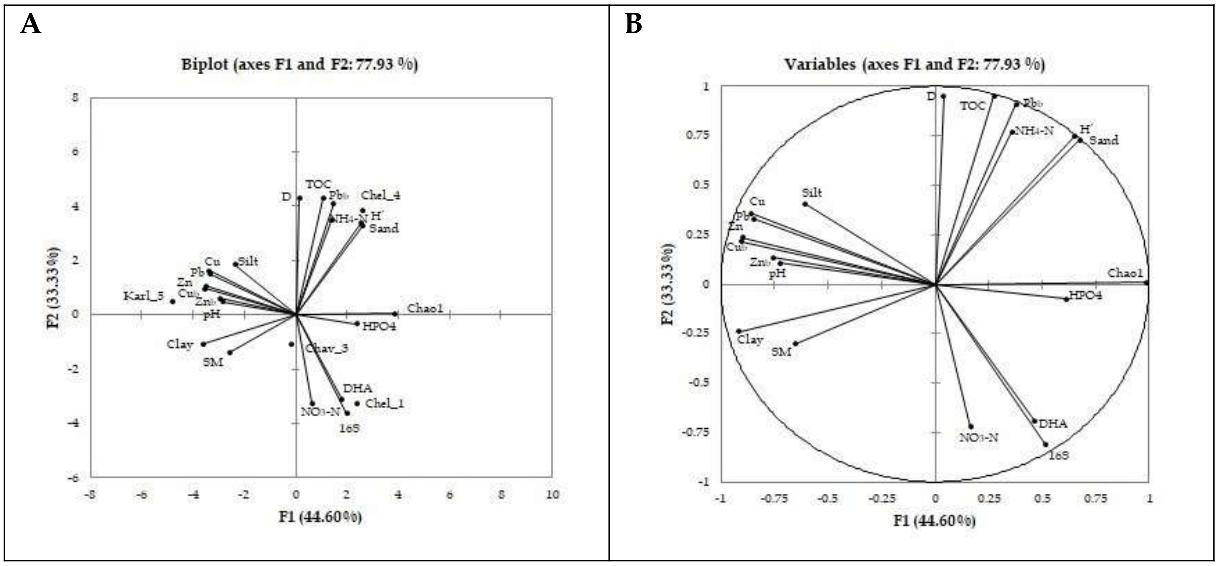

Fig. 3. Principal Component Analysis (PCA). (A) PCA (F1, F2) scores' biplot of sampling sites, based on their soil abiotic and microbial properties, shown in Table 1, and (B) two dimensional (F1, F2) PCA correlation circle, representing the correlation between any two soil variables.

PC ordination confirmed the results of cluster analysis, demonstrating the segregation of Chel_1 and Chav_3 from Chel_4 and Karl_5 along the axis F 1, which loaded 44.6% of the total variability (Fig. 3A). More distinctive for Chel_1 were the high values of dehydrogenase activity, bacterial abundance (16S rRNA gene copies) and genotype richness (Chao1), and soil NO_3-N. Chav_3's trait was strongly linked with edaphic variables such as clay content and soil moisture. Some edaphic (sand, TOC, NH_4-N and bioavailable Pb) and microbial (Shannon and Simpson genotype diversity) properties explained well the features of Chel_4, whereas Karl_5 was well distinguishable from the other sites according to the high content of soil HMs.

PCA analysis showed that DHA correlated significantly and positively with total bacterial abundance (16S rRNA gene copies) and NO_3-N, and positively but insignificantly with the bacterial genotype richness and HPO_4 (Fig. 3B). Significant negative relationships were found between DHA, and soil HMs (except bioavailable Pb), pH and silt (%). The positive correlations of microbial activity, and bacterial abundance and genotype richness supposed a dominant role of bacteria in soil organic matter transformation. Our results confirmed the findings of earlier studies, which revealed a strong significant positive correlation both between soil dehydrogenase and total microorganism count (copiotrophic and oligotrophic bacteria) (Wolinska et al., 2015), and between microbial biomass, and enzyme activities and selected soil properties (Reza et al., 2014). The positive relationships between DHA and soil nutrients (NO_3-N and HPO_4) indicated the nutrient limitation in soils and its crucial effects on soil microbial activity.

DHA correlated negatively with soil total and bioavailable (except Pb) concentrations of HMs. As we pointed above, the negative effects of HMs on microbial activity was evidenced by many authors (Murata et al., 2005; Wolińska & Stępniewska, 2012; Wiatrowska et al., 2015; Zhang et al., 2008), supposing decrease of intensity of many biological processes, including organic matter decomposition and turnover in contaminated soils. Also, the negative was relationship between DHA and soil pH. In general, the enzyme activity tends to increase with the increasing of soil pH (Błońska, 2010; Moeskops et al., 2010), although the investigations of Wolińska & Stępniewska (2012) indicated a case, where the DHA expressed high activity at pH 5.5 - 5.73, which was significantly inhibited at pH above 5.75. All these facts supposed that the studied soils were inhabited by well adapted to the local pH values acidophilic bacteria, whose metabolic activity was adjusted to the low pH values.

The results of the PCA indicated that bacterial genotype diversity strongly and positively correlated with TOC, sand (%), NH_4-N and bioavailable Pb. Chau et al. (2011) concluded that the light sandy soils are characterized by a larger porous space, which provide more microhabitats that microorganisms can colonized. This in turn increases the potential of many bacterial species to coexist in close proximity without competing for nutrients, and can increase bacterial diversity in soils (Chau et al., 2011). Therefore, the vast spaciousness which has loamy sand soils probably increases the diversity of the indigenous bacterial communities.

It is known that TOC is one of the most important factors affecting soil biodiversity (Fierer, 2017). Soil microorganisms act as "gatekeepers" for the exchange of carbon between the soil and the atmosphere, balancing the accumulation and decomposition of soil organic matter (Malik et al., 2018). Some authors showed positive correlation between the soil organic carbon and the abundance of phyla *Proteobacteria*, *Acidobacteria*, *Firmicutes* and *Verrucomicrobia*, applying the high-throughput sequencing (Trivedi et al., 2016). In our investigations, we evidenced also the dominance of *Proteobacteria*, *Actinobacteria*, *Acidobactria* and *Bacteroidetes* (assessed via 16S rRNA gene retrieval) in the soils of Zlatitsa-Pirdop valley, and changes in the structure of bacterial communities along the heavy metal contamination gradient (unpublished data).

In conclusion, the study demonstrated that: (1) long-term heavy metal contamination has a significant negative effect on soil dehydrogenase activity; (2) DHA correlates significantly and positively with total bacterial abundance and negatively with soil HMs; (3) bacterial genotype diversity is influenced mainly by soil organic matter and soil fraction of sand. This study extends our understanding of microbial activity and its relationships with bacterial diversity and environmental parameters along the heavy metals contamination gradient.

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Effect of Mixed Cropping Systems on Yield and Quality of Lettuce (Lactuca sativa L.)

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Abstract. The purpose of this work is to determine the quantitative and qualitative changes in biometric and biochemical parameters of lettuce plants when grown mixed with medicinal and vegetable species. The experiments were carried out in a growing house. The plants, used to create mixed crops, were lettuce (*Lactuca sativa* L.), cutivar "Bohemia", tagetes (*Tagetes* sp. L.), basil (*Ocimum basilicum* L.), calendula (*Calendula officinalis* L.) summer savory (*Satureja hortensis* L.) and arugula (*Eruca sativa* L. Cav.). The results of the analyses showed that the lettuce plants developed together with calendula had a significantly greater leaf and root mass. The calendula plants were 66.5% higher than the average height of the lettuces in the experiment and those developed together with savory and basil respectively 15.3% and 14.5%. The lettuce plants with the best vegetative development also had a high chlorophyll content. The highest value of the indicator Ch a + Ch b was measured in the variant of lettuce grown together with arugula. The lettuce in the containers with arugula had the highest solids content (10.68%) and total sugars (9.8%) in their leaves, while the vitamin C content was highest in the lettuce grown together with calendula (8.57 mg/100g fresh weight).

Key words: mixed farming, *Lactuca sativa*, container experiment.

Introduction

For most of the history of plant growing, the food has been produced from polyculture cropping systems. In nature, the plants always grow in a community, they support each other and complement each other. Combinations of plants prevent the emergence and development of potential pests, as well as increase yields and quality (Sunulahpašić et al., 2017). In their diversity, joint farming systems are close to natural phytocenoses and represent an attempt to create agrocenoses on the principle of differentiation of ecological niches. These complex non-natural systems have not yet been studied in detail. It has been empirically established that joint crops give a significantly higher yield per unit area than in separate cultivation (Poltoretskyi et al., 2019; Prykhodko et al., 2019), without sufficiently clarifying the mechanism of this phenomenon. It is generally accepted that the increased productivity of mixed crops is probably due to the differences between the components, in the way they use the growth factors of the habitat and in their competition

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for them. The relationships between the components of joint crops affect different aspects of plant life activity. The positive interaction between crops in joint crops is largely determined by the different timing and different activity of nutrient, uptake by their root system. Particularly favourable are those plants in which the minimum and maximum absorption of the elements of mineral nutrition are in different stages of the growing season (Kannan, 2010; Manolov & Manolova, 2013). To be biologically useful, mixed components must be carefully selected. The scientific principles for the selection of components for growing crops at the same place and the same time suitable for intensive farming conditions are not well developed. In this regard, research on interspecific and intraspecific relationships in the agrocenosis is essential, as individual crops at different stages of their ontogenetic development differ from each other in their needs for environmental factors - light, temperature, water, elements of mineral nutrition and other. The production of lettuce aims to increase yields and improve the quality of products that are environmentally friendly. The share of organically grown leafy vegetables is also increasing compared to conventional products. The study of the relationship between growing together cultivated species at different stages of their existence helps to find appropriate technologies for the creation and management of mixed crops, ensuring the sustainability of ecosystems and the potential of the respective habitat. Currently, there are numerous examples proving the higher efficiency of heterogeneous agrocenoses in intensive management conditions (Bevz & Toshkina, 2020; Zhulanova et al.; 2020). The joint cultivation of vegetables is also an old practice in Bulgarian horticulture.

The aim of the present work was to establish the quantitative and qualitative changes of biometric and biochemical parameters in lettuce plants in its joint cultivation with medicinal and vegetable species.

Materials and Methods

Characteristics of soil substrates and plant material

The experiment was planted in the second half of April in a growing house under controlled conditions, using a lettuce (*Lactuca sativa* L.) cultivar "Bohemia". The plants tagetes (*Tagretes* sp. L.), basil (*Ocimum basilicum* L.), calendula (*Calendula officinalis* L.), summer savory (*Satureja hortensis* L.) and arugula (*Eruca sativa* (L.) Cav.) were used to create mixed crops. The experiment was embedded in 3 L containers and contained 8 variants with 3 replicates. The soil was an alluvial-meadow soil (Fluvisol), suitable for growing leafy vegetables. The starting soil was poorly humus and was characterized by a neutral reaction, a very low content of mineral nitrogen and very good supply with mobile forms of phosphorus and potassium (Table 1). The used manure was bovine, well decomposed, meeting the phytosanitary and biological requirements of the crop (Shaban et al., 2014). The soil to manure weight ratio in the experiment is 5:1. The study also included a compost substrate variant (Rindstrup Group) with 28% organic "C" and a balanced macronutrient content.

The following variants were tested: 1. control variant: soil with lettuce plants (SL); 2. control variant: soil + manure with lettuce plants (SML); 3. soil + manure with lettuce plants + arugula (SML+ A); 4. soil + manure with lettuce plants + calendula (SML + C); 5. soil manure with lettuce plants + tagetes (SML + T); 6. soil + manure with lettuce plants + savory (SML + H); 7. soil + manure with lettuce plants + basil (SML + B); 8. compost substrate with lettuce plants (CSL). In the test containers, 3 lettuce plants and 3 tagetes, calendula, savory, basil or arugula plants were planted. In variants-1, 2 and 8 containing only soil, soil + manure and substrate, only lettuce plants are planted.

Growth and phenological observations

The studied vegetative parameters and biometric measurements of lettuce plants were performed: plant height (cm), root weight (g/plant), leaf mass (g/plant), total plant mass (g/plant), number of leaves (number) and stem diameter (cm).

Analysis of soil and plant samples

The soil samples from all variants of the experiment were taken and tested before setting the experiment and at the end of the growing season. The following indicators were reported - pH_{H2O}, pH_{KCl}, mobile forms of N, P, K, Ca, Mg. The hydrogen index (pH) was determined potentiometrically in H_2O and KCl (Arinushkina, 1962); the mineral N content was measured by the Bremner and Keeney method (Bremner & Keeney, 1965); the content of K_2O or P_2O_5 was determined in lactate extract (DPS-AL) (Ivanov, 1984); the soil organic C (humus) was determined by the Turin method (Vorobyova, 1998). The plant samples were analysed for N, P, K, Ca and Mg during the growing season and at the end of the active growing season. The content of macronutrients in plants was determined by the following methods: the content of K_2O and P_2O_5 spectrophotometrically by the method of Milcheva & Brashnarova (1975) and total N according to Kjeldahl (Horneck & Miller, 1998). The content of plastid pigments (chlorophyll-a, chlorophyll-b) was determined in fresh plant samples by the method of Zelenskiy & Mogileva (1980). The leaf dry matter content of lettuce was determined by drying at 60 ° C for 3 hours and then at 105 ° C to constant weight (Tomov et al., 1999); the content of vitamin C in lettuce leaves was determined reflectometrically by a method based on the reduction of phosphomolybdic acid to phosphomolybdenum blue complex.

After averaging and straining the samples, the resulting juice was diluted with oxalic acid and filtered. To the filtrate thus obtained was added polyvinylpolypyrrolidone (Divergan® RS) at pH >1. After mixing, the sample was filtered and the Vitamin C content was read on an RQflex® reflectometer. The sugar content of the lettuce leaves was determined using an RQflex reflectometer and test strips.

Statistical analysis

Statistical processing of the results obtained was prepared with the statistical product Statgraphics-18 (2017). Analysis of variance (ANOVA) was used to determine the influence of the test parameters. The mean values were compared by a Tukey test.

Results

Table. 2 shows the values of the studied biometric indicators of lettuce plants in different variants.

The lettuce plants were well developed after planting them into the pots with arugula and tagetes. The average survival of the lettuce plants picked in the experiment was very good - 2.54 pieces/pot. The lowest number of 1.67 lettuce plants was reported in the pots with the substrate variant. There are no statistically proven differences in the number of lettuce plants between the experimental variants.

The tallest were the plants in the variant with calendula – 21.33 cm, followed by those developed together with savory (14.77 cm) and basil (14.67 cm). At an average height of 12.81 cm for the experimental plants, the lettuce of the calendula variant were 66.5% higher, and those developed in the vicinity of savory and basil were 15.3 resp. 14.5%. The differences in heights were statistically proven between variant 4 (SML + C) and variants 6 (SML + S) and 7 (SML + B), while between the variants with savory and basil the differences in the heights of the lettuce plants were not significant. Except for the control variants 1 (SL), the variant 3 with arugula were with the lowest lettuce plants (9.67 cm). There were statistically proven differences in plant heights between lettuce plants in the control variant and all other variants except variant 3 with arugula.

At the expense of the lower height, the lettuce plants grown together with arugula were the most leafy (13.07 leaves/plant) or 25.6% more formed leaves than the average number of leaves for the experiment. A large number of leaves were formed by lettuce plants in pots with calendula (17.5% more than the average number for all experimental variants).

Table 1. Content of nutrients in soil, manure and compost before the experiment.

Substrate	pH		NH_4-N+NO_3-N	P_2O_5	K_2O	Ca	Mg	Humus
	H_2O	KCI	(mg/кg)	mg /100g				%
Soil	6.7	5.9	18.9	24.7	37.4	-	-	1.72
Manure	8.0	7.2	107.7	365.2	482.9	-	-	44.02
Compost	5.5	-	98.5	110	192	-	10	-

Table 2. Growth parameters of lettuce plants.

Variant	Number of plants per pot	Plant height/ cm /	Number of leaves per plant	Total plant mass (g/plant)	Leaf mass (g/plant)	Root weight (g/plant)
1.SL	2.33	8.30	8.97	6.65	5.46	1.19
2.SML	2.67	12.53	9.47	10.80	9.66	1.13
3.SML+A	3.0	9.67	13.07	13.06	11.24	1.82
4.SML+C	2.66	21.33	12.23	14.18	11.22	2.96
5.SML+T	3.00	10.97	10.27	7.80	6.84	0.95
6.SML+S	2.67	14.77	9.53	9.63	8.33	1.30
7.SML+B	2.33	14.67	9.23	6.41	5.78	0.63
8.CSL	1.67	10.27	10.50	10.05	8.71	1.34
Mean	**2.54**	**12.81**	**10.41**	**9.82**	**8.41**	**1.42**
F- ratio	2.26	23.96	5.34	13.69	7.10	10.83
P- value	0.0336	0.0000	0.0027	0.0000	0.0006	0.0000
LSD ≤ 95	0.865	2537	1.933	2.299	2.540	0.648
LSD ≤ 99	-	3.495	2.664	3.168	3.499	0.893

The lettuce leaves were similar in number in the variants with savory and basil, as well as in the containers of variant 2 (soil + manure). The differences in the number of leaves were significant between variant 3 (SML+A) and the variants with tagetes, savory and basil. The total average weight of plants in the study was 9.82 g/plant. The variant with calendula, which formed the highest plants, also had the largest total measured mass (leaves+root) 14.18 g or with 44.4% heavier plants compared to the average total mass of lettuce plants in the experiment. In pots grown with arugula, lettuces have large masses of 13.06 g (33% more than the average total weight), regardless of the small height, thanks to the large number of developed leaves. The lettuces in pots with co-growing arugula weighed 13.06 g (33% more than the average total weight) despite the small height, thanks to the large number of developed leaves. The lowest weight was of the lettuces from the version with basil 6.41 g (34.7% less than the average total weight for the experiment). The total weight of these plants was lower than that of lettuces (6.65 g) in the control version without manure. There were no proven differences between the variants, in which the lettuce plants is grown together with arugula and calendula. The differences (LSD ≤ 99) between the marigold variant and the peat, tagetes, savory and basil variants were statistically very well represented. The plants developed together with arugula (11.24 g/plant) and calendula (11.22 g/plant) had the highest leaf mass, significantly exceeding that in the other variants. With an average above-ground mass for the experiment of 8.41 g/ plant, the excess in variants 3 (SML+ A) and 4 (SML + C) was 33.7 and 33.4%. The leaf mass of the lettuces from the other mixed cultivation variants was between 5.78 and 8.71 g/plant. The differences between the variant with arugula and those with tagetes and savory were with a high degree of evidence (LSD ≤ 99), as well as between the pots of lettuces grown together with calendula and those with tagetes and savory. In accordance with the well-developed above-ground part, the lettuces of the variant grown together with calendula also had the best developed root system of 2.96 g/plant. The measured root mass of these plants was more than twice the average (1.42 g/plant) for the experiment. The lettuce plants from pots with basil had the least developed roots 0.63 g/plant. There were very strong differences (LSD ≤ 99) in the root masses of lettuces between the pots with calendula and the variants with tagetes, savory and basil.

The plastid pigments are involved in photosynthesis and play a role in plant growth and development (Table. 3). In the study, the chlorophyll content (Ch a + Ch b) varied between 6.943 and 10.425 mg%. The highest value of the indicator Ch a + Ch b was measured in the variant of lettuce grown together with arugula.

The values of the quality parameters of the lettuce production are presented in table (Table 4). In the experimental variants, the dry matter in the lettuces varied from 6.53 to 0.68%. The high dry matter content of the plants grown with arugula corresponded to both the high plastid content and the reported high yields of vegetative and root mass in this variant. The dry matter content of plants is genetically determined. Vegetable crops are characterized by a relatively low dry matter content, but their characteristic feature is their high content of vitamins (Stancheva et al., 2004). The content of ascorbic acid in the lettuce leaves, with the exception of the control variant (soil with lettuce plants, SL), varied in a narrow range from 6.26 to 8.57 mg/100g. The lettuce leaves grown next to calendula had the highest vitamin C content. The low vitamin content in the plants of the control variant (SL) corresponds to their weak vegetative development.

The nitrate content in the experimental variants ranged between 183.6 and 858.6 mg per 1000g of fresh mass and was significantly lower than the permissible contents (Stancheva et al., 2004; European Commission (2006). The high content of nitrates in lettuce leaves grown in containers with arugula corresponds to low yields and disturbed plant development. No nitrites were detected in the rest of the production, with the exception of lettuce plants grown alone on soil + manure in which were measured 8.77 NO_2 mg per 1000g of fresh mass. This probably has a direct or indirect connection with the mixed cultivation of crops.

The content of total nitrogen in the vegetable mass of lettuce varied between 0.86% and 1.45 % (Table 5). The high nitrogen content in lettuce pants of control variant (soil + manure with lettuce plants SML) is probably due to the lack of competing species, while the relatively high levels of total nitrogen in arugula and calendula variants could be explained by optimal nutrition conditions and consequently good vegetative development of lettuce plants. Despite the good mineral nitrogen supply of the substrate of variant 8 (compost substrate with lettuce plants, CSL), the lettuce plants had the lowest nitrogen content. The measured phosphorus contents in lettuces were between 0.89% and 0.46%. Apart from the high values of absorbed phosphorus in the plants in the variants without concomitant culture, high levels of absorbed phosphorus were present in lettuce plants grown together with arugula and calendula. In the plants of the variant with basil, where the reported vegetative mass was the lowest, there were obviously problems in the absorption of phosphorus as well as potassium.

Table 3. Content of plastid pigments. Legend: different letters = stat. difference; p<0.05; Tukey test.

Variant	Ch a [mg%]	Ch b [mg%]	Ch a+ Ch b	Ch a/Ch b	C car. [mg%]
1.SL	5.940bc	2.478a	8.418c	2.40b	2.157b
2.SML	6.042c	2.547a	8.589c	2.37b	2.182b
3.SML+A	6.744c	3.681b	10.425f	1.83a	2.155b
4.SML+C	6.308c	2.714a	9.022d	2.32b	2.183b
5.SML+T	4.802a	2.318a	7.120b	2.07ab	1.787a
6.SML+S	5.429b	2.324a	7.753b	2.34b	1.941a
7.SML+B	5.198b	2.789a	7.987b	1.86a	1.841a
8.CSL	4.826a	2.117a	6.943a	2.28b	1.886a

Table 4. Quality parameters in lettuce cultivar "Bohemia". Legend: different letters = stat. difference; p<0.05; Tukey test.

Variant	Dry matter (%)	total sugars (%)	Ascorbic acid mg/100 g f. m.	NO_3 mg/ 1000 g f. m.	NO_2 mg/ 1000g f.m.
1.SL	8.24b	6.2b	5.11a	397.8b	0.00
2.SML	6.53a	5.0a	6.26b	788.5f	8.77
3.SML+A	10.68d	9.8f	7.43a	539.7c	0.00
4.SML+C	8.50b	6.3b	8.57a	724.3f	0.00
5.SML+T	9.13c	9.8f	8.449a	692.9d	0.00
6.SML+S	7.72ba	5.0a	7.90a	657.2d	0.00
7.SML+B	8.40b	7.1c	7.02a	858.6e	0.00
8.CSL	8.20b	8.1d	6.83b	183.6a	0.00

Table 5. Nutrient content in 30-day-old lettuce plants. Legend: different letters = stat. difference; p<0.05; Tukey test.

Variant	Total N (%)	P (%)	K (%)	Ca (%)	Mg (%)
1.SL	1.17b	0.89c	6.3b	1.46c	0.35a
2.SML	1.45c	0.88c	6.2b	1.00b	0.38a
3.SML+A	1.24c	0.84c	6.3b	1.33c	0.37a
4.SML+C	1.13b	0.72b	6.4b	1.20bc	0.33a
5.SML+T	0.98b	0.67b	6.0b	1.02b	0.30a
6.SML+S	0.99b	0.70b	6.1b	0.95b	0.28a
7.SML+B	0.98b	0.46a	5.3a	0.72a	0.24a
8.CSL	0.86a	0.65b	6.1b	1.04b	0.25a

Table 6. Content of nutrients in the soil after the end of the experiment. Legend: different letters = stat. difference; p<0.05; Tukey test.

Variant	pH		NH_4-N+NO_3N	P_2O_5	K_2O	Ca	Mg	Hummus
	H_2O	KCI	(mg/κg)	mg/100 g				%
1.SL	6.9a	6.0a	16.1a	15.5b	33.3a	290b	42.5a	1.67a
2.SML	7.3a	6.5a	20.7b	99.0a	164.0f	365c	48.0a	4.43c
3.SML+A	7.3a	6.6a	16.7a	103.1a	138.4c	363c	58.0b	3.37b
4.SML+C	7.3a	6.6a	17.7a	103.8a	123.0b	368c	60.0c	3.78b
5.SML+T	7.2a	6.6a	20.2b	102.6a	135.0c	360c	61.0c	4.00c
6.SML+S	7.2a	6.5a	23.6b	103.3a	132.7c	343c	55.0b	3.54b
7.SML+B	7.2a	6.4a	23.6b	90.6a	129.5b	360c	59.0bc	3.45b
8.CSL	6.6a	5.7a	66.2c	17.1b	38.4a	175a	42.0a	41.37d

The soil analysis after harvesting the lettuce plants and the end of the experiment showed no differences in the soil reaction of the control variant: soil + manure with lettuce plants (SML), i. e. the crops associated with lettuce did not have a different effect on the soil reaction (Table 5). With the exception of a variant 8 (compost substrate with lettuce plants CSL), in which the mineral nitrogen content was already higher in the initial samples, in all other variants, the NH_4-N + NO_3-N content at the end of the study was very low regardless of the addition of manure. In the variants with arugula or calendula, the residual nitrogen contents in the soil were lower, probably due to the higher nitrogen export with the vegetative mass. The high residual levels after the completion of the experiment of mobile forms of phosphorus, potassium, calcium and magnesium in the soil of the manure variants apparently did not have a differentiated effect on the studied indicators (Table 6).

Discussion

The results indicate that the lettuce plants developed together with arugula and calendula were with the statistically proven highest rates of leaf and root mass of the plants from the variant with calendula were 66.5% taller, and those developed together savory and basil were 15.3% resp. 14.5% higher, than the average height in the experiment.

The lettuce plants that had the best vegetative development also had a high chlorophyll content. The highest value of the indicator Ch"a"+Ch"b" was measured in the variant of lettuce grown together with arugula. The obtained contents of plastid pigments in the performed study were higher than the values obtained in our other studies (Dinev & Mitova, 2011; Mitova & Marinova, 2012; Mitova et al., 2017) under similar controlled conditions (container experiment). The probable cause for this may be the cultivar characteristics, as well as favourable meteorological conditions in April and May. By comparing the data with those obtained from the biometric analysis, it can be seen that the plants (arugula, calendula) that have the best vegetative development also have a high chlorophyll content. Despite the small number of plants (1.67) in the containers with compost substrate with lettuce plants (variant 8, CSL), which implies better conditions for the development of lettuce plants, as well as the balanced content of macro-and micronutrients, the chlorophyll content of lettuces in this variant is the lowest. In this case, this low content of plastid pigments is probably related to the mixed culture. It is known that in conditions of mixed crop, significant changes in the chlorophyll content of maize leaves were found under the influence of grown together annual legumes, which had a significant impact on the intensity of photosynthesis and productivity of maize (Stancheva, 2011). According to some authors (Pochinok, 1976) the normal ratio of Ch a/Ch b should be 3:1. Berova et al. (2007) consider that the ratio between chlorophylls is in the range of 2-3:1, but it is not constant, but depends on a number of factors. In the present study, it was found that the ratios of Ch a/Ch b reported when harvesting the plants were close to those indicated in the literature as optimal. Only in the variants of mixed cultivation of lettuce with arugula and basil were the values of this ratio lower.

The results highlight that the lettuce plants in the pots with arugula had the highest dry matter content (10.68%) and total sugars (9.8%) in their leaves, while the vitamin C content was highest in lettuce leaves grown together calendula. The study confirmed the data obtained in other works with vegetable crops, namely that mixed cropping favours the synthesis of ascorbic acid (Wierzbicka & Majkowska-Gadomska 2012). The total sugars in the experimental variants ranged between 5.0 and 9.8%. The plants in the arugula and tagetes pots had the most total sugars in their leaves. The values obtained in the study for total sugars were higher than those in similar experiments with lettuce (Dinev & Mitova, 2011; Mitova & Marinova, 2012) and comparable with those obtained by Mitova et al. (2017) in experiments with fertilizing of lettuces with under increasing nitrogen fertilization norms.

The nitrogen contents of the plants obtained in the study are lower than those cited in the literature (Mitova & Marinova, 2012), which is probably due to both cultivar characteristics and the fact that the initial soil has a very low content of mineral nitrogen, and nitrogen from manure is more difficult to digest.

The amounts of potassium absorbed by lettuce plants were completely comparable with those indicated by other authors (Mitova & Marinova, 2012) and correlate with the values of phosphorus absorbed by plants in all variants. The variants in which lettuces were grown together with arugula (6.3%) and calendula (6.4%) also had high potassium content. The amounts of calcium and magnesium absorbed by the plants were comparable to calcium and significantly lower than those cited in the literature for magnesium. Absorption disturbances were observed in both elements in the basil variant. The variants with arugula and calendula were again with the greatest intake of calcium and magnesium in the plants.

The fact that lettuce plants in the compost substrate variant, which has a neutral soil reaction and a more balanced ratio of nutrients compared to the soil and manure variants, did not form high yields and quality indicators, could be explained with a favourable influence of the growth of mixed crop plants. The introduction into the crop of components with different rates of leaf formation and duration of the vegetation period significantly changes the photosynthetic potential of the agrophytocenoses. It, in turn, affects the growth of biomass and the net productivity of photosynthesis.

Our results are in line with information obtained by other authors (Ijoyah, 2012; Maseko et al., 2018), who prove that the effect of mixed intercropping of production and yield potential and quality in vegetables and leafy vegetables. In the study carried out by Guvenc & Yildirim (2006), cabbage was used as a main crop, and cos lettuce (*Lactuca sativa* L. var. *longifoila*), leaf lettuce (*Lactuca sativa* L. var. *crispa*) were used as intercrops under field conditions. The production was increased significantly when cabbage was intercropped with cos lettuce, leaf lettuce. This cropping systems did not significantly affect nitrogen, phosphorus, potassium, calcium, magnesium and iron content of cabbage.

The results obtained in this study pointed out that intercropping systems increase total yield, productivity and quality of the lettuce plants. The results obtained could be used as a basis of a wider study to determine the effectiveness and sustainability of the lettuce production under mixed cropping system.

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Stress Induced Damages in Psammophilic Bivalve Species: A Pilot Study on Wedge Clams from Black Sea Habitats in the Upper Subtidal Zone (Bulgaria)

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Abstract. The aim of the present pilot study was to estimate the presence of oxidative damages in wedge clams (*Donax trunculus* L.) from different sites in the upper subtidal zone along the Bulgarian Black Sea Coast. In response to this aim the levels of the oxidative stress biomarkers lipid peroxidation, protein oxidation and glutathione, together with the activity of the antioxidant enzymes superoxide dismutase, catalase and glutathione-S-transferase were measured. The obtained results indicated significant variations of the studied biomarkers, depending both on the sampling site and the specific marker. Increased concentrations of lipid peroxidation and protein oxidation, along with glutathione decrease, indicated the presence of stress and tissue damages in the wedge clams. For most of the studied sites a moderate level of oxidative stress in wedge clams was found. Significant oxidative stress was present in the wedge clams from Kabakum Bay. Here, highest values of lipid peroxidation, superoxide dismutase and catalase, along with low glutathione, which was probably depleted by the action of glutathione-S-transferase, were found and this clearly indicated the presence of environmental stress and cell damage. The analysis of samples taken from different seasons, allowed some preliminary conclusions which suggested that the oxidative stress in the wedge clams seemed to be higher in spring, somewhat lower in summer and then somewhat rising in autumn. In conclusion, *D. trunculus* is a suitable bio-indicator of the stressfulness of the environment in the sandy habitats of the subtidal Black Sea zone and can thus present an "early-warning" signal in biomonitoring studies.

Key words: Black Sea, *Donax trunculus*, antioxidant enzymes, lipid peroxidation, protein oxidation, glutathione.

Introduction

Human activity exerts increasing pressure on the marine environment through the deposition of a huge variety of xenobiotics, eutrophication, habitat changes, overfishing, introduction of alien species etc., resulting in ecosystem degradation, which in turn negatively affects important marine ecosystem services. This urgently requires research and systematic monitoring using reliable environmental indicators coupled with informative biomarkers to adequately assess the state of marine biodiversity and ecosystems. The use of bivalve marine species in monitoring programs as a tool to assess impacts and resilience of ecosystems is supported by the Water Framework Directive of the EU (EC, 2000).

Bivalves, as filter feeders, have the capacity to accumulate various xenobiotics and to concentrate them in their tissues. It is considered that the analysis of the substances accumulated in their soft tissues and shells brings more information on the effect of environmental pollution than the analysis only of abiotic environmental components (Baumard et al., 1999). In addition to their accumulation capacity, bivalves respond to contaminants through a number of physiological and biochemical reactions, which underline the biomarker concept (Hook et al., 2014).

One of the biomarkers recently most widely used in the study of effects of the aquatic environments on organisms is the oxidative stress (OS). OS occurs when pro-oxidant processes intensify as a result of overproduction of reactive oxygen species (ROS) and/or a decrease in the antioxidant defense, resulting in cell and tissue damage (Sies, 2015). A wide range of chemical pollutants can upset the redox balance and cause OS in aquatic organisms (Belcheva et al., 2015). OS in marine bivalves can be also caused by changes in environmental factors such as temperature, salinity, oxygen saturation etc. (Soldatov et al., 2014). The response of organisms to pro-oxidants is complex and causes a cascade of interrelated cellular responses with possible significant effect on higher levels of ecological organization (Tlili et al., 2013).

In addition, marine bivalves, in particular clams, are beginning to gain increasing commercial and economic importance as food resource. OS changes in bivalve tissues not only impair their taste and nutritional value (Amaral et al., 2018), but can have direct negative effect on human health (Estevez & Luna, 2017).

Studies of shellfish as bioindicators of the state of the Black Sea ecosystems mainly concerned the black mussel (*Mytilus galloprovincialis* Lam.). This studies are related to assessment of the effect of petroleum products on lipid composition (Nechev et al., 2002), comparisons of antioxidant activity between mussels from clean and contaminated habitats (Gorinstein et al., 2003; Moncheva et al., 2004), metal accumulation, oxidative and genetic status (Yakimov et al., 2017, 2018; Ivanov et al., 2019). Regarding the biological responses to pollution and changes in the habitats of clams in the Black Sea area, the available data are limited and concern mainly species of the *Anadara* genus (Gostyukhina & Andreenko, 2020).

The present study aimed to carry out an initial pilot assessment of the presence of oxidative stress damages in the tissues of *Donax trunculus* L. in response to the different environmental conditions of several typical sublittoral sandy habitats along the Bulgarian Black Sea Coast.

Materials and Methods

Specimens: The clams *D. trunculus* (length 23-40 mm) were gathered manually in different seasons (spring, summer, autumn) from their typical natural habitats in the upper subtidal zone along the Bulgarian Black Sea Coast (Fig. 1).

Tissue preparation. The soft tissues of 6-10 mussels for each site were excised and homogenized separately in 100 mM potassium phosphate buffer (pH 7.4). To obtain a post nuclear fraction for determination of lipid peroxidation and glutathione levels, the homogenates were centrifuged at 3000 g for 10 min. A portion of the fraction was re-centrifuged at 12 000 g for 20 min to obtain a post mitochondrial supernatant used for measurement of the enzyme activity. All work was carried out at 4°C.

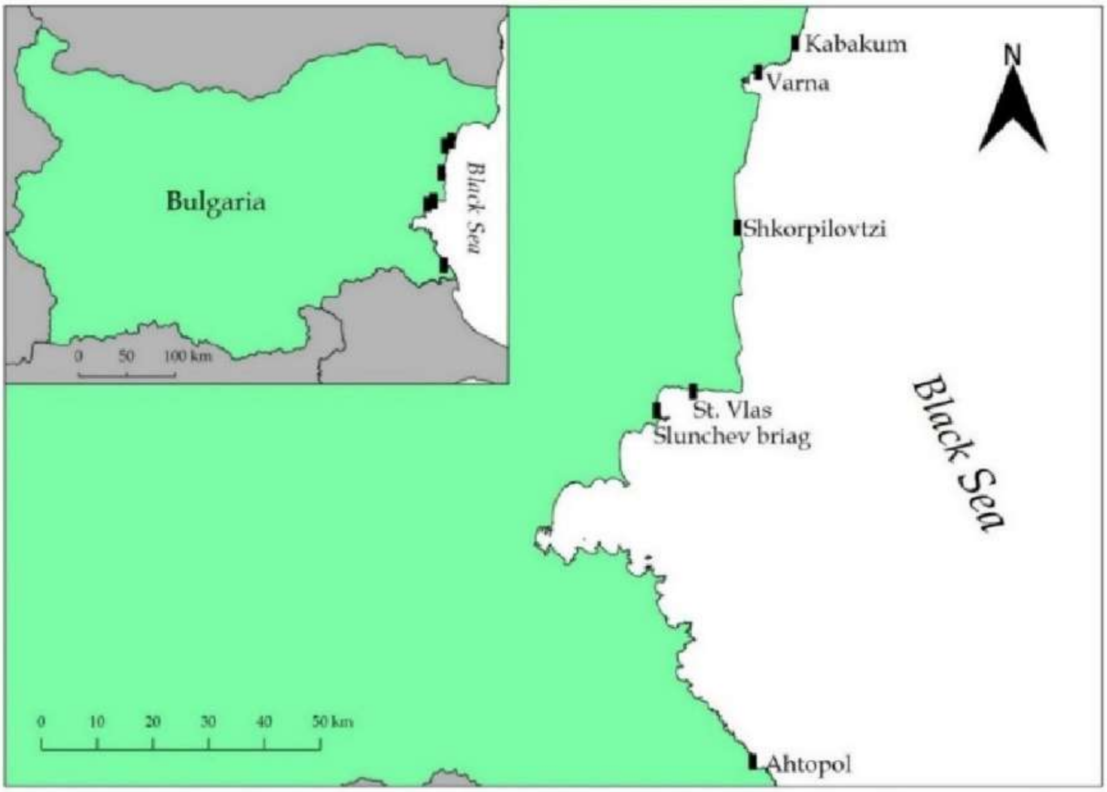

Fig. 1. Wedge clam sampling sites location along the Bulgarian Black Sea Coast.

Biochemical analysis. All biomarkers were measured spectrophotometrically using commercially available kits from Sigma-Aldrich Co. LLC, USA, in accordance to the manufacturer instructions.

Lipid peroxidation (LPO) was estimated by the content of thiobarbituric acid reactive substances (TBARS), by using Lipid peroxidation (MDA) assay kit MAK085. The amount of TBARS was expressed in nmoles malondialdehyde (MDA)/mg protein.

Protein oxidation (PO) was measured by using Protein Carbonyl Content Assay Kit MAK094. The method is based on the reaction of the protein carbonyls (PC) with 2,4-dinitrophenylhydrazine (2,4-DNPH). The carbonyl content was expressed in nmoles PC/mg protein.

Glutathione content (GSH) were measured by the absorption of the color product from reaction of reduced glutathione with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in presence of glutathione reductase and NADPH by using Glutathione Assay Kit CS0260. The amount of glutathione was calculated from the reference standard and expressed as ng/mg protein.

Superoxide dismutase (SOD) activity was determined by using SOD Assay Kit-WST 19160 and was expressed as U/mg protein. As the unit of activity is considered the amount of the enzyme needed for 50 % inhibition of nitroblue tetrazolium (NBT) reduction.

Catalase (CAT) activity was measured by the absorption decrease at 240 nm in result of H_2O_2 decomposition by using Catalase Assay Kit CAT100. Enzyme activity was expressed as U/mg protein.

Glutathione-S-transferase (GST) activity against 1-Chloro-2,4-dinitrobenzene (CDNB) was determined by using Glutathione-S-Transferase Assay Kit, CS0410. Enzyme activity was expressed as U/mg protein.

The protein content was measured according to Lowry et al. (1959) with bovine serum albumin as a standard. The absorbance was measured at 700 nm.

Statistical analyses. Data on the measured oxidative stress markers were subjected to statistical analysis by the Generalized Linear Model Procedure present in the statistical environment of the package STATISTICA 10 (StatSoft Inc., 2007). The factorial ANOVA design was applied. Principle component analysis (PCA) was used to study underlying gradients and grouping of sample sites by oxidative status.

Results

The OS indicators measured in the wedge clams showed significant variations among the studied sites (Table 1). The changes in the pro-oxidants markers LPO and PO indicated the oxidation potential (stressfulness) of the marine environment of the studied wedge clam habitats. In particular, the levels of LPO were lowest (0.36±0.04 nmoles MDA/mg protein) in the specimens from Shkorpilovzti Bay in summer and the highest (1.65±0.36 nmoles MDA/mg protein) were present in the sample from Kabakum Bay in Autumn (Table 1). The levels of PO also varied significantly being the lowest (3.07±0.87 nmoles MDA/mg protein) in clams from Varna Bay in autumn and highest (11.22±1.57 nmoles MDA/mg protein) at Shkorpilovtzi Bay in spring (Table 1).

The measured concentration of the antioxidant GSH was lowest (330.67±63.30 ng/mg protein) in the samples from Slunchev Briag Bay in spring and reached a maximum value (1457.00±184.20 ng/mg protein) in the wedge clams gathered from Ahtopol Bay in summer.

The study of the antioxidant enzyme system biomarkers in the wedge clams also showed that their values varied among the studied sites (Table 1).

The enzyme activity variations among habitats however, seemed to follow a very similar pattern. The lowest activity of the antioxidant enzymes SOD and CAT was present in wedge clams gathered in summer from the sandy sublittoral at Shkorpilovtsi Bay and Ahtopol Bay. The highest activity of these enzymes was registered in wedge clams sampled from Kabakum Bay in autumn. The GST also showed similar variation to the other antioxidant enzymes with lowest values measured in wedge clams gathered from Ahtopol Bay and Shkorpilovtsi Bay in summer and highest activity in the wedge clam samples from Kabakum Bay.

The interrelations between the measured values of the most important biomarkers LPO, PO and GST, indicating the level of OS in the wedge clams from the studied sites are presented in Fig. 2.

The data showed that the wedge clams sampled from Kabakum Bay (in autumn) were the most affected by OS. They had the highest LPO and also showed highly activated antioxidant enzyme defense compared to all others sites (Table 1). Here, the highest activity of GST together with low values of the non-enzyme antioxidant GSH was also present. However, the oxidative damage of the proteins (PO) was relatively low.

In wedge clams from the sites Shkorpilovtzi, Sveti Vlas, Slunchev Briag and Ahtopol (in spring) there was an almost identical and moderate degree of OS stress indicated by the intermediate values of LPO, GSH, SOD, CAT and GST. Similar results were obtained for wedge clams sampled in autumn from the region of Varna Bay, but here somewhat higher SOD activities were registered. In the wedge clams sampled in autumn from both Kabakum Bay and Varna Bay low levels of PO were present. In an attempt to study the significance of the observed variations in the examined OS biomarkers in the wedge clams from the different habitats and the responsible factors we applied factorial ANOVA analysis (Table 2).

The analysis showed that effects of both the site and the particular OS marker separately, as well as their interaction, were statistically significant. These results confirmed that the measured OS markers varied significantly among the studied sites and that the differences significantly depended both on the site (i.e. the local environmental conditions of the habitat) and on the specific interrelation of the OS markers (i.e. how the oxidative process develops in the wedge clams at the particular site).

In our study there were samples taken in different seasons from the studied sites, although not all sites were sampled in each season. Nevertheless, this allowed us to make some very preliminary conclusions on the presence of some seasonal differences. In general, OS in the studied wedge clams seemed to be higher in spring, as indicated by the measured markers, being somewhat lower in summer and then somewhat higher in autumn. This general observation was partly confirmed by the results from the two sites, i.e. Shkorpilovtzi and Ahtopol, where samples were taken both in spring and in summer (Table 1).

In the summer samples from Shkorpilovtzi Bay and Ahtopol Bay the lowest levels of LPO were measured against the background of very high values of the non-enzymatic antioxidant GSH. In the same samples, the antioxidant enzymes were not activated and showed the lowest activities compared to the wedge clams from the other sites, studied in this research. In contrast, in spring, higher values of LPO, SOD and CAT were present in the wedge clams from the same sites (Table 1). The most pronounced difference between the spring samples and the summer samples was the threefold increase in the concentration of the antioxidant GSH in the summer samples of wedge clams. Hence, it can be assumed that in summer the OS of the wedge clams from these sites was lower compared to the OS of the wedge clams in the spring samples from the same sites.

In order to study the overall pattern of the OS changes in the wedge clams from the different sites, which are indicative of tissue damages, a PCA analysis of changes in the values of the pro-oxidant markers LPO and PO was carried out (Fig. 3).

Table 1. Oxidative stress markers (mean ± SD) in wedge clams gathered from different sites and seasons. *Legend: Shk* – Shkorpilovtzi; *Aht* – Achtopol; *Kab* – Kabakum; *Var* –Varna; *Svlas* - Sveti Vlas; Slb – S*lunchev Briag* (*Spr* – Spring; *Sum* – Summer; *Aut* – Autumn).

Site (Season) / OS marker	Shk (Spr)	Slb (Spr)	SVlas (Spr)	Aht (Spr)	Shk (Sum)	Aht (Sum)	Kab (Aut)	Var (Aut)
LPO nmoles MDA/mg protein	0.84 ±0.08	0.89 ±0.12	0.99 ±0.15	0.71 ±0.07	0.36 ±0.04	0.43 ±0.11	1.65 ±0.36	0.89 ±0.11
PO nmoles PC/mg protein	11.22 ±1.57	11.17 ±2.49	9.07 ±1.14	10.40 ±1.65	7.43 ±0.79	8.98 ±1.14	4.72 ±1.58	3.07 ±0.87
GSH ng/mg protein	345.10 ±64.50	330.70 ±63.30	370.80 ±87.10	385.30 ±54.80	1436.80 ±152.90	1457.10 ±184.20	393.10 ±92.00	460.70 ±53.30
SOD U/mg protein	3.02 ±1.34	2.75 ±0.54	5.18 ±1.32	1.99 ±0.71	0.59 ±0.09	0.87 ±0.14	12.54 ±1.84	8.85 ±1.34
CAT U/mg protein	2.56 ±0.40	2.83 ±0.56	2.59 ±0.37	2.53 ±0.25	1.17 ±0.27	1.33 ±0.32	3.16 ±1.32	2.44 ±0.91
GST U/mg protein	126.90 ±22.70	197.30 ±60.60	153.30 ±17.50	119.50 ±15.30	114.90 ±10.30	112.10 ±21.80	303.90 ±31.90	162.10 ±31.60

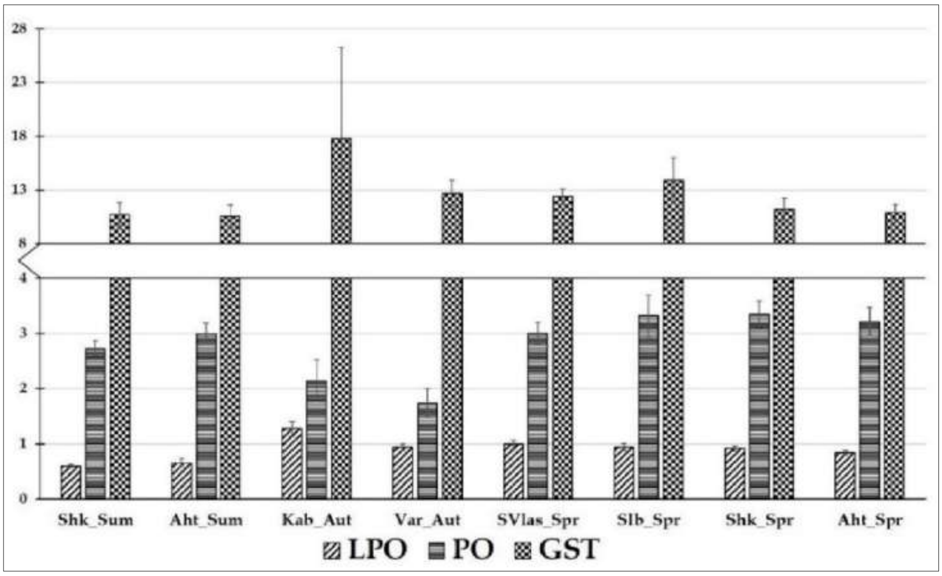

Fig. 2. Mean levels (±SD) of lipid peroxidation (LPO), protein oxidation (PO) and glutathione-s-transferase (GST) activity of *D. trunculus* from different locations (for abbreviations see Table 1). The data was square root transformed (n=6-10).

Table 2. Analysis of variance (ANOVA – factorial design) of the studied oxidative stress biomarkers in wedge clams from different sites.

	SS	DF	MS	F	P
Intercept	5649386	1	5649386	644.81	<0.0001
Site	1522310	7	217473	24.82	<0.001
Biomarker	16555132	5	3311026	377.91	<0.0001
Site*Biomarker	9372483	35	267785	30.56	<0.0001
Error	2304231	263	8761		

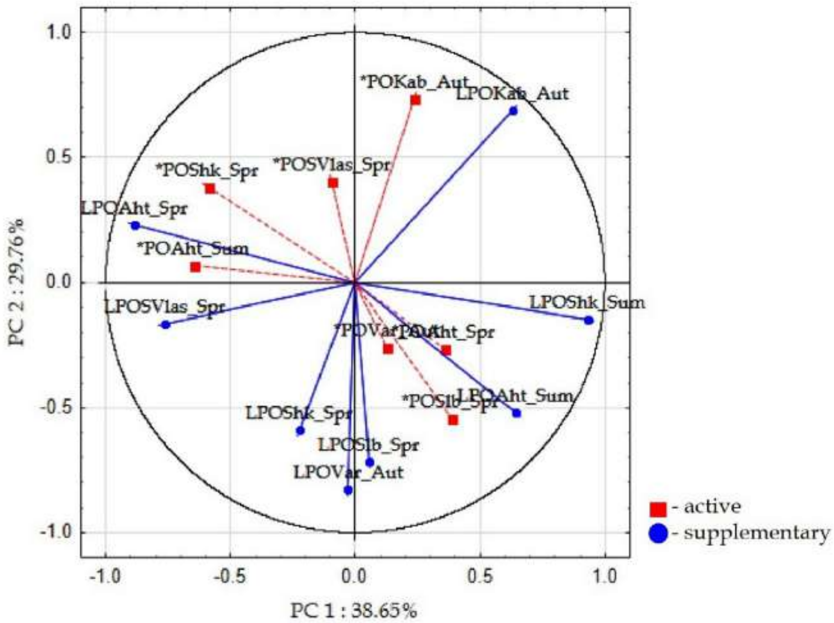

Fig. 3. Ordination graph of PCA analysis of LPO (active variable) and PO (supplementary variable).

The first two principle components (PC) explained 68.41% of the total variation (PC1 - 38.65% of the variation and PC2 -29.76% of the variation). The analysis demonstrated the presence of a gradient along PC1 from sites with relatively high LPO levels in wedge clams together with relatively high PO levels (on the left) towards sites with low LPO levels and relatively high PO levels in the wedge clams (on the right of the diagram). Along the second main PC axis the sites with intermediate LPO and high values of PO in the wedge clams (bottom of figure) are separated from the site with very low PO and with relatively high LPO (top of figure), i.e. the Kabakum Bay.

Discussion

The present paper reports result of the first preliminary comparative study of the level of OS in the wedge clams from several representative sites of the sandy sublittoral habitats along the Bulgarian Black Sea coast.

The wedge clam *Donax trunculus* L. is common in the Black Sea (Petrova & Stoykov, 2010) and is also widely spread in the Mediterranean Sea and the Atlantic coasts of western Europe. The wedge clams are used in monitoring programs for assessment of marine environmental pollution through direct measurement of several OS biomarkers in them (Amira et al., 2011; Sifi et al., 2013). OS is the result of misbalance of the pro-oxidant and antioxidant processes in organisms which is indicated by specific biomarkers. Oxidative processes in marine bivalves can be induced by different environmental pressures on their habitats and can result in OS cell damage. The ratio of the levels of markers of pro-oxidant processes and the antioxidant system activity can indicate the severity of the OS in the wedge clam tissues and hence the potential damages. One of the most sensitive OS markers is considered to be the lipid peroxidation (LPO), especially in bivalves, because of the high content of polyunsaturated fatty acids (Rudneva, 1999). Among the various products resulting from the peroxidation of lipids, the aldehydes (MDA) and hydroxynonenal (HNE) are the most significant and also most studied (Ayala et al., 2014). In the present study, highest rates of MDA were found in the tissues of wedge clams from Kabakum Bay. The oxidative modification of lipids leads to impairment of membrane fluidity and permeability causing damage of cellular metabolism and ultimately disruption of membrane and cell dead (Ayala et al., 2014). Increased LPO in *D. trunculus* has been specifically associated with urban, harbor, agricultural and industrial pollution of sea waters (Amira et al., 2011; Sifi et al., 2013).

Overproduction of ROS in response to environmental stress can lead not only to high LPO in cell membranes, but also to increased protein oxidation (PO). As a marker of PO, protein carbonyls (PC) are widely used, also in aquatic organisms (Merad & Soltani, 2015). Carbonylation of proteins is the most commonly occurring oxidative protein modification. A significant increase in PC has been demonstrated in clams after exposure to metals (Merad et al., 2016) and organic compounds (Xiu et al., 2016). Since the carbonyl groups are chemically stable, the carbonylation is irreversible and unrepairable. Carbonylation of proteins leads to alteration of protein functions, incl. inhibition of enzyme activities or increase of their susceptibility to proteolytic attack. Interestingly, in our study low PC values were found in the samples from Kabakum Bay and Varna Bay. On the other hand, our results showed high level of OS in the clams from Kabakum Bay. It could be assumed that the reduction of PC levels in the wedge clams from this site could have been the result of activation of metabolic pathways to remove the damaged proteins (Grune et al., 2011). In support of this assumption is the reported observation that during the depuration period after Cd intoxication, lower levels of PO were present in *D. trunculus* (Merad et al., 2016).

The induction and development of OS depends not only on the activity of pro-oxidants but also on the strength and activity of the antioxidant cell defense system, which includes enzymatic and non-enzymatic components. A major cellular non-enzymatic antioxidant is accepted to be GSH and its depletion is considered as a marker of OS development. Reduction of this tripeptide has been found in a number of bivalves exposed to pollutants and toxicants (Sifi et al., 2013). The importance of GSH for the antioxidant protection of cells is essential as it is involved not only in the direct scavenging of ROS, but is also a co-substrate of important enzymes. One such enzyme is GST, which, in addition to being involved in the elimination of OS products, is an important enzyme in phase II of xenobiotic detoxification (Wojtal-Frankiewicz et al., 2017). Induction of GST activity in different mussels has been established after exposure to PAHs, PCBs, dioxins (Van der Oost et al., 2003) and metal pollution (Vidal-Liñán et al., 2014).

Our results suggested that the very low GSH content observed in wedge clams from Kabakum Bay was probably related to its depletion upon the action of GST, which in turn showed excessively high activity in these samples. On the other hand, the high GSH levels measured during the summer were probably a prerequisite for the detected low LPO levels. Similar reciprocal relationship between these two OS markers has been often reported (Gostyukhina & Andreenko, 2020). High levels of GSH in different bivalves in summer have been established also in earlier studies (Power & Sheehan, 1996).

The antioxidant enzymes SOD and CAT are also recognized indicators of OS. They are being activated as an adaptive response to toxicants effects, allowing partial or complete overcoming of the OS in a polluted environment. Such effects have been reported in *M. galloprovincialis* (Serafim et al., 2011), *Chamelea gallina* (Rodríguez-Ortega et al., 2002), *Perna perna* (Jourmi et al., 2015) and *D. trunculus* (Amira et al., 2011). However, prolonged exposure to toxicants can lead to inhibition of the antioxidant enzymes due to deterioration in the state of organisms caused by chronic stress (Regoli et al., 2003). High doses of xenobiotics have a similar inhibitory effect (Trevisan et al., 2014). Thus, the antioxidant enzymes exhibit a "bell-shaped" response curve with increasing the dose or the exposure time (hormesis effect) (Marigomez et al., 2013). The presence of such hormesis effects has been reported in *M. galloprovincialis* (Tsangaris et al., 2008) and *Mytilus edulis* (Yaqin et al., 2008). This dose-response phenomenon is recently recognized as the basis of adaptive mechanisms and resistance to environmental stress in organisms.

Conclusions

The results of this study strongly indicated that the wedge clam *D. trunculus* is a suitable biological model which can be used to assess the stressfulness of the marine environment of its habitats and can thus present an "early-warning" signal in ecological biomonitoring.

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Recent Observations on the Size Structure of Donax trunculus Linnaeus, 1758 and Chamelea gallina (Linnaeus, 1758) in the Bulgarian Black Sea as Status Indicators of Commercially Exploited Shellfish under the Marine Strategy Framework Directive (MSFD)

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Abstract. Healthy stock of commercially exploited fish and shellfish are determined by MSFD as one of the marine environmental status descriptors (D3). The clams *Donax trunculus* and possibly *Chamelea gallina* have become commercially exploited shellfish in the Bulgarian Black Sea since 2012. Mixed catches due to habitat range partial overlap and lumped landings statistics create uncertainty about the catch's species composition and ratio but personal communication with clam catchers suggests predominant harvest of *D. trunculus*. Rapidly increasing landings to a maximum of 819 t in 2017 dropped to 506 t as soon as 2019. This study examines the wild population status of *Donax trunculus* in front of Chernomorets Beach (Varna) by investigating the size and weight structure, and the condition index as observed in February 2020. The predominant size class is 22 mm (37% of the sample), as the smallest and the largest observed specimens were 14.69 mm and 38.81 mm respectively. The b-value of the length – weight relationship was 2.82 ($p < 0.0001$), which was indicative of a negative allometric growth. The good status thresholds of the indicators 95th percentile of the Length (L95) and Height (H95) defined under MSFD D3 Criterion 3 were not reached with values calculated at 28.26 mm and 18.00 mm, respectively. The average condition index was 15.5. Overall deterioration of the population status is possibly associated with harvesting pressure. Year-round monthly surveys are planned to study the annual population dynamics with the objective to devise improved indicators and thresholds for better assessment of the population status.

Key words: *Donax trunculus*, commercially exploited species, size structure, length – weight relationship, Bulgarian Black Sea, MSFD.

Introduction

The clams *Donax trunculus* Linnaeus, 1758 and *Chamelea gallina* (Linnaeus, 1758) represent dominant species in the marine benthic habitat type "infralittoral sand" across the world. *D. trunculus* is distributed from Senegal to the northern Atlantic coast of France (Tebble, 1966), the Mediterranean and the Black Sea (Bayed & Guillou, 1985), as well as along the Marmara Sea (Deval, 2009). Whereas *Chamelea gallina* is found from the Lofoten Isles, south to the Iberian Peninsula, the Mediterranean and the Black Sea, along the Atlantic coast of Morocco and to Madeira and the Canary Isles (Marine Species Identification Portal, 2020).

In the Bulgarian Black Sea *D. trunculus* dominates the exposed to wave action upper infralittoral medium and fine sands usually between 1 and 6.5 m (but is also observed from 0.9 to 9 m), where wide fluctuations in abiotic factors are present. This limits the species diversity, but the characteristic species can reach a high density (Todorova, 2017; Todorova et al., 2015). *Donax* physiological and behavioural adaptations, in particular its excellent burrowing, enable the bivalve to maintain its position in the surf zone and avoid the risk of stranding and desiccation (Gaspar et al., 1999; Neuberger-Cywiak et al., 1990).

On the contrary *Ch. gallina* lacks such adaptations and prefers greater depths from 5 to 18 m (rarely till 25+ m), of the infralittoral medium and fine sands and muddy sands in the Bulgarian Black Sea, (Todorova, 2017; Todorova et al., 2015).

Both species have become commercially exploited shellfish in the Bulgarian Black Sea since 2012. Mixed catches due to habitat range partial overlap and lumped landings statistics create uncertainty about the catch's species composition and ratio but personal communication with clam catchers suggests predominant harvest of *D. trunculus*. Rapidly increasing landings to a maximum of 819 t in 2017 dropped to 506 t as soon as 2019.

Healthy stock of commercially exploited species are determined by the EU Marine Strategy Framework Directive – MSFD (EC, 2008) as one of the total 11 marine environmental status descriptors (D3), according to which: "Populations of all commercially exploited fish and shellfish are within safe biological limits, exhibiting a population age and size distribution that is indicative of a healthy stock". A key criterion for assessment of the progress towards achieving good environmental status is the population age and size structure to include a high proportion of adults/large individuals and limited adverse effect of exploitation on genetic diversity.

At national level the Bulgarian monitoring program for commercially exploited fish and shellfish established the 95th percentile of the observed species size structure during marine scientific surveys (L95 for the length parameter and H95 for the height respectively) as a quantitative indicator for their environmental status (Todorova, 2017).

This study examines the wild population status of *Donax trunculus* and *Chamelea gallina* in front of Chernomorets and Pasha Dere Beaches (Varna) by investigating the size and weight structure, and the condition index as observed in February 2020. The obtained size structure values are interpreted according to the thresholds in the above-mentioned national monitoring program. Additionally, a comparison between all examined parameters and those reported for other populations at different geographical locations is made.

Material and Methods

Sampling methods

In February 2020 around 200 specimens of *D.trunculus and Ch.gallina* were sampled by dredging with a fishing dredge. Its metal frame was 80 cm wide, 60 cm long with mouth of 18 cm height and 30 cm long teeth spaced by 0.8 cm. The attached net was with 1 cm mesh size. The dredge was operated from boat in front of Chernomorets and Pasha Dere Beaches, located south of Galata Cape, near Varna (Fig. 1). The tows were performed parallel to the shoreline in depths from 2 to 6 m with a duration of 5 minutes each. For each individual, the shell length (maximum distance along the anterior posterior axis) and shell height (maximum distance from the umbo to the ventral margin) were measured to the nearest 0.01 mm accuracy with a digital caliper. The total weight (TW) of each individual, together with shell weight (SW), tissue wet weight (TWW) and tissue dry weight (TDW), obtained after oven drying at 60°C for 48 hours were determined by a digital balance with a precision of 0.0001 g.

Statistical analysis

Length–frequency distribution was calculated per 2 mm size-classes. Statistical analyses (95th percentile and regressions) were performed using MS Office-Excel 365 ProPlus® software.

The relationship between length and weight was estimated by regression analysis, using the logarithmic model:

$$\log Y = b. \log X + \log a,$$

which could easily be turned into a power curve type:

$$Y = a.X^b,$$

where Y is the total weight or the tissue dry weight and X is the shell length, a is the intercept and b is the slope.

The regression line was calculated using the least squares method to test the existence of an isometry or allometry between the correlated variables. Comparing the value of the slope of the fitted line with a theoretical value, three cases are possible: b=3 (isometric), b>3 (positive allometry) and b<3 (negative allometry).

The coefficient of determination R^2 together with Significance F and P-value were used as indicators of the quality of the regression.

The condition index (CI) was calculated through two different approaches:

CI = tissue dry weight/shell weight * 100 (Beninger, 1984; Walne & Mann,1975)

CI= [tissue dry weight/(total weight - shell weight)]*100 (Crosby & Gale, 1990)

total weight - shell weight=shell cavity weight.

Results and Discussion

We consider as relevant only the collected data for *D. trunculus*, taking into account that the sample of *Ch. gallina* was taken from a site which was not representative habitat for the species. It turned out to be a difficult task to find a representative place with enough living specimens, even at greater depths. Nevertheless, the estimated values for the 95th percentile of the length and height data distribution, length-weight relationship and condition index were given at the end of this section.

Length-frequency distribution

The predominant size class for *D. trunculus* was 22 mm (37% of the sample), as the smallest and the largest observed specimens were 14.69 mm and 38.81 mm respectively (Fig. 2).

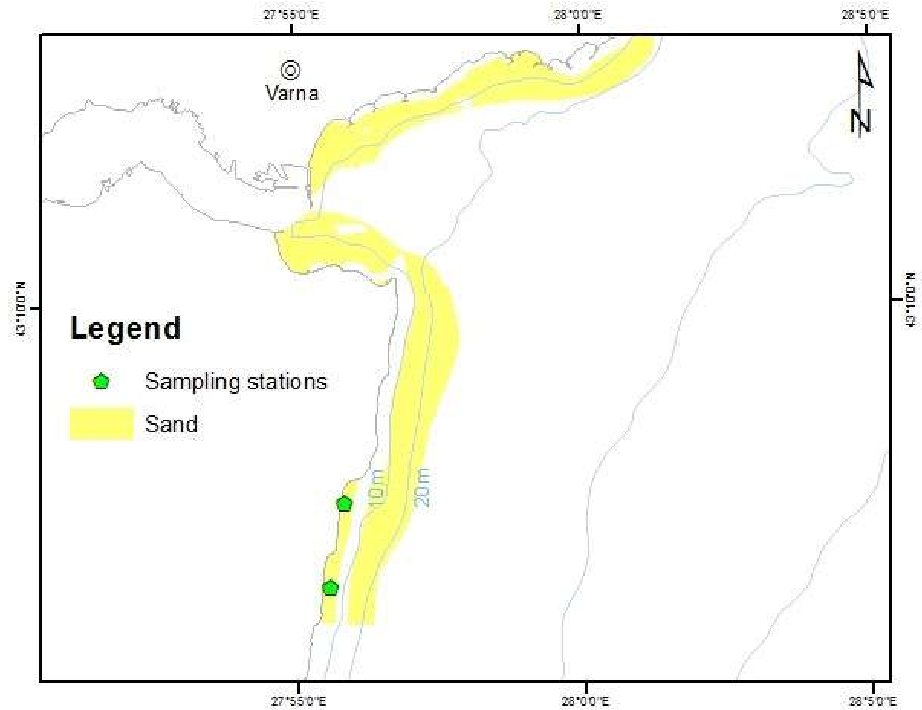

Fig. 1. Indicative map of the sampling area.

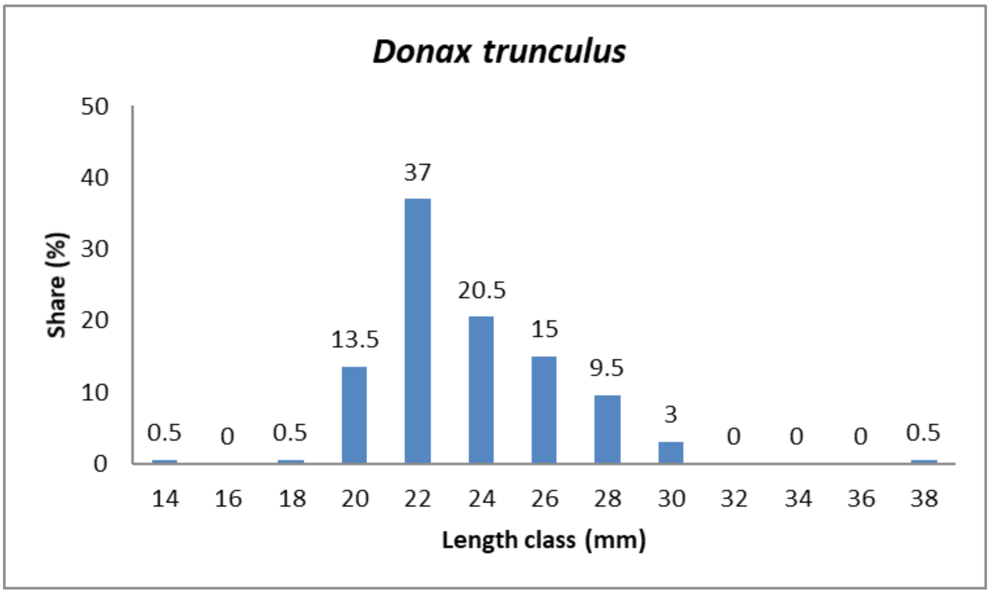

Fig. 2. *Donax trunculus* length-frequency distribution in February 2020.

The minimum and maximum shell length values empirically reported to the whole distribution area ranges from 3 to 45 mm [Northern Atlantic: (Ansell & Lagardère, 1980; Bayed & Guillou, 1985; Gaspar et al., 2002; Mazé & Laborda, 1990;); Mediterranean: (de la Huz et al., 2002; Hafsaoui et al., 2016; Mouëza & Chessel, 1976;); Adriatic: (Zeichen et al., 2002;); Marmara Sea: (Çolakoğlu, 2014; Çolakoğlu & Tokaç, 2011; Deval, 2009); Black Sea: (Aydın et al., 2020).

The minimum shell length values obtained from the present study were higher than those reported by Aydın et al. (2020) for the Southeastern Black Sea coast due to the fact that a different sampling gear was used. The larger mesh size of the fishing dredge that was used did not allow an appropriate interpretation of this parameter, which made the maximum shell length more indicative for the population structure. Its estimation was comparable with the reports from the Mediterranean and Adriatic where the values were between 37 and 40 mm (Mouëza & Chessel, 1976; Zeichen et al., 2002), while larger specimens 42-45 mm were observed from the Atlantic and the Marmara Sea (Çolakoğlu, 2014; Delgado et al., 2017; Deval, 2009). From the Turkish Black Sea coast lower maximum length of 35.5 mm was reported, despite the large number of the sample taken from 0-1.5 m depth (Aydın et al., 2020). This dissimilarity could be caused by the different-sized vertical distribution pattern described by Zeichen et al. (2002) for the Adriatic, where the ecological conditions are relatively closer to those in the Black Sea. Juveniles from the Adriatic population were distributed mainly at least depth and down to 1 m with decreasing densities. Adults were conspicuously found in the deepest bathymetric layers from 0.7 m down to the distribution limit of the species. A possible explanation is the 'sink-source' diffusion model in *D. trunculus*, with the least depths functioning as source and the greatest ones as sinks. This phenomenon could be explained by currents and hydrodynamism passively pushing larvae to colonize the shallower areas on the shore. As they increase in size and in their capacity for active movement, individuals migrate deeper, possibly in order to decrease food competition and population density (Ansell & Lagardère, 1980). Observations from the Thyrrhenian Sea (Voliani et al., 1990) and the Atlantic coast populations (Ansell & Lagardère, 1980) confirmed the increase in size with depth trend and it has been explained as due to the intraspecific competition between juveniles and adults.

D. trunculus in the Bulgarian Black Sea is distributed mainly between 1 and 6.5 m (but is also observed from 0.9 to 9+ m) (Todorova, 2017; Todorova et al., 2015). In the present study the species was sampled from 2.5 m depth.

The shell length of 38.8 mm could be to an individual aged from 2 to 4 years (Mazé & Laborda, 1990; Mouëza & Chessel, 1976; Zeichen et al., 2002), but a further investigation on the growth rate in the specific conditions of the Black Sea is needed. Various factors such as temperature, trophic conditions and population density (Ansell et al., 1980; Ansell & Bodoy, 1979; Neuberger-Cywiak et al., 1990) seem to regulate growth processes.

The good status thresholds of the indicators 95th percentile of the Length (L95≥33.78 mm) and Height (H95≥20.91 mm) defined under MSFD D3 Criterion 3 were not reached with values calculated at 28.26 mm and 18.00 mm, respectively.

The majority of the individuals from the sample (95.5%) fell into 5 size classes from 20 to 30 mm shell length. The very low percent of the smaller size classes was due to the selectivity of the sampling gear, which was purposed to a commercial use. Furthermore, the absence of the larger size classes could be indicative for the impact of the intensive fishing pressure on the species in the last years.

Length-weight relationship

The b-values of the length-weight relationships were 2.82 for the total weight and 2.68 for the tissue dry weight, ($p<0.0001$). That was indicative of a negative allometric growth (Fig. 3-a,b).

Differences in allometry allow life-history comparisons between populations from different habitats. Intersite differences depicted in weight–length relationships could be related to differences observed in growth (Tlili et al., 2010) and reproduction between the sites of origin of bivalves.

The estimated values of the slope were slightly lower than that reported by Aydın et al. (2020) for the Southeastern Black Sea population, as similar negative allometry was observed.

Negative allometric growth of *Donax trunculus* was commonly reported across its distribution area (Aydın et al., 2020; Çolakoğlu, 2014; Çolakoğlu & Tokaç, 2011; Deval, 2009; Mazé & Laborda, 1990).

The obtained zero b-value for the shell length-condition index (estimated by both ways) relationship showed that no relationship was found, which indicated that the condition of the individuals was not related to their size (Fig. 3-c,d).

Condition Index

The average condition index estimated by the second method as a ratio between tissue dry weight and shell cavity weight was 15.5. ± 2.5 (SD). Its value calculated using the first method (ratio between tissue dry weight and shell weight) was 9.5±1.5 (SD).

Condition indices are usually regarded as useful measurements of the nutritive status of bivalves (Crosby & Gale, 1990) and are influenced by many factors including food availability, temperature, and most importantly the gametogenic cycle (Boscolo et al., 2003).

A similarity in the patterns of variation of the condition index and the degree of gonadal development was reported. Aydın et al. (2020) observed a steady increase in the condition index after the initiation of the gametogenesis in February. This increasing trend continued until the beginning of spawning. The condition index decreased sharply as the percentage of spawning individuals reached maximum in May (121.3 ± 18.2 SD). During the inactive stage the condition of specimens increased slightly until August (42.8 ± 19.82 SD), when there was a decrease in the index. Since the spawning period showed parallelism with the period of maximum condition index value, it could be assumed that the reproduction season in the Black Sea is between May and August. Such trend was also identified for the adjacent Marmara Sea

(Deval, 2009). Researches conducted in other areas also reported that the reproduction season is between May and August (Gaspar et al., 1999; Zeichen et al., 2002). Therefore *D. trunculus* was categorized as an opportunistic species in which growth and reproductive activities are restricted to the favourable environmental conditions of the spring-summer season (Delgado et al., 2013).

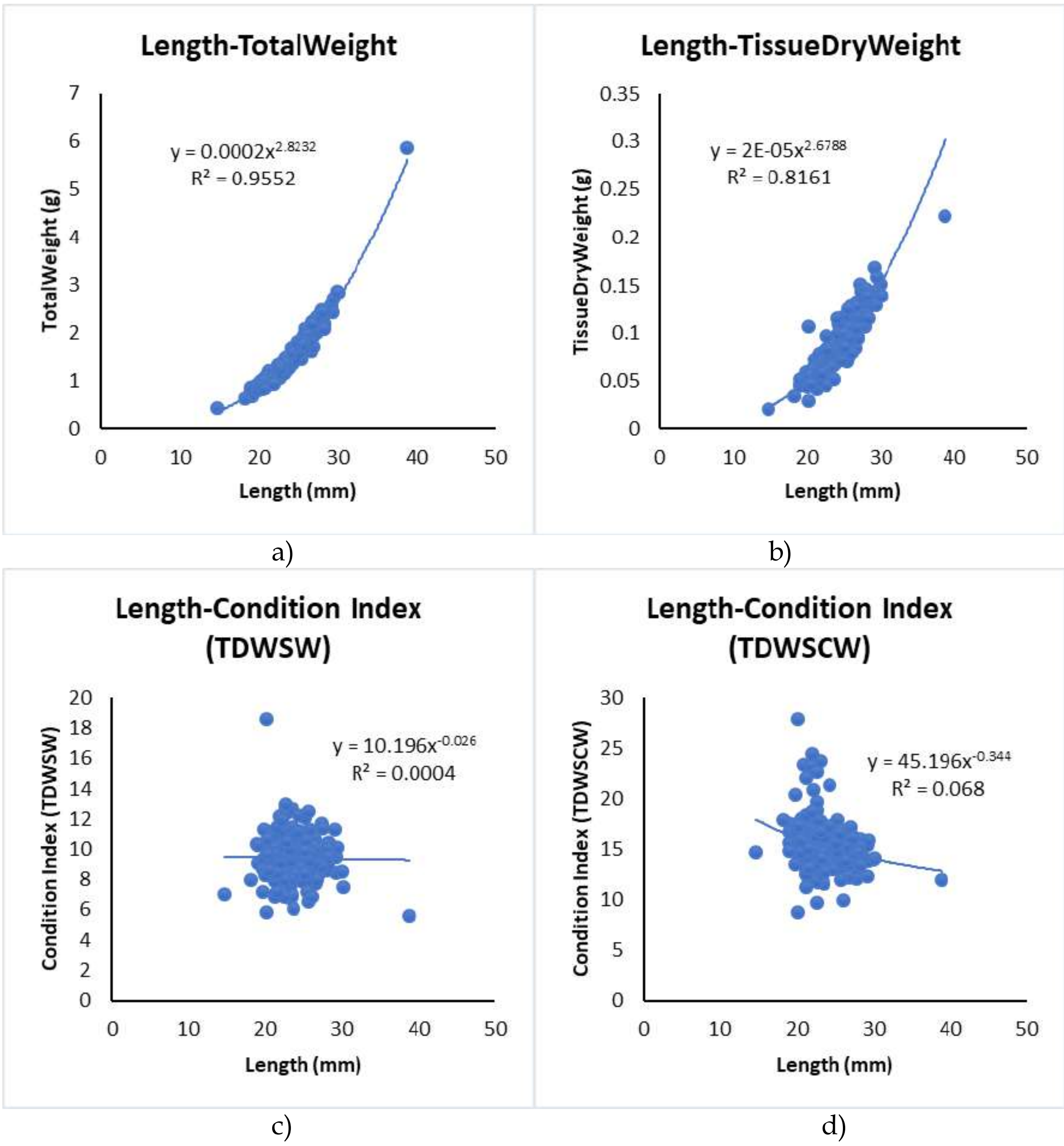

Fig. 3. Estimated relationships of *Donax trunculus* for: a) Length-Total weight; b) Length-Tissue dry weight; c) Length-Condition index (as a ratio between TDWSW-Tissue dry weight and Shell weight); d) Length-Condition index (as a ratio between TDWSCW-Tissue dry weight and Shell cavity weight).

In the present study an analogy could be examined between the estimated by Aydın et al. (2020) values of the condition index for February (average 53.8, min. 11.5 and max 260.0) in Southeastern Black Sea and those from the population in front of

Chernomorets Beach, Western Black Sea. The average condition index value 15.5 obtained from the Western Black Sea was 3.5- fold lower than that from the southeastern part and its value was relatively close to the calculated minimum border. This contrast could be mainly caused by a methodological difference between both studies and precisely the imported value in the formula for tissue weight, which could have accounted for different results. Other factors that influence the condition index of the bivalves are the food availability and temperature. Several authors highlighted the importance of beach morphodynamics, and particularly, the sediment grain-size as main factor controlling the distribution of *D. trunculus* populations (de la Huz et al., 2002; La Valle et al., 2011).

The predominant size class for *Ch. gallina* was 14 mm (38,5% of the sample), as the smallest and the largest observed specimens were 10.47 mm and 19.32 mm respectively. The b-values of the length-weight relationships were 2.62 for the total weight and 3.27 for the tissue dry weight, ($p<0.0001$). That was indicative of a negative allometric growth of the total weight with increasing body length. On the contrary a positive allometric growth of the tissue dry weight with increasing body length was reported. The obtained 0.76 and 0.36 b-values for the shell length-condition index relationship (estimated by both ways)" showed that no relationship was found, which indicated that the condition of the individuals was not related to their size.

The good status thresholds of the indicators 95th percentile of the Length (L95≥23.92) and Height (H95≥22.22) defined under MSFD D3 Criterion 3 were not reached with values calculated at 18.06 mm and 16.53 mm, respectively. The average condition index was 10.3 ± 1.4 (SD).

Conclusions

Overall deterioration of the population status of *Donax trunculus* is possibly associated with harvesting pressure since 2012. Year-round monthly surveys are planned to study the annual population dynamics and biological processes with the objective to devise improved indicators and thresholds for better assessment of the population status.

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Assessment of Marine Cetacean Populations in Bulgarian Black Sea in 2017 According to Indicators of the EU Marine Strategy Framework Directive

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Abstract. Three species - harbour porpoise, common dolphin and bottlenose dolphin - represent the cetacean fauna in the Black Sea and require monitoring and protection by EU member states under the Habitats and Marine Strategy Framework Directives. Assessment of cetacean populations state was based on data, collected during a ship-based visual survey by line transect method, carried out during 24.11.2017 – 22.12.2017 in the whole Bulgarian coastal and shelf areas. Threshold values for population abundance (D1C2) and density (D1C4) indicated in the national monitoring program for Descriptor 1 of MSFD were applied and preliminary data for distributional range (D1C4) are reported. The three cetacean species - *P. phocoena*, *T. truncatus* and *D. delphis* were observed during the study with the total number of 123 sightings and 360 observed individuals. The abundance of Bottlenose dolphin was estimated at 1365 animals, density - 0.113 ind.km^2 and distributional range of 745.81 km^2. The common dolphin was less abundant – 963 individuals and with lower density - 0.0796 ind.km^2, but widely distributed (992.99 km^2). The most abundant was Harbour porpoise with an estimate of 6474 individuals, a density of 0.536 and a range of 2145.09 km^2. The integrated assessment of the status within species and to species group of marine mammals in coastal and shelf areas in 2017 showed that the two of the three species of cetaceans are in „Not good" status and the good environmental status is not achieved by species group.

Key words: cetaceans, abundance, distribution, monitoring, indicators, Bulgarian Black Sea, MSFD, Descriptor D1.

Introduction

The cetacean fauna in the Black Sea is represented by three species – the Black Sea Harbour Porpoise (*Phocoena phocoena*), Short-beaked Common Dolphin (*Delphinus delphis*) and Common Bottlenose Dolphin (*Tursiops truncatus*). All three marine mammal species are listed in Annex IV of the European Habitats Directive (HD 92/43/EEC) and require strict protection by EU member states. Two of the species - Common Bottlenose Dolphin and Harbour Porpoise - are listed in Annex II requiring member states to designate sites of community interests (NATURA 2000) to ensure the conservation of their habitat. The Marine Strategy Framework Directive (Directive 2008/56/EC) establishes the basis of integrated marine management taking into account the state of biological elements and corresponding pressures. In the case of marine mammals, the assessment of

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conservation status of the species is under Descriptor 1 - Biodiversity and shall be based on the information on status and trends of species populations and the main pressures and threats. Good environmental status (GES) is monitored through a list of indicators and reference levels that are suggested at the national level. The indicators of D1 are grouped within five criteria - mortality rate per species from incidental by-catch, population abundance, population demographic characteristics, species distributional range and pattern and habitat for the species. The functional group of marine mammals is very sensitive to human-driven alteration in the marine environment as a result of fisheries, shipping, tourism and other maritime and land-based activities.

The present state of the Black Sea cetacean populations is not certain despite research and conservation measures during the last twenty years. The scientific information is lacking or insufficient about the trends in the population abundance, distributional range and patterns, migrations, critical habitats and anthropogenic and natural threats. The information about the population abundance and distribution of the cetacean species along the Bulgarian Black Sea coast is scarce. Most of the data were derived from stranding and opportunistic sightings during research cruises with other goals rather than cetacean sighting (Nikolov, 1963; Stanev, 1996; Raykov & Panayotova, 2012; Panayotova & Todorova, 2015a; b). Recent research surveys dedicated to estimating the cetacean abundance and distribution were carried out in 2015 in the pilot area enclosed between cape Galata and cape Emine up to 100m depth (Panayotova et al., 2017) and in SCI Strandzha (Popov et.al, 2020). During these surveys, visual or combined visual and acoustic observations were applied. Large scale aerial and ship-based surveys in the Western Black Sea area were carried out in 2013 (Birkun et al., 2014) which provided basic information about cetacean abundance, density and distribution in the Bulgarian Black Sea area.

The current study is aimed to assess for the first time the state of cetacean populations in front of Bulgarian Black Sea coast based on indicators under criteria D1C2 and D1C4 of Descriptor 1 of MSFD. Assessment was made on basis of data, collected during a ship-based visual line transect survey, executed in 2017 under the national monitoring program of MSFD.

Material and Methods

The study area, covered by the monitoring survey encompasses the Bulgarian coastal (up to 30 m) and shelf areas (30-200 m) – Fig.1, including seven marine reporting units (MRU). Data were collected during a ship-based visual survey within the period 24.11.2017 – 22.12.2017 over an area of 12 090 km^2 and the total length of transects of 684 km. Pre-determined track-lines were designed using Distance software following the principles of line transect sampling (Buckland et al., 1993; Thomas et al., 2010). Visual observations were carried out during the daylight hours using the single platform method. For each observation, the following data is recorded: date, time, platform, distance, angle, species, behavior and group size. Data on weather conditions (Beaufort's sea state, reflections and glare, wind direction and force) are also recorded. Observations were not conducted at reduced visibility (below 1000 m) or strong waves (> 4 at Beaufort). The abundance and density were estimated using the DISTANCE 7.0 software package (Thomas et al., 2010). During the observations, a constant vessel' speed of about 6-7 knots was maintained.

Assessment is based on the indicators and thresholds, available for criteria D1C2 and D1C4 and stated in the Monitoring program of Descriptor 1 (Marine mammals) for Bulgaria (bsbd.org), according to the new GES Decision (Commission Decision (EU) 2017/848), as follows:

- D1C2 — Primary: The population abundance of the species is not adversely affected due to anthropogenic pressures, such that its long-term viability is ensured.

Indicator: abundance (number of individuals) per species and MRU

- D1C4 — Primary for species covered by Annexes II, IV or V to Directive 92/43/EEC and secondary for other species: The species distributional range and, where relevant, pattern is in line with prevailing physiographic, geographic and climatic conditions.

Indicators: distributional area by species (GIS layer); Density of distribution (ind.km^{-2}).

Threshold values for the population abundance (D1C2) and density (D1C4) indicated in the national monitoring program for Descriptor 1 of MSFD for the combined coastal and shelf MRU zones, were applied – Table 1.

The final assessment of the state of cetacean' population is done according to the following principles:

- The integration of individual indicators by species and MRUs for each criterion was carried out under the "One Out All Out (OAAO)" rule.
- The integration between criteria for each species - under the "One Out All Out (OAAO)" rule.
- The final assessment for the Descriptor 1 Biodiversity Mammals regarding the group of marine mammals was formed by the percentage of species in "Good" status. The threshold value is 100%.

Results and Discussion

All three cetacean species were observed during the survey - *P. phocoena*, *T. truncatus* and *D. delphis*, but in different numbers and distributional patterns. Totally, 123 sightings were recorded (Table 2), from which 24 of *T. truncatus*, 81 of *P. phocoena* and 18 - of *D. delphis*. The total number of observed individuals amounts to 360.

Population parameters of small cetaceans (abundance, density, group size) were calculated by Distance 7.0 software separately for each species observed. The data matrix includes all observations in coastal and shelf areas combined, due to the low number of sightings by marine reporting unit (MRU). In the case of cetaceans, which are highly mobile, it is reasonable, because they perform long distance movements related to the feeding and wintering (Birkun, 2008). The results of the analysis for the three cetacean species are presented in Table 3.

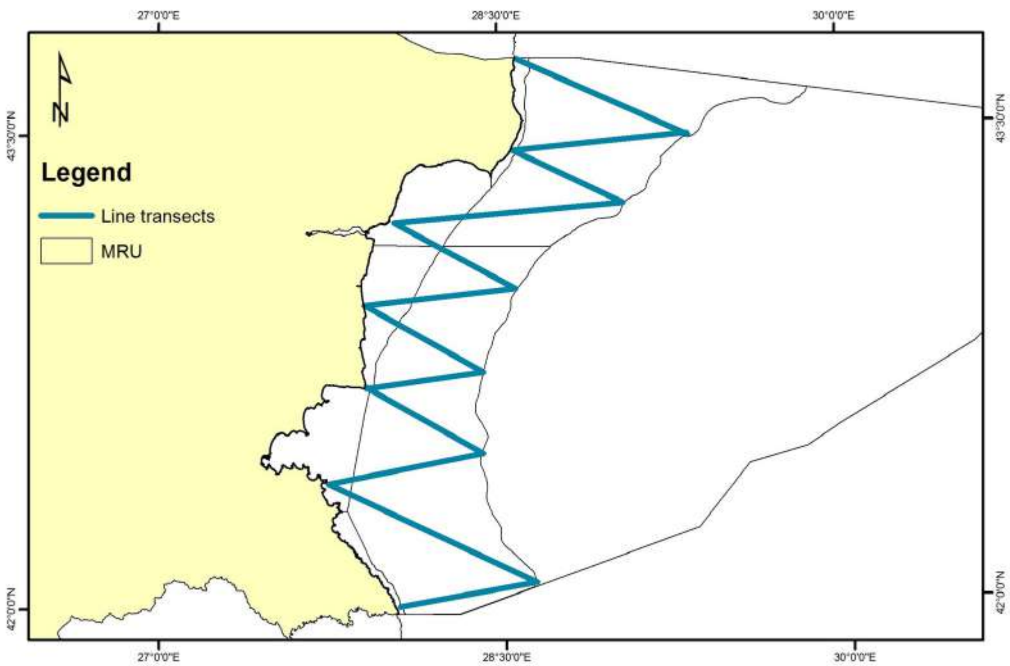

Fig. 1. Map of the study area in the Bulgarian coastal and shelf zones.

Table 1. List of criteria, indicators and thresholds currently applied in Bulgaria (coastal and shelf zone combined).

Feature	Element assessed	Criterion	Indicator	Threshold value	Unit
D1 Biodiversity (Mammals)	*D. delphis*	D1C2	Abundance	5019	Count
		D1C4	Density	0.718	$ind.km^{-2}$
		D1C4	Distributional range	Not yet set	km^2
	T. truncatus	D1C2	Abundance	4861	Count
		D1C4	Density	0.696	$ind.km^{-2}$
		D1C4	Distributional range	Not yet set	km^2
	P. phocoena	D1C2	Abundance	1003	Count
		D1C4	Density	0.144	$ind.km^{-2}$
		D1C4	Distributional range	Not yet set	km^2

Table 2. Number of marine mammals sightings and observed individuals by species and MRUs in 2017.

MRU (area)	Number of sightings	Number of sightings (observed individuals)		
		T. truncatus	*D. delphis*	*P. phocoena*
Sivriburun – Kaliakra (156 km^2)	-	-	-	-
Kaliakra – Galata (825 km^2)	1	-	-	1 (1)
Galata – Emine (699 km^2)	4	-	1 (2)	3 (7)
Emine – Maslen Nos (856 km^2)	8	4 (4)	3 (4)	1 (3)
Maslen Nos – Rezovo (155 km^2)	-	-	-	-
Northern shelf (3878 km^2)	4	1 (2)	-	3 (5)
Southern shelf (5522 km^2)	106	19 (51)	14 (36)	73 (245)
Total	**123**	**24 (57)**	**18 (42)**	**81 (261)**

Table 3. Estimated population parameters of three cetacean species in coastal and shelf areas.

Parameter	Species		
	T. truncatus	*P. phocoena*	*D. delphis*
Area, km^2	12090	12090	12090
Effort (total length of transects, *L*), km	684.176	684.176	684.176
Number of observations (individuals or groups)	24	81	18
Encounter rate (ER; *n/L*)	0.035	0.118	0.026
Coefficient of variation (CV), %	28.67	19.32	26.63
95% Confidence Interval (CI)	0.01979 - 0.0622	0.0809 - 0.1732	0.0154 - 0.0450
Value of pdf* at zero for line transects, *f(0)*	0.0027097	0.0031364	0.003151
Standard error	0.000374	0.0001934	0.000424
Coefficient of variation (CV), %	13.81	6.17	13.45

95% Confidence Interval (CI)	0.002039 - 0.00360	0.00277 - 0.00355	0.002375 - 0.00418
Probability of observing an object in defined area *(p)*	0.53649	0.24962	0.45903
Standard error	0.074078	0.015394	0.061756
Coefficient of variation (CV), %	13.81	6.17	13.45
95% Confidence Interval (CI)	0.40374 - 0.71290	0.22081 - 0.28219	0.34603 - 0.60892
Effective strip width (ESW), m	369.05	318.84	317.36
Standard error	50.957	19.662	42.697
Coefficient of variation (CV), %	13.81	6.17	13.45
95% Confidence Interval (CI)	277.73 - 490.39	282.04 - 360.44	239.24 - 420.99
Estimate of density of clusters/groups *(DS)*	0.0475	0.1857	0.04140
Standard error	0.0151	0.117	0.124
Coefficient of variation (CV), %	31.82	20.28	29.84
95% Confidence Interval (CI)	0.02543 - 0.0888	0.12469 - 0.27643	0.022971 - 0.074794
Estimate of expected value of clusters/group size *(E (S))*	2.1703	2.8843	1.9208
Standard error	0.299	0.2315	0.26117
Coefficient of variation (CV), %		8.03	13.6
95% Confidence Interval (CI)		2.4591 - 3.3831	1.4417 - 2.5591
Average cluster size	2.375	3.2222	2.3333
Standard error	0.3798	0.29502	0.3025
Coefficient of variation (CV), %	15.99	9.16	12.96
95% Confidence Interval (CI)	1.7096 - 3.2993	2.6865 - 3.8648	1.777 - 3.0639
Estimate of density of animals (*D*, individuals/km^2)	0.1129	0.53548	0.0796
Standard error	0.0402	0.117	0.0261
Coefficient of variation (CV), %	35.62	21.81	32.79
95% Confidence Interval (CI)	0.0565 - 0.225	0.34964 - 0.82016	0.041935 - 0.15115
Estimate of the number of animals in the surveyed area (*N*)	1365	6474	963
Standard error	486.16	1411.9	315.75
Coefficient of variation (CV), %	35.62	21.81	32.79
95% Confidence Interval (CI)	684.00 - 2722.0	4227.0 - 9916.0	507 - 1827

Common bottlenose dolphins – *T. truncatus*, (24 sightings, 57 animals) were observed in the Northern and Southern shelf areas, while in the coastal zone – only in Emine – Maslen Nos Region (Table 2). This species was unequally distributed, with the highest number of sightings and the highest relative number of observed groups (0.005 groups.km^{-2}) in the Emine – Maslen Nos area. Most of the observations were recorded in the shelf area at depths below 50 m. Bottlenose dolphins have been observed as single animals (8 observations; 14.04% of the total number of individuals) or groups of two (7 sightings; 24.56% of the total number of individuals), three (7 sightings; 36.84% of total individuals) or more animals (2 sightings; 24.56% of total individuals). The largest observed group consists of 10 animals. The estimated value of the expected group size (E (S)) is 2.17 individuals – Table 3. The estimated values for the density of *T. truncatus* groups (DS) and for single animals (D) were low, respectively 0.0475 groups.km^{-2} and 0.113 ind.km^{-2} (Table 3). Absolute abundance (N) was calculated at 1365 individuals in the study area and the encounter rate (ER) - as 0.0351 ind.km^{-1} (Table 3).

The state of the *T. truncatus* population along the Bulgarian coast was assessed using the indicators and thresholds for criteria D1C2 and D1C4.

• Criterion D1C2: The estimated abundance of 1365 individuals (Table 3) is lower than the threshold (Table 1), and the population is assessed as in "Not Good" state.

• Criterion D1C4: the estimated species density (D) of 0.113 ind.km^{-2} for the whole coastal and shelf areas is lower than the threshold value (Table 1) and the population status is assessed as in "Not Good" state. The distributional range of bottlenose dolphin was estimated at 745.81 km^2.

The data and the final assessment across the indicators and criteria are presented on Fig. 2. The species status was assessed as "Not Good".

Short-beaked common dolphins – *D. delphis*, (18 sightings; 42 animals) were observed in the Southern shelf area and in the coastal zone – in Galata – Emine and Emine – Maslen Nos Regions (Table 2). Species were unequally distributed, with the highest number of sightings and the highest relative number of observed groups (0.004 groups.km^{-2}) in the Emine – Maslen Nos Area. Most of the observations were recorded in the shelf area at depths below 50 m. Common dolphins have been observed as single animals (5 sightings; 11.90% of the total number of individuals), groups of two (6 sightings; 28.57% of the total number of individuals), groups of three (5 sightings; 35.71% of total individuals) or more animals (2 sightings; 23.82% of total individuals). The largest observed group consists of 6 animals. The estimated value of the expected group size (E (S)) is 1.92 animals – Table 3. The estimated values for density of *D. delphis* (DS) groups and for single animals (D) are very low - 0.0414 groups.km^{-2} and 0.0796 ind.km^{-2}. Absolute abundance (N) was calculated at 963 individuals in the study area and the encounter rate (ER) - as 0.0263 ind.km^{-1} (Table 3).

The state of *D. delphis* population along the Bulgarian coast was assessed using the indicators and thresholds for criteria D1C2 and D1C4.

• Criterion D1C2: The estimated abundance of 963 individuals (Table 3) is lower than the threshold (Table 1), and the population was assessed as in "Not Good" state.

• Criterion D1C4: the estimated species density (D) of 0.0796 ind.km^{-2} for the whole coastal and shelf areas is lower than the threshold value (Table 1) and the population status was assessed as in "Not Good" state. The distributional range of Short-beaked common dolphin is estimated at 992.99 km^2.

The data and the final assessment across indicators and criteria are presented on Fig. 2. The species status was assessed as "Not Good".

Harbour porpoises – *P. phocoena*, (81 sightings; 261 animals) were observed in all marine regions except the coastal areas of Sivriburun - Kaliakra and Maslen Nos - Rezovo (Table 2). Individuals were unequally distributed, with the highest number of sightings and the highest relative number of observed groups (0.013 groups.km^{-2}) in the Southern shelf area. Harbour porpoises have been observed as single animals (16 sightings; 6.13% of the total number of individuals), groups of two

(25 sightings; 19.16% of the total number of individuals), groups of three (16 sightings; 18.39% of the total number of individuals) or more animals (24 sightings; 56.32% of the total number of individuals). The largest group observed consists of 20 animals. The estimated value of the expected group size (E (S)) e 2.88 individuals – Table 3. The estimated values for density of *P. phocoena* (DS) groups and for single animals (D) are - 0.186 groups.km^{-2} and 0.536 ind.km^{-2}. Absolute abundance (N) was calculated at 6474 individuals in the study area and the encounter rate (ER) - as 0.118 ind.km^{-1} (Table 3).

The state of *P. phocoena* population along the Bulgarian coast was assessed using the indicators and thresholds for criteria D1C2 and D1C4.

- Criterion D1C2: The estimated abundance of 6474 individuals (Table 3) is above the threshold value (Table 1) and the population is assessed as in "Good" state.
- Criterion D1C4: the estimated species density (D) of 0.536 ind.km^{-2} for the whole coastal and shelf areas is above the threshold value (Table 1) and the population status is assessed as in "Good" state. The distributional range of harbor porpoise is estimated at 2145.09 km^2.

The data and the final assessment across indicators and criteria is presented on Fig. 3. The species status was assessed as "Good".

The final integrated assessment of the functional group of marine mammals by indicators, criteria and at Descriptor 1 level, is summarized on Table 4. The state of the functional group of marine mammals in front of the Bulgarian Black Sea coast in 2017 is assessed as not in GES, because only one of the three species is in "Good" state according to the applied criteria.

According to the results shown in Table 3, the harbor porpoise was the most abundant species in the Bulgarian area. Birkun et al. (2014) reported that in 2013, the common dolphin was the most abundant cetacean species in Bulgarian waters. For the Romanian coast, assessments show that the total number of cetaceans are around 1800 individuals for the period 2001 – 2004 and 1710 individuals for 2013 (Tiganov et al., 2017). According to the results of Birkun et al. (2014), the *P. phocoena* was the most abundant species in the Romanian Black Sea area.

The marine areas inhabited by cetaceans are subject to multipurpose usage from fisheries, tourism and marine transport. All these activities generate a number of threats as habitat degradation, pollution, the introduction of alien species, over-exploitation of fishery resources, but the incidental entanglement in fishing nets is the major source of human-induced mortality of Black Sea cetaceans (Birkun et al., 2014). Profound knowledge of the spatial and temporal distribution of marine mammals is fundamental for the implementation of effective conservation measures and spatial planning of human activities in the conflict areas.

The three species are from regional importance and for the improvement of their population status regional cooperation is essential. All Black Sea coastal countries take measures to protect and conserve cetaceans. Coastal states have ratified commitments protecting biodiversity (e.g., the Convention on Biological Diversity, 1992) and endangered marine species through responsible fishing practices (e.g., the Code of Conduct for Responsible Fisheries, FAO, 1995). All coastal states also have national legislation in place that prohibits killing or injuring cetaceans. EU countries - Bulgaria and Romania, are obliged to take measures to establish a system of strict protection in their natural range, as the Black Sea cetacean species are listed in Annex IV of Directive 92/43/EEC (Habitats Directive). The NATURA 2000 network in Bulgaria currently includes a total marine area of 245 227 ha, designated in 14 marine sites, in which *T. truncatus* and *P. phocoena* are subject of conservation. Until the total regional ban on hunting was introduced in 1983, commercial hunting was the principal anthropogenic threat to the Black Sea cetacean populations.

Table 4. Assessment summary of the status of the functional group of marine mammals under Descriptor D1 - Biodiversity.

Element assessed	Criterion	Indicator	Threshold value	Values achieved	Unit	Criterion status	Status of population (element)	Extent to which GES is achieved
Delphinus delphis	D1C2	Abundance	5019	963	Count	Not good	Not good	Proportion of populations in good status: 33.33% (1 out of 3 populations)
	D1C4	Density	0.718	0.0796	ind.km^{-2}	Not good		
	D1C4	Distributional range	Not yet set	992.99	km^2	Not assessed		
Tursiops truncatus	D1C2	Abundance	4861	1365	Count	Not good	Not good	
	D1C4	Density	0.696	0.113	ind.km^{-2}	Not good		
	D1C4	Distributional range	Not yet set	745.81	km^2	Not assessed		
Phocoena phocoena	D1C2	Abundance	1003	6474	Count	Good	Good	
	D1C4	Density	0.144	0. 536	ind.km^{-2}	Good		
	D1C4	Distributional range	Not yet set	2145.09	km^2	Not assessed		

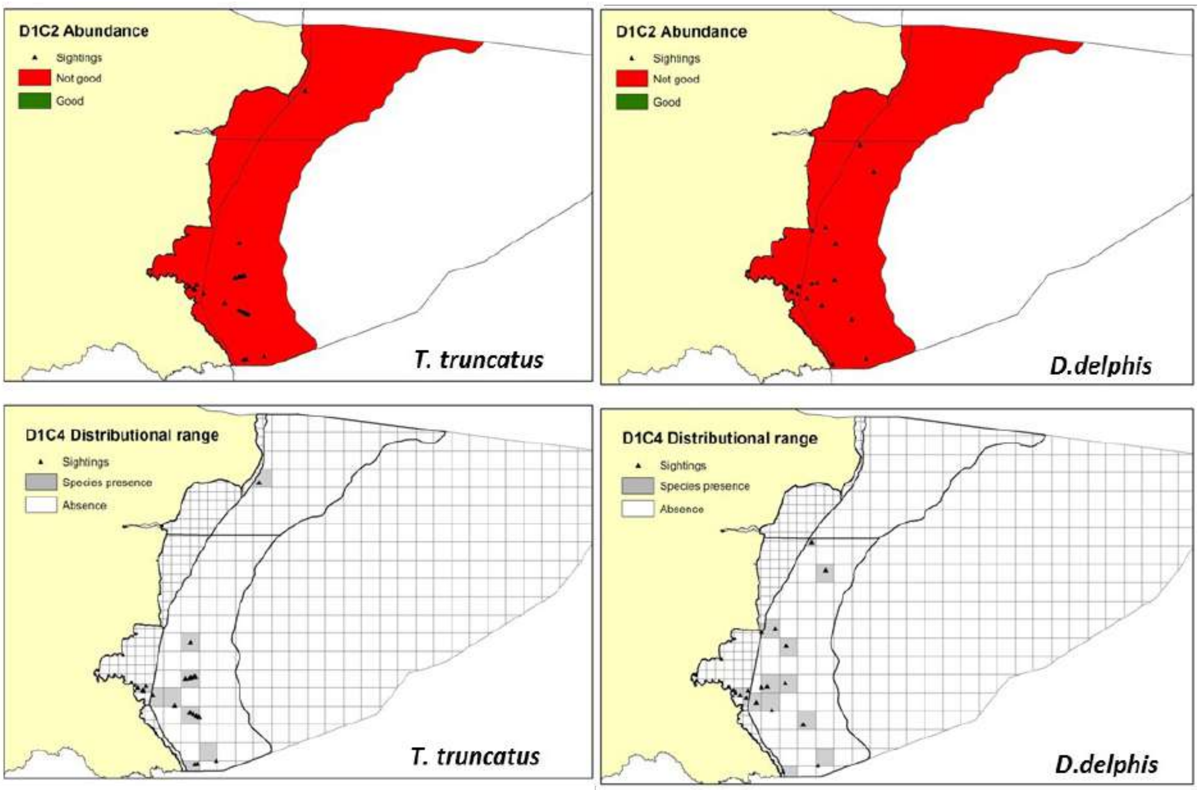

Fig. 2. Final assessments of *T. truncatus* and *D. delphis* in 2017, according to criteria D1C2 and D1C4 of Descriptor 1 Marine mammals.

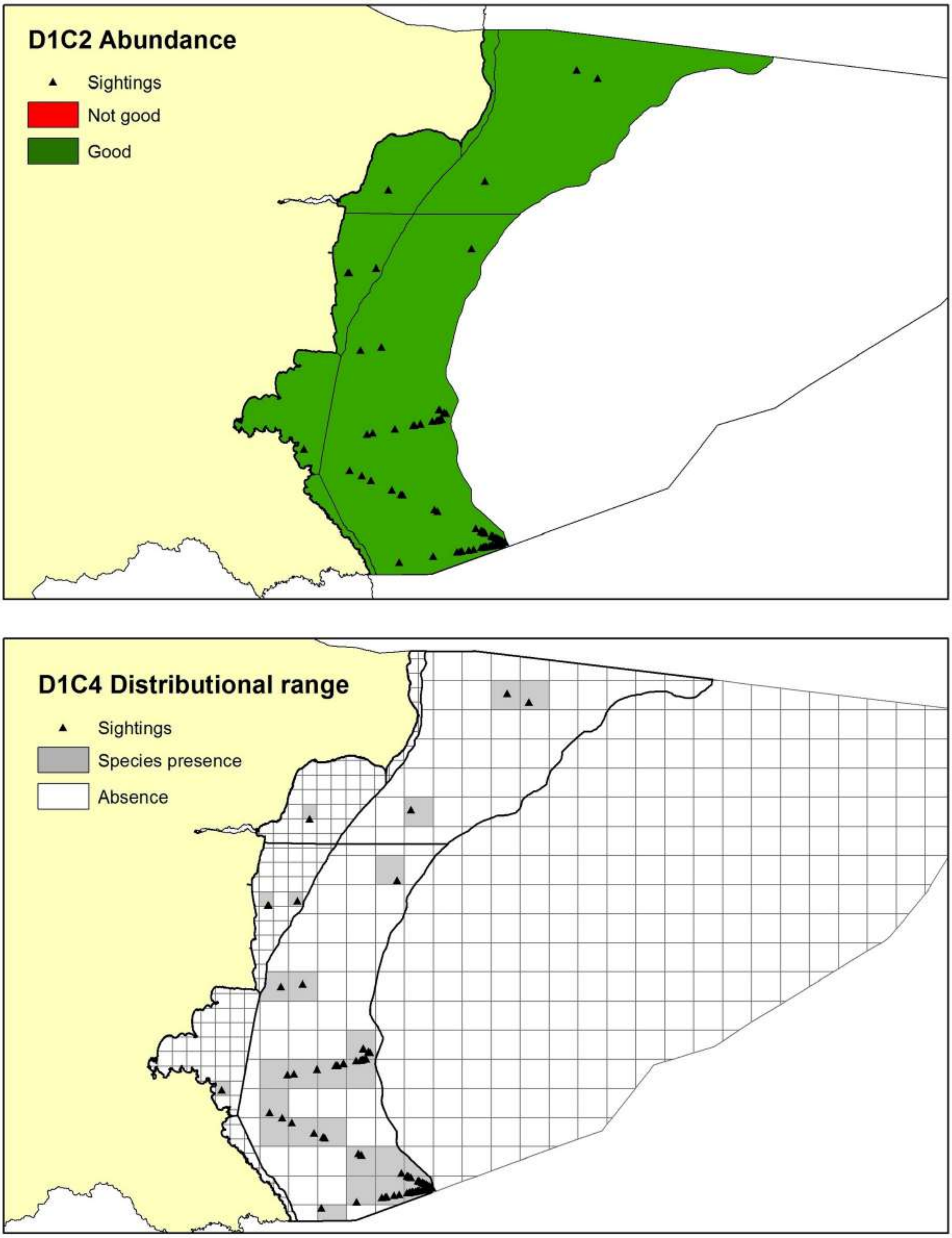

Fig. 3. Final assessment of *P. phocoena* in 2017 according to criteria D1C2 and D1C4 of Descriptor 1 (Marine mammals).

Nowadays, the mortality and non-mortal injuries in fishing gears are the most important threat to marine mammals. Harbour porpoises almost always represented the major part of cetacean by-catches around the Black Sea (Birkun et al., 2014), mainly in passive fishing, predominantly in spring, from March to May. Introduction of mitigation measures for reducing or preventing cetacean by-catch and their conservation in the Black Sea are very important and necessary. For the other side, the regular scientific surveys dedicated to marine mammals research will provide crucial information for updating of indicators and thresholds and success in achieving GES.

Conclusions

Assessment of the status of the functional group of marine mammals according to criteria, indicators and thresholds of Descriptor D1 Biodiversity - Marine Mammals was carried out in 2017. Obtained results revealed that the populations of the Short-beaked Common Dolphin and the Black Sea Bottlenose Dolphin along the Bulgarian coast are in "Not Good" state and the Good Environmental status for the whole group is not achieved, because only the Black Sea Harbour Porpoise is in "Good" status. For improvement of the status of their populations and achieving GES, adequate management of human activities affecting marine mammals and conservation measures are required. Scientific surveys are essential for the monitoring of the populations of marine mammals and for updating of indicators and thresholds, used in assessment.

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Seasonal Composition and Density of Marine Litter on Asparuhovo Beach, Varna, Bulgaria

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Abstract. Marine litter is a growing environmental problem affecting oceans and seas worldwide. Waste created by humans on land or at sea has been discharged into coastal or marine environments. Marine Strategy Framework Directive establishes the basis of integrated marine assessment taking into account the human pressures and their environmental impacts, including marine litter under Descriptor 10. In this study the composition and density of coastline debris were analysed as indicators of marine litter. Two monitoring campaigns for collection and identification of marine litter took place at Asparuhovo Beach (Varna) in 2019, covering the surveyed area of 8814 m^2. In the spring campaign, 3608 items of artificial polymer materials, rubber, textile, paper, processed wood, metal and glass with a total weight of 19.591 kg were collected. Litter density was estimated at 0.41 $items.m^{-2}$ in abundance and at 0.002 $kg.m^{-2}$ in mass. In the autumn survey, the number of collected items decreased to 1461 items and the weight – to 4.189 kg. Compared to spring, the results for beach litter density manifested 2.4 fold decrease in abundance (0.17 $items.m^{-2}$) and 4 fold decrease in mass (0.0005 $kg.m^{-2}$). In both surveys, the artificial polymer materials prevailed in abundance – 87% and 86% respectively. Cigarette buts, plastic/polystyrene pieces, industrial packaging, plastic cups and rings were predominant in marine litter composition. The Clean Coast Index classified the Asparuhovo Beach as "Moderate" beach in spring and as "Clean" in the autumn season.

Key words: marine litter, beach monitoring, Bulgarian Black Sea, MSFD, Descriptor 10.

Introduction

Marine litter is a waste created by humans that have been discharged into coastal or marine environments, resulting from human activities on land or at sea. Marine Strategy Framework Directive – MSFD (EC, 2008) establishes the basis of integrated marine assessment and it is aimed at achieving Good Environmental Status (GES) in the European seas by 2020. The marine environment is assessed based on 11 qualitative descriptors (characteristics) taking into account the state of the marine environment, types of pressures and impacts on it. Marine litter (D10) is one of the Descriptors related to the types of pressures on the marine environment. The MSFD requires the EU Member States to be certain that by 2020, the marine litter available in the coastal and marine environments will do no harm marine life (Directive 2008/56/EC; Commission Decision (EU) 2017/848). The marine debris has been assessed on beaches, sea surface and seabed. Monitoring activities related to coastline litter should evaluate and describe litter pollution and provide data to national assessments of marine debris and support to assess the level of threat to biota and ecosystems.

Numerous studies on monitoring methods for assessing litter in the marine environment have been published over the last decades, but the most comprehensive are those, published by Cheshire et al. (2009) and Galgani et al. (2013a). Studies examine the existing methods for marine litter surveys, monitoring methods and protocols used during the beach surveys. Marine litter can be categorized as plastic, metal, wood, rubber, glass or paper. Of all categories of litter, artificial polymer (plastic) waste is the most damaging. The threat from plastic litter is one of the most dangerous ones since it is insoluble and non-degradable in the marine environment (Zarfl & Matthies, 2010; Cole et al., 2011; Engler, 2012; Galgani et al., 2013b). The impact of plastics on the environment is significant. Some of the marine species swallow small pieces of plastics, which leads to internal damage and even death. Some of the marine species are entangled in ALDFG (Abandoned, Lost or Discarded Fishing Gear). Entanglement kills and injures animals, while ghost nets continue trapping sea life (Brown& Macfadyen, 2007; Kühn et al., 2015; Matsuoka et al., 2005; NOAA Marine Debris Program, 2015; IUCN, 2016). Other environmental impacts are habitat destruction (Chiappone et al., 2005; Yoshikawa & Asoh, 2004), consequences of chemical transport and introduction (Lithner et al., 2011; Mato et al., 2001; Ogata et al., 2009) and spread of invasive species (Kiessling et al., 2015). Persistent plastics, with an estimated lifetime for degradation of hundreds of years in marine conditions, can break up into a micro- and nanoplastics over shorter timescales, thus facilitating their uptake by marine biota throughout the food chain (Urban-Malinga, 2018). There are also economic impacts, social impacts and human health and safety (Lang et al., 2008; Campanale et al., 2020).

Marine litter related information in the Black Sea and along Bulgarian coast remains limited, inconsistent and fragmented (Topcu et al., 2013; Ioakeimidis et al., 2014; Moncheva et al.,2015; Simeonova et al., 2017). Within this context the need for accurate scientific data on marine litter in the Black Sea is evident in order to address marine litter problems effectively, ensuring the sustainable management and use of the marine and coastal environment.

The current study is aimed to conduct two seasonal surveys on Asparuhovo Beach (Varna, Bulgaria) in 2019 to collect and assess the composition and density of coastline marine litter pollution.

Material and Methods

Two monitoring campaigns for collection and identification of marine litter were conducted at Asparuhovo Beach (Varna) in spring (on 17th April 2019) and in autumn (25th October 2019) seasons. The selected Asparuhovo Beach is situated in the suburbs of Varna. The city of Varna is the third biggest town of Bulgaria, numbering 471 252 citizens in 2019 and important touristic city. The local city beach is vast, tranquil and not attended by many tourists – Fig. 1. Human activities on the beach include restaurants, fishing village and port of Varna close to the beach.

For all surveys, the methodology recommended by Galgani et al. (2013a) in Marine Strategy Framework Directive Guidance was applied. The sampling unit represents 100m fixed section, randomly selected on the Asparuhovo Beach, covering the entire area from the water's edge and the area where the sand ends and the asphalted part begins. The total surveyed area was 8814 m^2. The same sampling area was used during the spring and autumn surveys. The monitored section of 100 m length was marked by permanently marked GPS points and by rope for efficient waste monitoring. All artificial objects with size more than 2.5 cm, stranded on the monitoring unit were collected and counted. Collected marine litter was separated and classified according to the main types of materials (plastic, metal, paper/cardboard, glass, rubber, wood, textile, other), placed in the separate disposal bags and weighted by type. The amount per each category was recorded. The collected debris was analyzed and separated to subcategories under laboratory conditions, according to the Master List of Categories of Litter Items, following the Guidance of MSFD Technical Subgroup on Marine Litter. During the laboratory processing, some items with size less than 2.5 cm (>2 cm) were registered and also included here as a result. Observed litter densities were estimated as the number of items per square meter, and in terms of mass - as kilograms per square meter. All data were uploaded and available at Marine Litter Watch Portal.

Beach cleanliness was assessed through the Clean Coast Index (CCI) according to Alkalay et al. (2007). CCI was estimated by formula:

$$CCI = (\text{Total litter on transect}/\text{Total area of transect}) \times K$$

where: K (constant) = 20.

Beach condition by seasons was classified from “Clean” to “Extremely dirty” according to Alkalay et al. (2007) scale – Table 1.

Table 1. Clean Coast Index classification (after Alkalay et al., 2007).

CCI	Very clean	Clean	Moderate	Dirty	Extremely dirty
Value	0 - 2	2 - 5	5 - 10	10 - 20	20+
Definition	No litter is seen.	No litter is seen over a large area.	A few pieces of litter can be detected.	A lot of debris on the shore.	Most of the beach is covered with plastic debris.

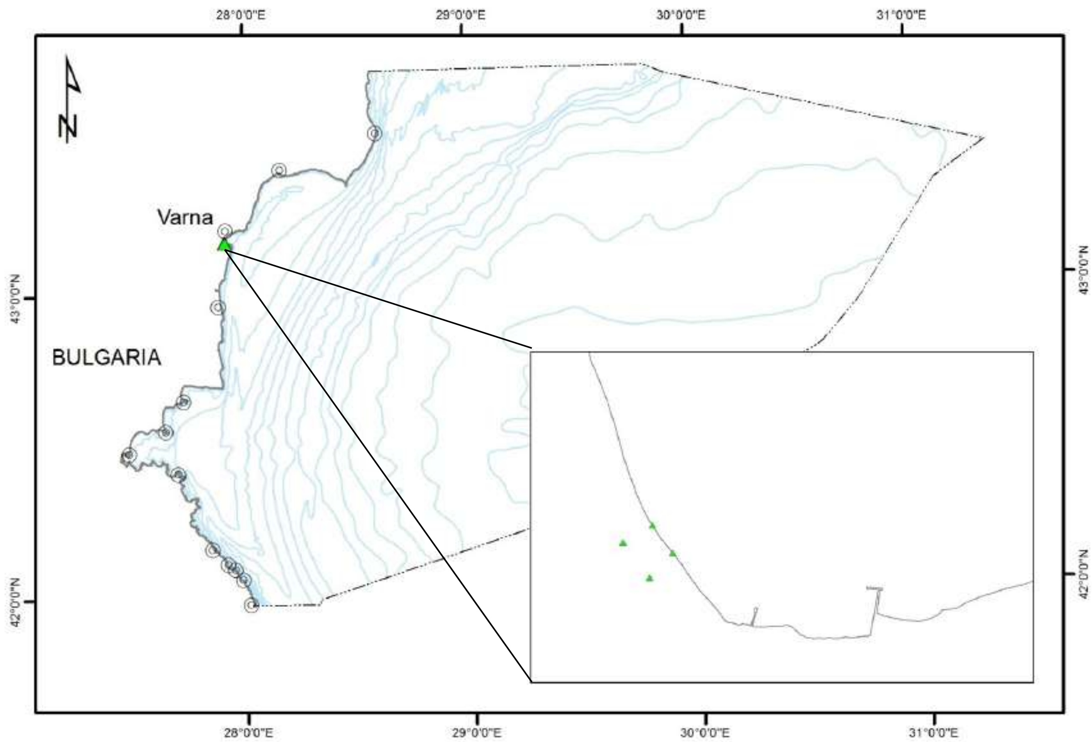

Fig. 1. Sampling area (Asparuhovo Beach) during spring and autumn monitoring campaigns for the collection of marine litter in 2019.

Results and Discussion

The results of the conducted two marine litter monitoring surveys in 2019 on Asparuhovo Beach showed a total number of 5069 items and 23.78 kg collected beach litter. The number of items was highest in spring (3608 items, 0.41 items.m^{-2}) and lower in autumn (1461 items, 0.17 items.m^{-2}). A similar tendency was observed in terms of weight – highest in spring (19.591 kg, 0.002 kg.m^{-2}) and lower in autumn (4.189 kg, 0.0005 kg.m^{-2}).

Collected marine litter was classified into 7 categories and 102 subcategories. The most abundant type of debris during both seasons was category "Artificial polymer materials" with shares of 87% (spring) and 86% (autumn) of the total number (Fig. 2), followed by paper/cardboard (5%, 3%), glass/ceramics (3%, 4%) and processed/worked wood (2%, 3%). In terms of weight, plastics prevail in spring survey (47%), but in autumn season – the "Glass/ceramics" category overcome in the total weight, representing 39% of total weight, followed by wood (29%), artificial polymer materials (28%) and metal (2%) - Fig.2. The shares of the rest of the categories were around 1% or less.

Artificial polymer materials prevailed with 58 subcategories in spring and 29 subcategories in autumn. In April, most of the inventories were plastic pieces, packets and cigarette buts – Fig. 3. In October, the most abundant were cigarette buts, plastic/polystyrene pieces 2.5 cm, industrial packaging and plastic cups and rings (Fig. 3).

„Paper/Cardboard" was the second abundant category, presented by 5 subcategories, constant in April and October. Inventories include cardboard, newspapers, tetra pack, cigarette packets, paper fragments and other paper items – Fig. 4. „Glass/ceramics" was the third category concerning the accumulation of marine litter, presented by 3 subcategories in April and by 4 – in October. Inventories include tableware, light tubes, glass items, bottles, pieces etc – Fig. 4.

„Processed/worked wood" category was presented by 4 subcategories in April and 2 – in October. Inventories include processed timber, ice-cream sticks and other wood – Fig. 5. „Metal category" was presented by 8 subcategories in April and 5 – in October. Inventories include wire, aerosol/spray cans, cans, fishing items, cables, bottles, caps etc. – Fig. 5.

„Cloth/textile" category was presented by 6 subcategories in spring and 4 – in autumn. Inventories include clothing, shoes, bags, carpet & furnishing, rope, string, nets and other textiles – Fig. 6. „Rubber" category was presented by 4 subcategories in spring and 2 – in autumn. Inventories include balloons, tires, rubber bands and other rubber pieces – Fig. 6.

A Clean Coast Index (CCI) was developed and applied as a tool for evaluation of the beach cleanness (Alkalay et al., 2007). It measures marine debris as a beach cleanliness indicator. The CCI was used to increase public awareness regarding coast cleanliness and motivate the authorities to clean their beaches (Alkalay et al., 2007). The Clean Coast Index classified the Asparuhovo Beach as a ''Moderate" beach (CCI = 8.19) in spring and as "Clean" (CCI = 3.32) beach in autumn. Visual descriptions of beach cleanliness provided by Alkalay et al. (2007) were consistent with the CCI values obtained.

Artificial polymer material was the dominant debris during the study as had already been reported from Black Sea (Topçu et al., 2013; Simeonova et al., 2017), Adriatic (Munari et al., 2016), Mediterranean (Asensio-Montesinos et al., 2019), USA waters (Hardesty et al., 2017) and in many other studies worldwide (Derraik, 2002; Ryan et al., 2009). We estimated the top 10 most common items, observed during both studies in 2019 (Fig. 7). Plastic pieces, cigarette butts, food packets and wrappers, plastic caps, and lids are all common items in both datasets due to touristic activities and different beach uses. The main reason for this is that plastic is used in almost all human activities, together with its long persistence in the marine environment.

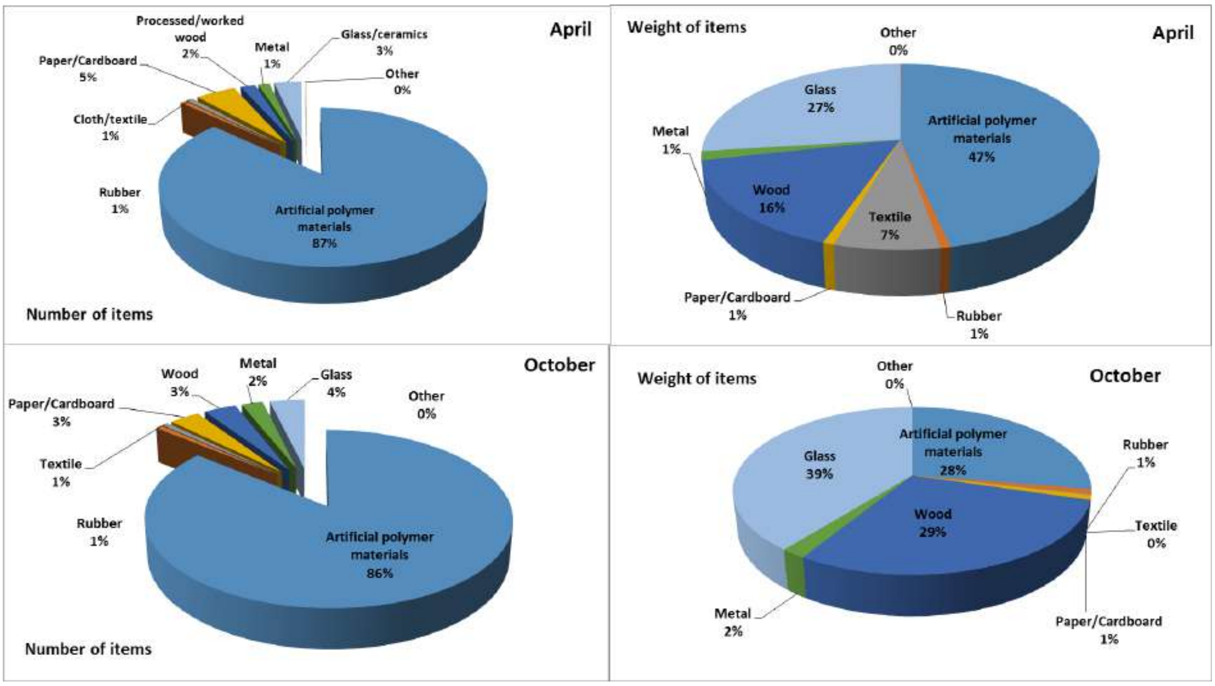

Fig. 2. Shares (in numbers and weight, kg) of collected items per categories depending on the material.

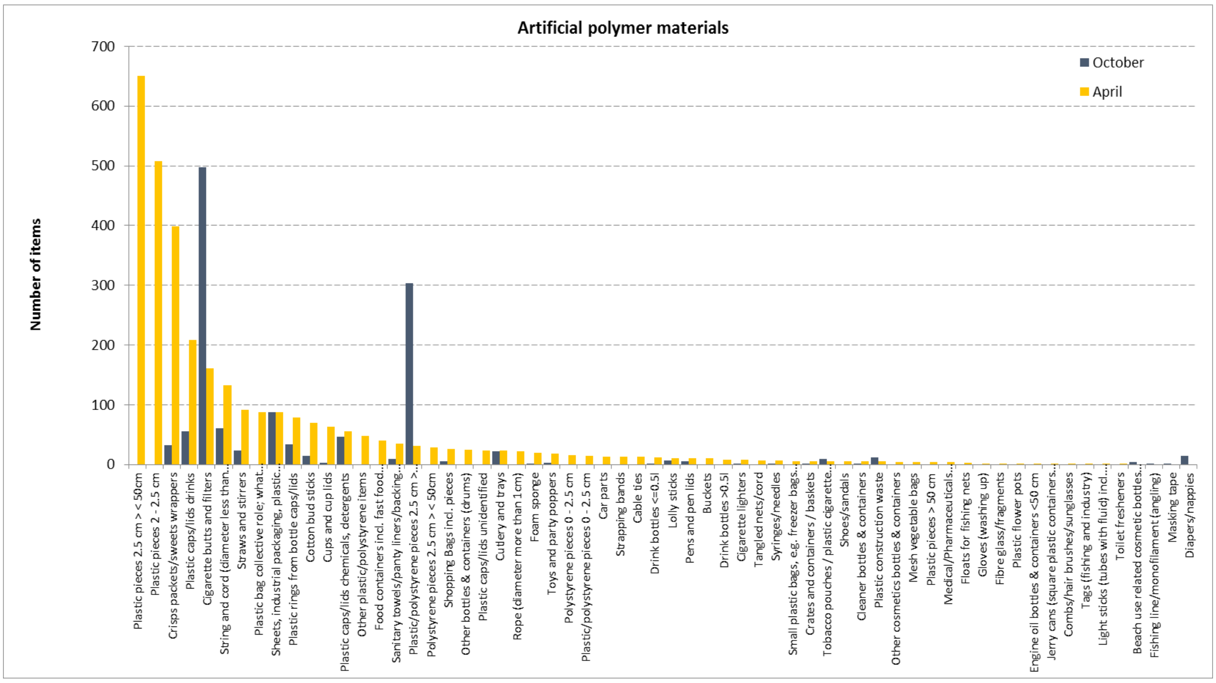

Fig. 3. Subcategories of Artificial polymer material, present in the surveyed area in 2019.

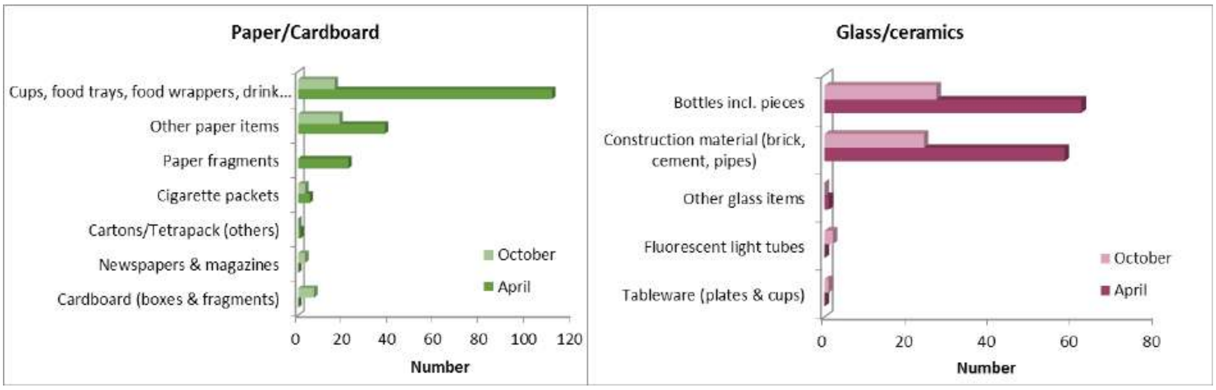

Fig. 4. Subcategories of „Paper/Cardboard" and „Glass/ceramics", present in the surveyed area in 2019.

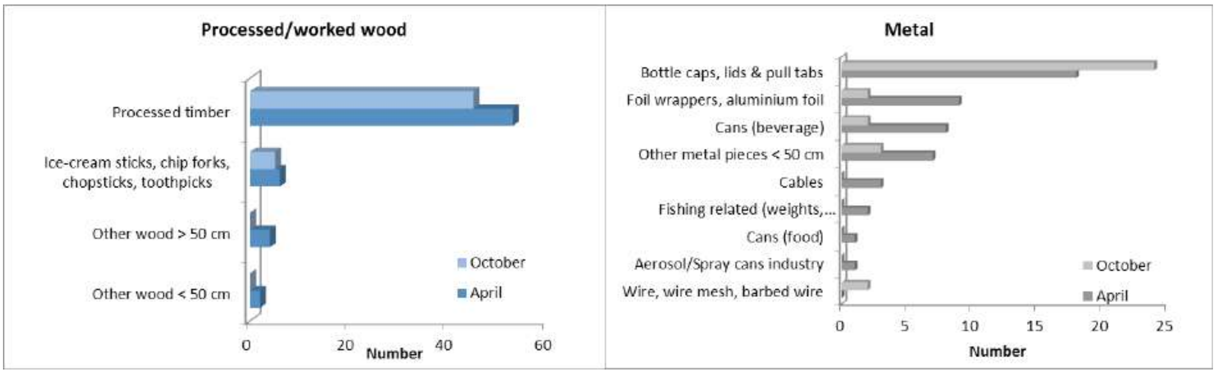

Fig. 5. Subcategories of „Processed/worked wood" and „Metal", present in the surveyed area in 2019.

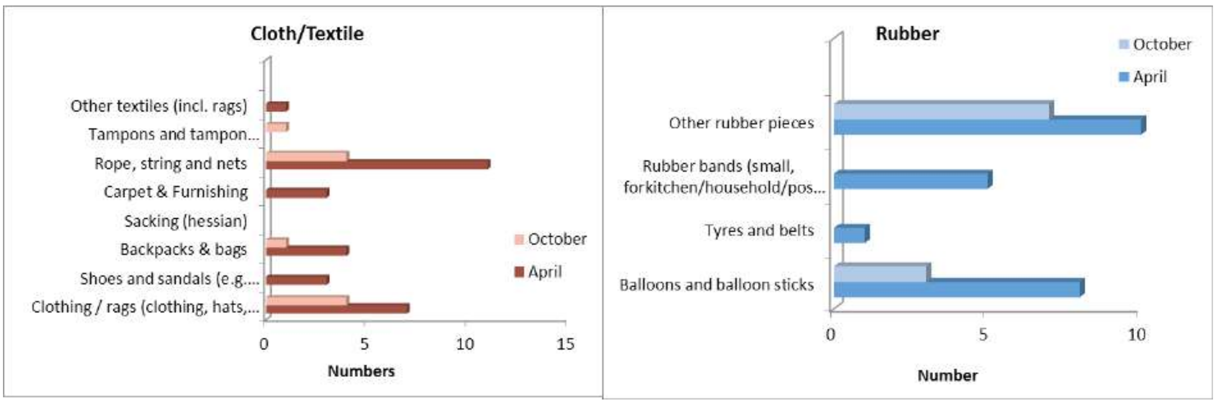

Fig. 6. Subcategories of „Cloth/Textile" and „Rubber", present in the surveyed area in 2019.

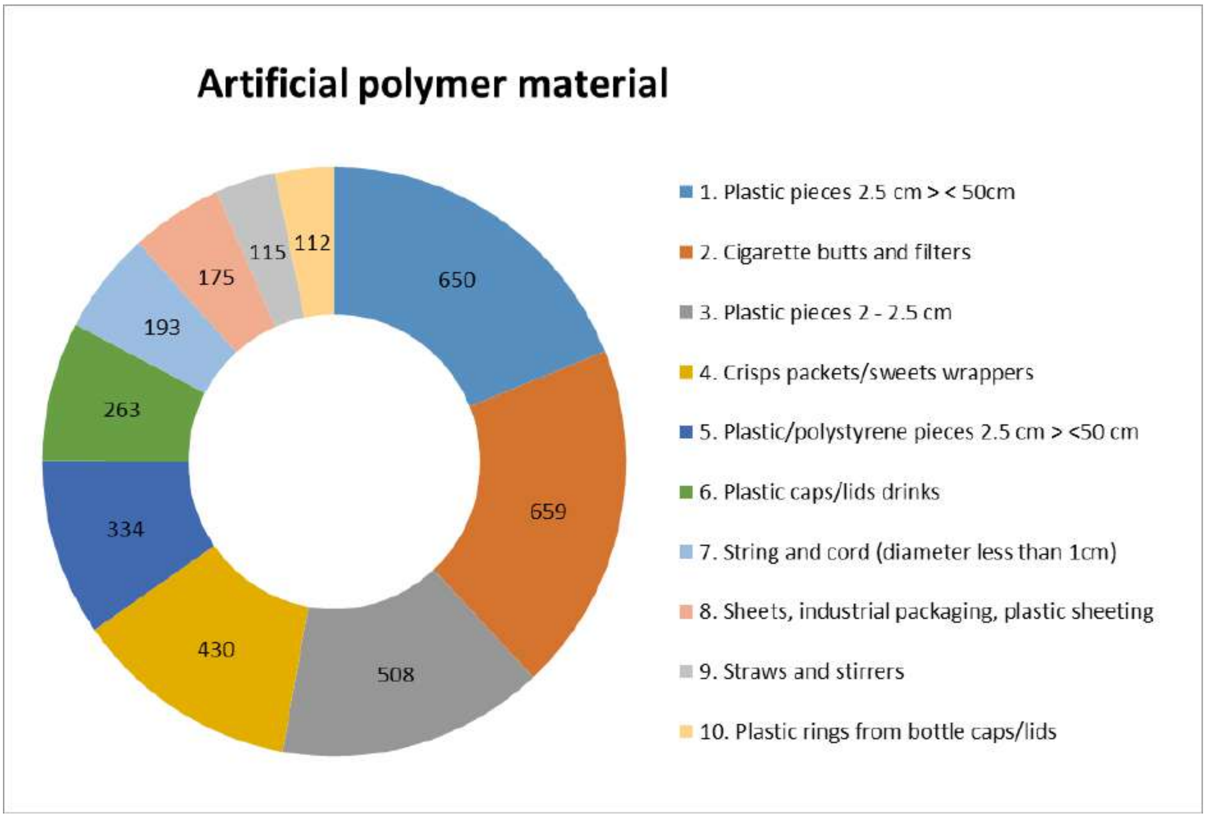

Fig. 7. The top 10 most commonly found plastic items from coastal surveys on Asparuhovo Beach in 2019. The total number of collected items in 2019 is given on the figure.

For the Bulgarian coast, our results confirm that the plastics, followed by paper/cardboard are the most abundant categories (Simeonova et al., 2017) in beach litter composition. For the Asparuhovo Beach area, Simeonova et al. (2017) reported lower densities of marine litter (0.1343 items.m^{-2}), compared to our results in 2019 and increasing trend in coastal beach pollution was observed during the years. The data collected during the monitoring campaigns allow tracing the seasonal dynamics in marine debris abundance. Comparison between results from spring and autumn monitoring campaigns showed that the number of items per square meter reduced 2.4 times fold from spring to autumn and in terms of mass – 4 times fold. The main reason probably is that after the touristic season in September, large quantities of marine litter are acumulated on the beach by winds and currents during the winter season. On the opposite side, during the summer, due to an increasing number of beach visitors and intensive recreational activities, the owners of beach restourants and tenants put efforts for beach cleanup and in early and mid-autumn (October) the beach is still clean (CCI=3.32). The main sources for the observed composition of marine litter on the Aspruhovo Beach are shoreline and recreational activities, smoking-related activities, fishing activities and medical/personal hygiene. The presence of marine litter on the beach, exept the loss in aesthetic value, could cause an economic harm, such as loss from tourism and additional cleaning costs. Ecological harm resulting from the ingestion of litter, including the uptake of microparticles (mainly microplastics), could seriously affect marine fauna.

Monitoring of marine litter and prevention of pollution of marine environment by adequate legislation and measures is the main challenge in Bulgaria. Current monitoring activities are irregular and there is an insufficiency of data for assessment of the current state and development of thresholds by different criteria and indicators of D10. Collection of new data is required for formulation of the basic state of macro- and microlitter, elaboration of respective thresholds and assessment of progress in achieving GES by criteria D10C1 and D10C2 (quantity of marine litter on the coast/beaches; litter on the sea surface and on the seabed). It can be assumed that GES is achieved when the litter and its degradation products present in and entering in marine waters do not cause harm to marine life and habitats, do not pose direct or indirect risks to human health and not lead to negative socio-economic impacts.

The increase of public awareness and involvement of stakeholders and citizens in common activities related to marine litter is important for the successful implementation of measures reducing coastal pollution.

Conclusions

The results of the conducted monitoring surveys for marine litter on Asparuhovo Beach in April and October 2019 revealed moderate beach pollution. The most significant level of pollution was due to the category of Artificial polymer materials – 86 - 87%. With the highest frequency in this category, including 58 subcategories, were cigarette buts, plastic/polystyrene pieces, industrial packaging and plastic cups and rings. The obtained results showed a reduction in the number of marine litter items in the autumn season compared to the spring season in 2019. The Clean Coast Index classified the Asparuhovo Beach as ''Moderate" beach in spring and as "Clean" in autumn. The conducted monitoring on the Bulgarian Black Sea coast could contribute to collecting new data about the composition and abundance of marine litter, distributed on the beaches and development of thresholds regarding indicator 1 (D10C1) of MSFD and achieving good environmental status of the marine environment.

Acknowledgements

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Comparative Analysis of Anthropogenic Transformations of Landscapes in the Lands of the Settlements of Belozem (Plovdiv Province) and Opalchenets (Stara Zagora Province) with Remote Sensing

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Abstract. In this study, a remote analysis of the anthropogenic activity, which plays a fundamental role in the formation of the contemporary landscapes in part of the Pazardzhik-Plovdiv lowland area - the lands of the Belozem (Plovdiv Province) and Opalchenets (Stara Zagora Province) villages - is carried out, as well as of the landscape pattern, determined by the complex interaction of natural and anthropogenic factors. The study area was selected based on the registration of some differences in the character of the land use in the lands of the two villages. Remote sensing contributes for more profound investigation of the spatial pattern and dynamics of anthropogenic landscapes in the study area. Satellite data, as well as landscape metrics analysis of the landscape pattern in the study area are used. A multi-temporal analysis was conducted to study the dynamics of landscapes over different periods. Some differences have been registered in the pattern of agricultural landscapes of the two studied lands, which could be explained by the different type of organization of the agricultural process on the territory of Plovdiv and Stara Zagora provinces. The manifestation of versatile by its character anthropogenic activity is a major factor in the formation of specific types of land use, which depend to a large extent on the socio-cultural and economic features of the environment, which in turn influences the formation of the landscape pattern. This is precisely what necessitates the study of these transformations and interactions and in a temporal aspect.

Key words: landscape pattern, anthropogenic effect, landscape metrics, satellite data, remote sensing, NDVI, NDWI.

Introduction

The landscapes in the Pazardzhik-Plovdiv field are a product of the millennial interaction between nature and people. The anthropogenic impact on the landscapes in this densely populated region of Bulgaria is characterized by significant by its intensity and stability over time manifestation. This has had a fundamental influence on the formation of the contemporary landscape pattern. However, the genesis and characteristics of the anthropogenic impact are not the same in all parts of the lowland area. The main factors that influence the specifics of the Land Use in the Pazardzhik-Plovdiv lowland area are the

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natural preconditions, the historical development, but last but not least the peculiarities of the traditions in agriculture and the distinctive features of the organization of the economy in regional and administrative-territorial aspect. Precisely because of the nuances and differences in the nature of the landscape-forming factors we consider it necessary to carry out a comparative analysis of anthropogenic transformations of the landscapes in two neighboring lands on the territory of Pazardzhik-Plovdiv lowland area - the lands of the villages of Belozem and Opalchenets. The choice of the study area is motivated by the differences in the specifics of Land Use on the territory of the two lands.

The present study examines the anthropogenic transformations of landscapes in the lands of two neighboring settlements - Belozem (Plovdiv Province) and Opalchenets (Stara Zagora Province). The study area is part of the Pazardzhik-Plovdiv lowland area and the agricultural activity is traditional. Agricultural landscapes predominate. They are the main manifestation of anthropogenic transformations within the study area. For this reason, they are subject of landscape-ecological studies in terms of anthropogenic impact on the territory.

The research is based on remote sensing. An analysis of the land cover was made using satellite data and calculating the NDVI (Normalized Difference Vegetation Index) and NDWI (Normalized Difference Water Index) indices. Classification of contemporary landscapes in the studied territory is made after differentiation of landscape forming factors. A comparative analysis of the peculiarities of the landscape pattern in the two lands in spatial and temporal aspect is made to reveal the temporal dynamics of the studied landscapes during different political and economic periods and to carry out the analysis of the landscape-forming factors using satellite images from 1985, 2005 and 2019. Landscape metric indicators is used to reveal the spatial features of the studied contemporary landscapes.

Material and Methods

For the purpose of the study, the lands of two neighboring villages located in two different provinces were selected - the lands of the villages of Belozem (Rakovski Municipality, Plovdiv Province) and Opalchenets (Bratya Daskalovi Municipality, Stara Zagora Province). Although located next to each other and characterized by similar natural geographical conditions, the lands of the two villages show some differences in terms of traditions and organization of the Land Use. These differences are due to different historical, demographic, economic and cultural preconditions, which have a direct impact on the landscape pattern in general.

The total area of the surveyed area is about 62 km^2. Forty two km^2 is the area of the land of Belozem, and 20 km^2 is the area of the land of Opalchenets. The area is calculated in ArcGIS 10.1 (ESRI, 2012).

For the purpose of the study LANDSAT 5 MSS, LANDSAT 5 TM and LANDSAT 8 OLI/TIRS satellite images (path 183, row 031) were used, acquired respectively on May 19, 1985, on June 27, 2005 and on June 18, 2019, were used (USGS, 2020b). All images have the same coordinate system - WGS84 / UTM zone 35N, with spatial resolution for LANDSAT 5 MSS 1985 image of 60 m and spatial resolution of the LANDSAT 5 TM and LANDSAT 8 OLI/TIRS images of 30 m. The USGS Digital Elevation Model (DEM) (USGS, 2020a) was used to determine the slope values in GIS, necessary for the classification of landscapes. Topographic maps of the studied area, Geology map of Bulgaria, Soil map of Bulgaria and CLC2018 layer were also used in order to carry out the differentiation and classification of the landscapes.

In the processing of satellite images, some preliminary activities need to be carried out to facilitate the research process and to improve its representativeness. Geometric correction of the 1985 satellite data was performed for the purposes of the present study in order to unify the spatial resolutions of the three satellite images. The spatial resolution of the 1985 image was changed from 60 m to 30 m, using the "nearest neighbor technique" for resampling the satellite image in ArcGIS 10.1 (ESRI, 2012). Georeferencing of the DEM raster layer was also performed in ArcGIS in order to adjust the relief layer to the WGS84 / UTM zone 35N coordinate system used for the purpose of the study.

The research is based on remote sensing of the temporal structure of the Land Use and Land Cover, as well as on landscape-metric analysis of the spatial landscape pattern in the scope of the studied territory. To study the anthropogenic transformations of landscapes, it is necessary to pay special attention to the Land Use and Land Cover (LULC), which can be considered as one of the landscape-forming factors. The structure and dynamics of the LULC in spatial and temporal aspect is a major reflection of the type and characteristics of the anthropogenic impact on the landscapes. It is for this reason that the study begins with an analysis of the LULC for the three time points in the three years - 1985, 2005 and 2019. The analysis was conducted by calculating the NDVI and NDWI indices in GIS, in order to establish the values of the reflectance of the vegetation, and hence determination of the genetic and spatial characteristics of the elements of the specific material-substantial manifestations of the anthropogenic activity, located on the earth's surface - settlements, agricultural landscapes, infrastructure facilities, etc.

The Normalized Difference Vegetation Index (NDVI) was calculated by combining the spectral bands of the individual satellite images and is used to differentiate vegetation from anthropogenic objects and soils. The values obtained of the NDVI were grouped into separate categories, in order to achieve maximum representativeness of the obtained results and to get a clearer notion of the objects on the earth's surface.

The NDVI index is calculated by the following formula:

$$NDVI = \frac{(NIR - R)}{(NIR + R)},$$

where NIR is the Near-infrared band and R is the Red band of the satellite image. However, different combinations of individual spectral bands are required to calculate the NDVI index. For example, the combination of NIR (band 4) and the visible (band 2) bands was used to calculate the index of for LANDSAT 5 MSS satellite image (Gallo et al., 1987). For the LANDSAT 5 TM satellite image the combination of band 4 and band 3 was used (Markogianni et al., 2016). For the LANDSAT 8 OLI / TIRS satellite image the combination of band 5 and band 4 was used (USGS, 2020a). The NDVI values range from -1 to +1. The highest values of the index are associated with the presence of areas occupied by dense vegetation. Lower values are typical for areas with sparse vegetation, as well as for plowed soils. The lowest values are an indicator for anthropogenic units or water bodies. The interpretation of the NDVI index values depends on the different phenological and biological characteristics of the vegetation, as well as the specifics of the climatic factors. The seasonal climatic dynamics directly affect the development of the plants, and hence the values of the plant leaves and stems reflectance.

The Normalized Difference Water Index (NDWI) was calculated to facilitate the differentiation of water bodies from agricultural areas and areas with natural vegetation. It should be noted that the implementation of the NDWI index in the analysis of the Land Use and Land Cover should be considered not as a main but as an additional tool in the set of remote sensing methods in the present study. The NDWI index is calculated by the combination between band 1 (Green) and band 3 (NIR) for the LANDSAT 5 MSS satellite image proposed by McFeeters (1996). Rogers et al. (2013) propose the combination between band 3 and band 5 for the LANDSAT 5 TM satellite image. And for the LANDSAT 8 OLI / TIRS satellite image a combination of band 3 (Green) and band 7 (SWIR2) was proposed by Özelkan (2019). Following the example of Özelkan (2019), threshold values of 0 for the results of 1985 and 2005 and of 0.12 for the results for 2019 have been set. Values above 0 for the results for 1985 and 2005, and above 0.12 for the results for 2019, are indicator for the presence of surface water bodies - micro-dams or rice fields in the initial stage of development.

A key stage in the present study is the analysis of the natural landscape-forming factors and their interrelations with the anthropogenic impact on the territory. On the basis of the complex of landscape-forming factors and the analysis of their landscape-forming role, differentiation and classification of the contemporary landscapes in the studied territory have been carried out. For this purpose the European Landscape Classification (LANMAP) (Mücher et al., 2010) was used. According to the experience of previous research of the author (Tamburadzhiev, 2020), as well as according to the specifics of the study territory, some additions of the classification system were implemented. The specific additions are described by Tamburadzhiev (2020).

Cartographic images were generated, visualizing the distribution of NDVI and NDWI values within the territory of the two lands, as well as of the contemporary landscapes within the scope of the studied territory.

Finally, landscape metrics at the landscape level was implemented through the vector-based Landscape Analysis Tools Extension (vLATE) for ArcGIS in order to analyze the structure of the contemporary landscapes on the territory of the lands of the villages of Belozem and Opalchenets.

Results and Discussion

The study area is part of the western part of the Upper Thracian lowland – the Pazardzhik-Plovdiv lowland area. The parent material is represented by sedimentary deposits (*s) - gravel, sands and clay. The relief is a lowland (*l) and is slightly indented. Based on previous research by the author and based on data for Plovdiv station (Tamburadzhiev, 2020) we can assume that the studied area in the present study is part of a territory with a semi-humid continental (*ShC) climate according to the classification of De Martonne and according to the differentiation of Mücher et al. (2010). The hydrological features are characterized by the presence of significant amounts of groundwater, as well as surface water bodies - Rahmanliyska River, Srebra River and Maritsa River, whose flow is part of the southern boundary of the study area. The soils are represented by the groups of Fluvisols (*F), Luvisols (*L), Vertisols (*V), as well as by the Rendzic type (*R) of the Leptosols group. The study area is part of the Upper Thracian biogeographical region according to the classification of Assenov (2006). Vegetation is represented mainly by agrophytocenoses. Natural vegetation is distributed fragmentary along the rivers. Along the Maritsa River it is represented by transitional tree-shrub vegetation. There are two forest massifs with secondary tree and shrub vegetation in the land of the village of Opalchenets. The territories adjacent to the Maritsa River are part of the Protected Zone „Maritsa" under the Council Directive 92/43/EEC (EC, 1992) on the Conservation of natural habitats and of wild fauna and flora, as well as part of the „Maritsa-Parvomay" Protected Zone under the Council Directive 2009/147/EC (EC, 2009) on the conservation of wild birds (NATURA 2000, 2013).

From the point of view of the analysis of the anthropogenic transformations of the landscapes it is very important to specify the peculiarities of the Land Use and the Land Cover (LULC) in the studied territory. For the purpose of differentiation and classification of the landscapes, a combination of data from CLC2018 (Copernicus Land Monitoring Service, 2020) and the use of remote methods for determining the features of LULC were performed. There are 10 types of LULC. According to the nomenclature of CLC2018, these are non-irrigated arable land (*nal); rice fields (*ri); pastures (*pa); complex cultivation patterns (*ccp); land principally occupied by agriculture, with significant areas of natural vegetation (*anv); broad-leaved forest (*blf); transitional woodland-shrub (*tws); water bodies; discontinuous urban fabric (*rural settlement); industrial or commercial units (*industrial units). The types of LULC are a diagnostic criterion for Level 5 of the landscape classification system.

In brackets are shown the designations of the individual levels of the landscape classification system on the maps of the contemporary landscapes of the lands of Belozem and Opalchenets, shown respectively in Fig. 4. and Fig. 5.

Maps are presented in Fig. 2., visualizing the values of the NDVI index for the surveyed territory, respectively for May 19, 1985, June 27, 2005 and June 18, 2019. The significantly greater homogenization of the spatial structure of LULC in the land of Belozem Village in 1985 compared to 2005 and 2019 is obvious. Socio-political changes after 1989 are the main reason for the reorganization of the agricultural production. The return of the land ownership of the citizens has led to the division of the arable land into smaller, in terms of area, and different, in terms of the characteristics of the cultivated crops, agricultural plots. Regarding the land of Opalchenets Village, the differences in the spatial structure of the different types of LULC are less pronounced for the three studied time points. This can be explained by the preservation of some cooperative practices in the organization of the agricultural process on the territory of Bratya Daskalovi Municipality and Stara Zagora Province, as a whole, part of which is the same land. The predominantly lower values of the NDVI index for both lands in 2005 compared to the other two time points are due to the fact that this time point is the latest of all the others - June 27. Therefore, we can assume that for some of the crops - wheat, for example, has passed the harvest period and the soils have remained exposed to the surface. The road-bed of the Trakia Highway is clearly visible in the images from 2005 and 2019. This main road artery is a prerequisite for fragmentation of the landscape pattern in the studied area. From the point of view of the patch-corridor-matrix paradigm, the road-bed of the highway facility can be considered as a buffer corridor, which actively influences the processes of normal functioning of the adjacent landscapes.

The NDWI index is calculated to distinguish areas that are covered with vegetation or with cover of anthropogenic origin (road infrastructure, buildings, etc.) from water bodies. No lakes have been registered in the study area. Water bodies are represented by micro-dams, rivers, irrigation canals and rice fields during the initial stage of rice crop development. Due to the specifics of the scale of the study, irrigation canals and rivers were not taken into account because they occupy an insignificant area within the study area.

The results of the NDWI calculation are shown in Fig. 3.

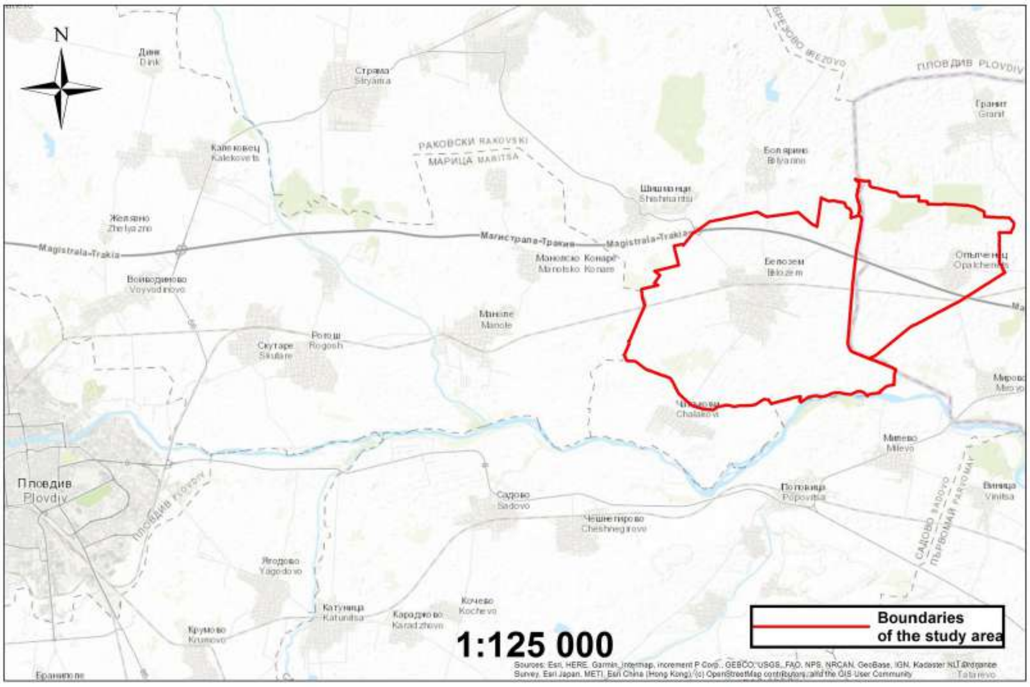

Fig. 1. Indicative map of the study area.

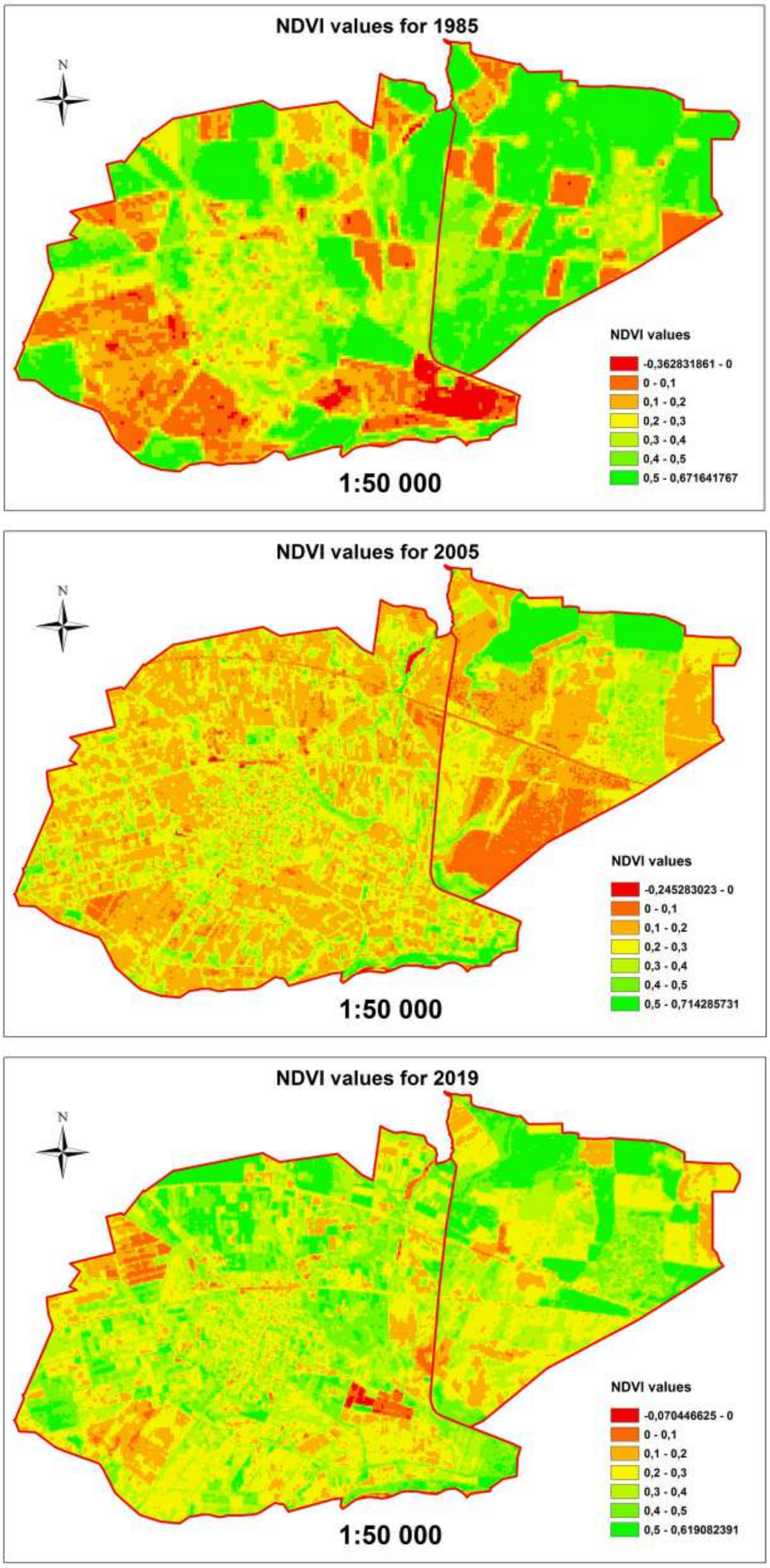

Fig. 2. NDVI values for 1985, 2005 and 2019.

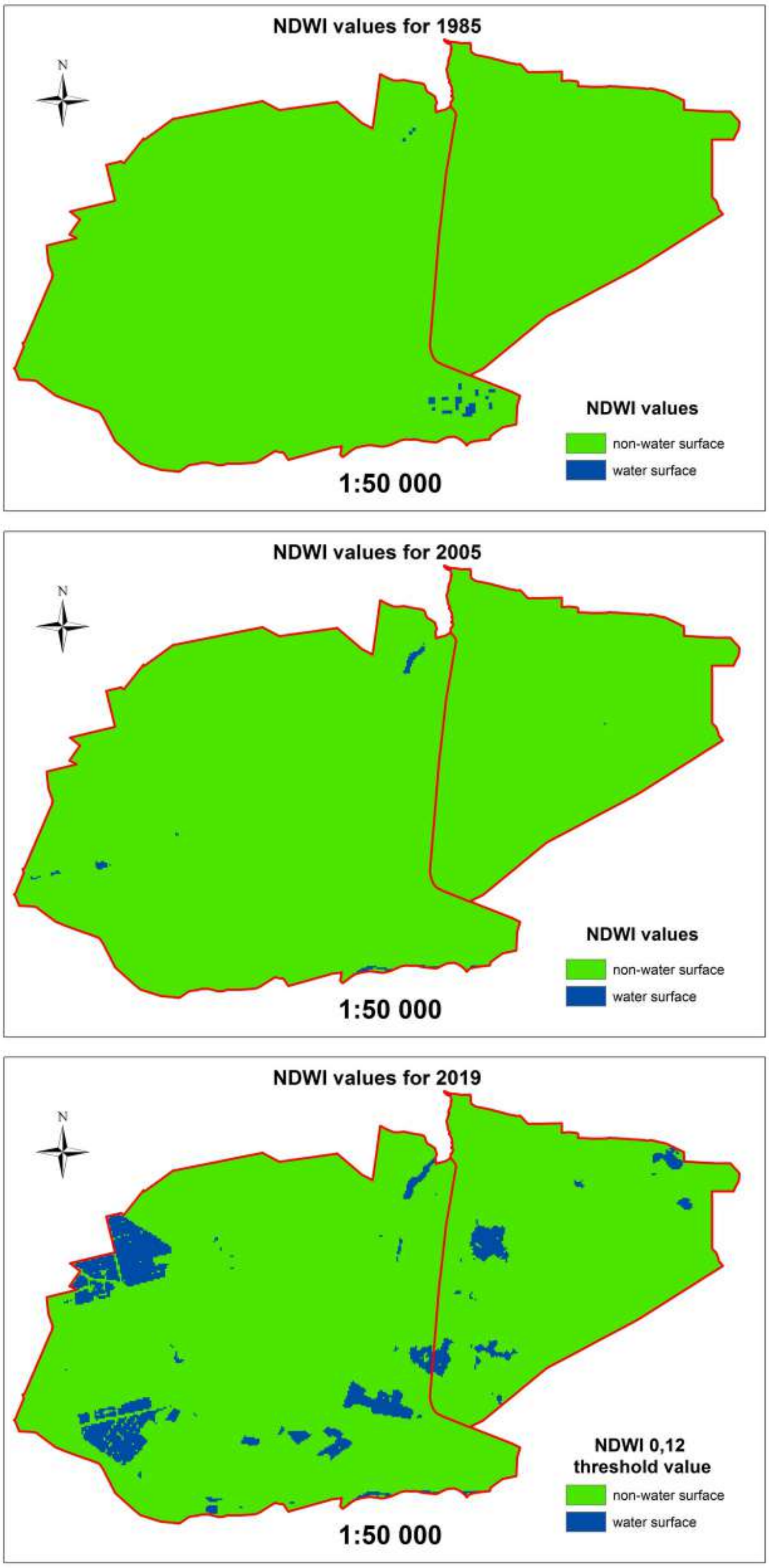

Fig. 3. NDWI values for 1985, 2005 and 2019.

The presence of surface water bodies is significant in the land of Belozem for the study period in 2019, as well as to a lesser extent for the land of Opalchenets during the same period. During the other two considered periods the surface water bodies on the territory of the two lands are insignificant in terms of spatial scope. This is probably explained by some features of the sub-branch specification of agricultural production in 1985 and 2005. Of the three time points considered, 2005 has the latest date - June 27. This could be one of the factors due to which no surface water bodies were registered during this period in the calculation of the NDWI index. It is quite possible to link the results to the fact that during the later periods of vegetative development of the rice crop the water layer in the rice fields is smaller compared to the water layer during the initial stage of development.

Based on the complex interrelations and interactions between natural factors and land use, the differentiation of the contemporary landscapes in the studied territory has been carried out. The diagnostic criteria of the used classification system are shown in Table. 1.

There are 23 different types of landscapes within the two lands. 17 of them are located on the territory of the land of Belozem and 15 of them are located on the territory of the land of Opalchenets.

The contemporary landscapes in both lands are shown in Fig. 4. and Fig. 5.

The results of the landscape metric analysis show that the landscape heterogeneity in the territory of Belozem village is more pronounced. In the Belozem land the fragmentation of the landscape structure is characterized by higher values, based on higher TE and MPE values. The landscape diversity of the Belozem land is more significant than that of Opalchenets based on higher values of Shannon's Diversity and Shannon's Evenness in Belozem. In this case, the higher degree of landscape diversity is due to the anthropogenic impact on the landscapes and not to natural preconditions. Results of landscape metrics analysis are shown in Table 2.

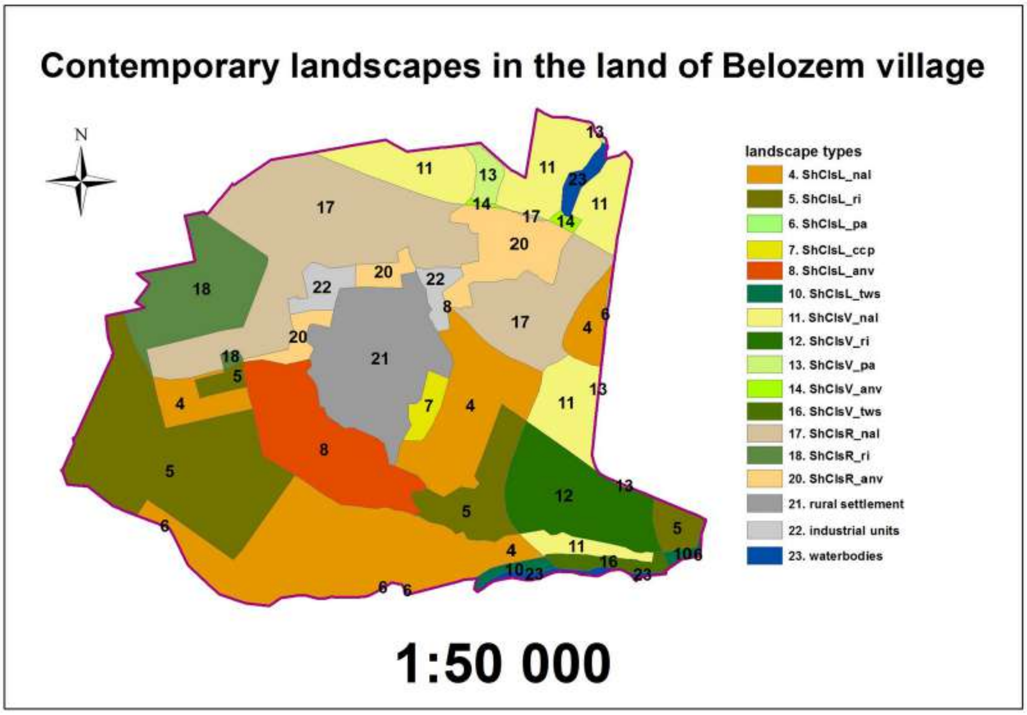

Fig. 4. Map of the contemporary landscapes in the land of Belozem Village.

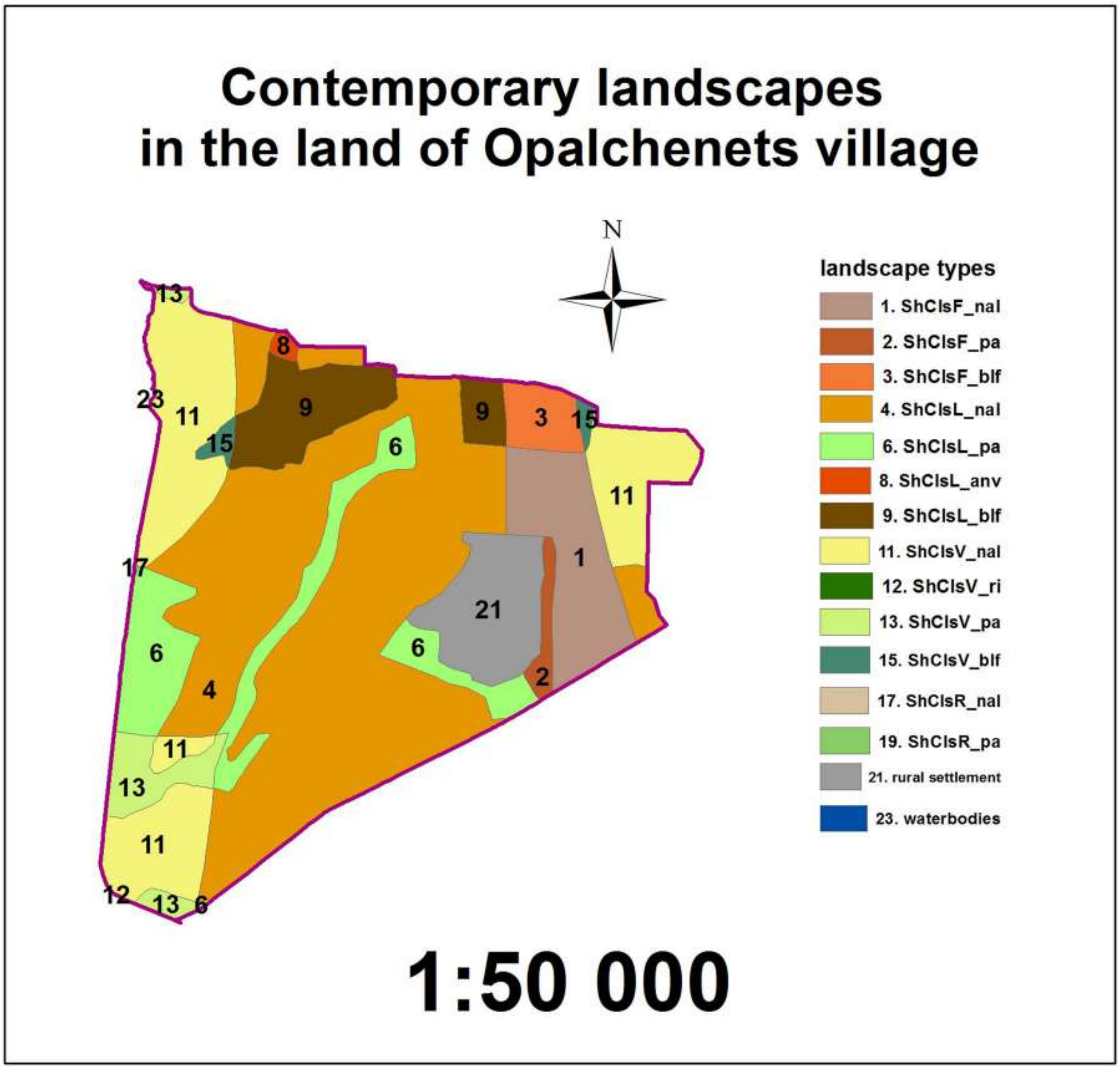

Fig. 5. Map of the contemporary landscapes in the land of Opalchenets Village.

Table 1. Diagnostic criteria and specifics of the typology classification system of the landscapes (after Tamburadzhiev, 2020).

Typology level	Diagnostic criteria	Symbol
Level 1	Climate	The 1st, the 2nd and the 3rd letters of the name (example: **ShC**lsF_nal)
Level 2	Slope	The 4th letter of the name (example: ShC**l**sF_nal)
Level 3	Parent material	The 5th letter of the name (example: ShCl**s**F_nal)
Level 4	Soil group	The 6th letter of the name (example: ShCls**F**_nal)
Level 5	Land use/ Land cover	The last letters of the name (example: ShClsF_**nal**)

Table 2. Results of landscape metrics analysis. *Legend:* Total Area (TA), Total Patches (NP), Edge Density (ED), Total Edge (TE), Mean Patch Edge (MPE), Mean Shape Index (MSI), Mean Perimeter-Area Ratio (MPAR), Mean Fractal Dimension (MFRACT).

Land	TA	NP	ED	TE	MPE	MSI	MPAR	MFRACT	Shannon's Diversity	Shannon's Evenness	Dominance
Belozem	41707669.50 sq. meters	34	44.66 meters per hectare	186281.45 meters	5478.87 meters	2.099	0.045	1.337	2.273	0.802	0.560
Opalchenets	19556636.87 sq. meters	21	53.12 meters per hectare	103887.23 meters	4947.01 meters	1.911	0.143	1.381	1.720	0.635	0.988

However, there are indicators that are characterized by higher values for the land of Opalchenets Village compared to the land of Belozem Village. These are, for example, the ED indicator, indicative of the degree of landscape fragmentation, and the Dominance indicator, indicative of the degree of landscape diversity. This should show that on the territory of both lands there are active processes of anthropogenization, which to one or another degree have an impact on the pattern and functioning of the landscapes.

Conclusions

The landscape pattern on the territory of the lands of Belozem Village and Opalchenets Village, shows some differences in spatial and temporal aspect. This is primarily due to the historical, socio-cultural, political and economic conditions affecting Land Use and Land Cover, and hence the formation of the entire landscape pattern. The temporal dynamics in Land Use and Land Cover is a major indicator of the interrelationships between the landscape pattern and the socio-political processes taking place in the field of agricultural production. The analysis of LULC is supplemented by calculating the NDVI and NDWI indices. The territories occupied with agricultural or natural vegetation are differentiated, as well as the water bodies and some infrastructural units. Significant differences were found in the spatial characteristics of the different types of Land Use in the land of Belozem for the three considered time points and relatively smaller differences for the spatial characteristics of the different types of Land Use in the land of Opalchenets for the three considered time points. After defining the main landscape-forming factors, differentiation and classification of the landscapes on the territory of the two lands was carried out. Twenty three different types of landscapes were differentiated within the study area. Seventeen of them are located on the territory of the land of Belozem and 15 of them are located on the territory of the land of Opalchenets Village. The landscape pattern of the land of the village of Belozem Village is characterized by a higher degree of fragmentation and landscape diversity, due to the active anthropogenic impact, compared to the landscape pattern of the land of the village of Opalchenets.

The study of anthropogenic transformations of landscapes through the use of remote sensing methods is an important stage in the overall process of analysis of the anthropogenic impact on a given area. Remote sensing is undoubtedly a necessary tool in the study of temporal dynamics and spatial features of landscapes. However, it is important its use to comply with the fundamental principles of landscape-ecological analysis in order to achieve maximum representativeness of the results obtained.

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Alkanna tinctoria: An Approach Toward Ex situ Cultivation

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Abstract. *Alkanna tinctoria* (L.) Tausch (Boraginaceae) is a perennial herbaceous medicinal plant species, with limited distribution and small populations. It is included in the Red Data Book of Bulgaria as endangered species, and protected by the Biodiversity Act. *Ex situ* cultivation is recommended as conservation measure for *A. tinctoria*. Seed germination rate under laboratory conditions: *in vivo*, *in vitro*, and in soil, was very low, up to 1%. The present study deals with stimulation of seed germination, acceleration of plants' growth using hydroponic technologies, and assessment of the photosynthetic apparatus of the adapted plants. Seeds gathered from 4 natural populations were treated by gibberellic acid (GA_3), and irradiation with red, blue, or infrared light emitting diodes, in order to stimulate their germination. Seedlings were grown on Cutting board hydroponic system for 6 weeks, than potted in soil substrate. Photochemical activity of adapted plants was characterized by Pulse-Amplitude-Modulated chlorophyll *a* fluorescence. Germination rate was enhanced mainly by GA_3 ($p < 0.001$) although seed origin and light quality influenced germination as well; furthermore, interaction of the 3 factors was also observed. In the best variant, 20% of the seeds germinated. No significant differences were noticed in the maximum quantum yield of primary photochemistry in dark-adapted state Fv/Fm between plants obtained from seeds germinated under different light quality. A pilot agriculture was established on the experimental field plot of IBER. Owing to the application of hydroponic technologies, plants' growth was accelerated and time from seed germination to plant flowering was twice shortened.

Key words: dyer's alkanet, seed germination, hydroponics, fluorescence of PS II, ABC analysis.

Introduction

Alkanna tinctoria (L.) Tausch is a perennial herbaceous medicinal plant species from Boraginaceae family. Its general distribution includes the Mediterranean area of Europe and Africa, from Spain and Morocco to Asia Minor and Jordan, the southern part of Central Europe (Hungary and Slovakia), and Caucasus (Pre-Caucasus and Transcaucasia) (Kozhuharov, 1989; Hoskovec, 2014). The species has a limited distribution with small populations in Bulgaria (Fig. 1). It is included in the Red Data Book of Bulgaria (Evstatieva, 2015) and the Red List of Bulgarian vascular plants (Petrova & Vladimirov, 2009) as endangered species, and protected by the Biological Diversity Act (2002).

A. tinctoria plants are harvested from its natural habitats for use as a red dye to give colour to wines, alcoholic tinctures, vegetable oils, and varnishes since antiquity in the Mediterranean region (Grieve, 1995-2020). In folk medicine it is applied as an excellent remedy for treating abscesses and inflammations (Duke, 2002). Plant extracts have expressed antiviral, antibacterial, anticancer, anti-inflammatory, anti-skin aging, and antiradical activities (Assimopoulou & Papageorgiou, 2005; Genova et al., 1995; Khan et al., 2015; Ozer et al., 2010; Tung et al., 2013).

In nature, reproduction of *A. tinctoria* is realized through seeds enclosed in monospermic achenes (Gerardi et al., 1998), however, the germination rate of its seeds is very low (Qi et al., 1993). The data on cultivation of *Alkanna tinctoria* are scantly. Attempt to its introduction in culture was done by Pluhar et al. (2001) who studied the germination rate and the propagation possibilities of the species. It was reported that alkanet could be propagated by raising seedlings, sowing the seeds in cold beds in autumn, or in heated equipment in early spring (Nemeth, 2009). *A. tinctoria* plants are maintained in the Botanical Garden of the Slovak Agricultural University in Nitra, grown by seeds sowed into prepared seed-bed on the experimental field (Becarova et al., 2012). The species is cultivated as a dye plant in Central and Southern Europe (Grieve, 1995-2020). Experimental work for *ex situ* cultivation is among the recommended conservation measures for *A. tinctoria* in the Red Data Book of Bulgaria.

The present study deals with stimulation of seed germination, acceleration of plants' growth using hydroponic technologies, and assessment of the photosynthetic apparatus of the adapted plants of *A. tinctoria*. The study was carried out within a research project supported by the Bulgarian National Science Fund. It is related to the development of effective protocols for cultivation of the species, as a part of a larger investigation on the possibilities for initiation of agricultures of medicinal plants with resource deficit from the Bulgarian flora.

Fig. 1. *Alkanna tinctoria* (L.) Tausch *in situ*.

Material and Methods

Plant material. Seeds were gathered from 4 Bulgarian natural populations in early June 2019, close to the villages Ilindentsi (N 41.65349, E 23.23062), Mikrevo (N 41.63887, E 23.17440), General Todorov (N 41.45800, E 23.28071), and Struma (N 41.55849, E 23.23130), all along the valley of the Struma River.

Seed germination. First tests of seed germination were carried out in different ways in parallel, under laboratory conditions: *in vivo*, *in vitro* as well as in soil substrate, and on a hydroponic system, 30 seeds per population. *In vivo* test was done on moistened filter paper in Petri dishes, at 25°C, while *in vitro* test was performed after standard seed disinfection, in plastic containers with basal MS medium, in a culture room at 23 ± 2 °C and 16/8 h photoperiod. For the soil test, seeds were put in a terrine with light soil and sand in proportion 1:1, in a room phytotron under natural daily light and temperature between 20 and 26 °C. The Cutting board hydorponic system (GHE), with 27 meshy pots with peat cubes on keramzit (5 seeds per meshy pot), was in the same phytotron; the nutrient solution, agitated by an aquarium pump and airstones, consisted of water and equal quantities of Flora Micro, Flora Grow, and Flora Bloom (GHE), its pH was maintained between 5.5 and 6.5, and the electrical conductivity (EC) – between 0.40 and 0.98 $mS.cm^{-1}$.

In order to stimulate seed germination, 2 factors were tested: soaking in gibberellic acid (GA_3) and irradiation with monochromatic light emitting diodes (LED). Seed origin (population) was considered as third factor with potential influence on seed germination. A total of 180 seeds per population were used, distributed in 2 consecutive trials (first one beginning in December 2019); half of the seeds were treated with 0.35% GA_3 for 4 h prior to germination, and the others were soaked in water for the same time, noticed as 'control'. Both GA_3-treated and control seeds were placed in Petri dishes, in 3 cameras at constant temperature of 21±1 ºC, under 12 h daily illumination with red (660 nm), blue (469 nm), or infrared (730 nm) light of the same quantum fluence rate of 110 mol/(m^{-2} s^{-1}). As a result, 24 variants were compared, 30 seeds per variant. Seeds were light-stimulated as long as one week after the end of seed germination.

Statistical analyses. The germination rate was assessed 4 weeks after the beginning of the tests, and expressed as percentage of germinated seeds. Seeds with hypocotyl were considered as germinated. The effect of GA_3 on seed germination was evaluated using Excel t-test paired. The output variable assumes values in the standard unit interval (0; 1). The values of seed germination were classified in 3 groups according to ABC analysis (Ultsch & Lötsch, 2015), and presented graphically using ggplot2 package functions (R Core Team, 2020; Wickham, 2016).

Soilless cultivation and plants' adaptation to soil. Seedlings were grown on the same Cutting board hydroponic system (GHE), described above, in mesh pots with peat cubes on keramzit, one per pot, for 6 weeks. Rosettes with diameter about 10 cm were potted in soil substrate consisting of light mix and sand, in proportion 2:1, and grown in the room phytotron.

Analysis of plant photochemical activity. After developing large rosettes, diameter of 15-20 cm, plants were analysed for their photochemical activity. They were dark-adapted for 5 min, than the induction of Chlorophyll *a* fluorescence was measured by Pulse-Amplitude-Modulated (PAM) fluorimeter (model 101/103, H. Walz, Effeltrich, Germany) as described by Fedina et al. (2003). The initial fluorescence level (F_0) was measured at frequency of 1.6 kHz. To evaluate the maximal fluorescence level (Fm), saturating flashes of 3000 μmol m^{-2} s^{-1} PFD with duration of 1s were provided by Schott lamp KL 1500 (Schott Glaswerke, Mainz, Germany). The maximum quantum yields of PSII photochemistry (Fv/Fm) were calculated according to Kitajama and Butler (1975). Five measurements were done for each plant.

Results and Discussion

Seed germination rate without stimulation, under *in vivo* and *in vitro* laboratory conditions, as well as in soil, was very low, from 0% in the soil terrine and *in vitro* conditions, up to 1% for the *in vivo* test in the Petri dishes. On the Cutting board hydroponic system, only one seed from Mikrevo population germinated i.e. 0.74% of all 135 seeds (Fig. 2).

Similar results were reported by Becarova et al. (2012) for *A. tinctoria* seed germination, where 51 seedlings were obtained from 925 seeds, diasporas being sowed in special seed-bed in the Botanical garden of the SAU in Nitra, Slovakia. Extremely low seed germination was reported not only for *A. tinctoria* but also for the whole Boreginaceae family (Gerardi et al., 1998). Presence of seed dormancy was proved for *Alkanna orientalis* during a testing of the seed germination behaviour of this species (Moustafa, 2002). Very likely, the dormancy is one of the reasons for the low seed germination in *A. tinctoria*, as well.

In all tested populations seed germination began at the end of the first week, first in variants with GA_3-treated seeds, and prolonged until the end of the third week. Germination rate was enhanced mainly by GA_3 ($p < 0.001$, t-test paired). Best results were noticed for seeds from Mikrevo population, treated with GA_3 and irradiated with blue light: 20% of 30 seeds germinated (Fig. 3). The stimulating effect of GA_3 on the germination of *A. tinctoria* seeds was demonstrated by Pluhar et al. (2001) as well, who reported up to 50% germination rate after seeds treatment with 400 ppm GA_3 for overnight before sowing.

Fig. 2. Germination of non-stimulated seeds on the Cutting board hydroponic system.

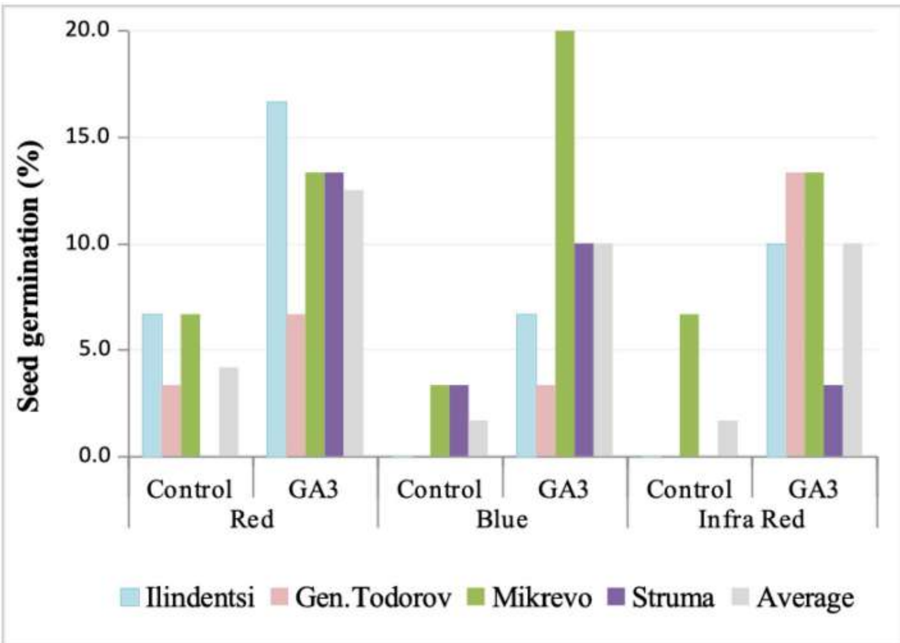

Fig. 3. Seed germination in all tested variants.

According to the germination rate, variants were grouped into 3 groups, comprising 6, 7, and 11 variants, respectively (Fig. 4) group A (count = 6, median = 13.4); group B (count = 7, median = 6.6); and group C (count = 11, median = 0). In 6 variants of the control seeds, belonging to 3 of the populations, no germination was observed at all (Fig. 3). Detailed analysis revealed relation between the seed origin and the germination rate (Table 1). Population near Mikrevo village was the only one with seeds germinating in all tested variants, and those close to villages of General Todorov and Struma manifested the worst germination potential. This might be genetically determined and additionally influenced by some differences in the environmental conditions such as soil characteristics, habitat exposure, and microclimatic features.

All of the 3 tested factors: GA_3, light quality, and seed origin, influenced seed germination; furthermore, interaction of the factors was also observed. Thus, red light was the best one for the seeds originating from Ilindentsi and Struma populations, blue light – for seeds from Mikrevo, and infrared light – for seeds from Gen. Todorov. The effect of different monochromatic lights on seed germination was reported in other species too (Costa et al., 2016; Nasrullah, 2001; Strydom et al., 2017). It could be specific and stimulating or inhibiting, as for ex. in chilli, where the red light was proved to increase seed germination in highest degree, while the violet light slacked it (Nasrullah, 2001). The red light enhanced also seed germination and survival in seagrass *Halophila ovalis* (Strydom et al., 2017).

The growth of 82% of the plants was accelerated on the Cutting board hydroponic system owing to the well-developed axial root and numerous root branches (Fig. 5). Plants were successfully adapted to soil substrate in pots, and after 2 months in the room phytotron (Fig. 6-A) they were transferred for a month to the greenhouse where they strengthened, formed ramified stems, and the biggest ones began to flower (Fig. 6-B). In mid-May, 27 plants were transferred outdoors, in the *ex situ* collection of IBER (Fig. 6-C), where the first flowering individuals were observed at the end of May 2020 (Fig. 6-D).

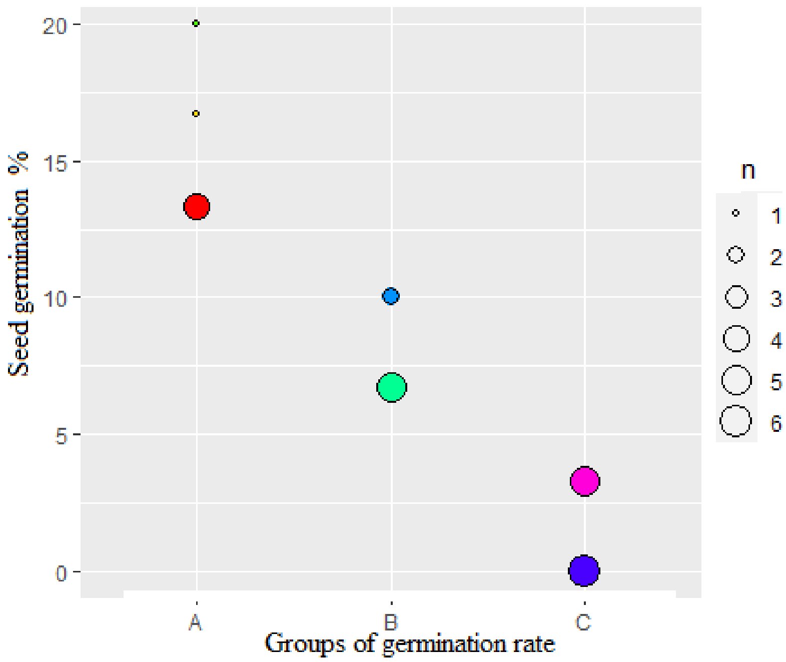

Fig. 4. Groups of variants with relatively high (A), medium (B) and low (C) germination rate. (*Note*: **n** indicates the number of variants with equal values, circle size corresponding to the number of variants with the same values, concerning seeds from all tested populations).

Table 1. Relation between the seed population origin and the germination rate of the seeds: groups with relatively high (A), medium (B) and low (C) germination rate.

Population	Number of participation in the ABC-groups		
	A	**B**	**C**
Mikrevo	3	2	1
Ilindentsi	1	3	2
Gen.Todorov	1	1	4
Struma	1	1	4

Fig. 5. Plants with vigour root system, on the Cutting board hydroponic system: **A**) top view; **B**) bottom view; **C**) entire plant before the transfer from the hydroponic system to pots.

Fig. 6. Plant acclimation: **A**) Well developed potted plant rosettes in the room phytotron; **B**) Plant with ramified stems in the greenhouse; **C**) Plants on the *ex situ* collection of IBER; **D**) Beginning of flowering in late May 2020.

Our results showed the advantages from the application of the hydroponic techniques in the propagation of *A. tinctoria*: in the present study, the whole cycle, from the sowing seeds to the plants flowering in open air, took 6 months, while in the experiment conducted by Becarova et al. (2012) flowering plants were obtained after one year from its onset.

Usually, seeds and cuttings are used as initial material for acceleration of plants' growth by hydroponic techniques (Texier, 2013). Concerning *A. tinctoria*, seedlings were proved to be the only appropriate starting material. To our knowledge, in the literature there are reports on few medicinal plant species cultivated hydroponically, among them *Mentha, Stevia, Arnica, Ocimum* (Giurgiu et al., 2014). The present experiment was one more successful application of the soilless cultivation in the propagation of medicinal plants with resource deficiency.

Photochemical activity of adapted plants was characterized by PAM chlorophyll *a* fluorescence. The use of monochromatic light can be perceived by the plant as a stress factor; therefore, we compared the photochemical activity of the potted plants grown from seeds, germinated under red, blue, and infrared light. To avoid the possible influence of population based features, only plants from one and the same population (Mikrevo) were tested. No significant differences were noticed in the maximum quantum yield of primary photochemistry in dark-adapted state Fv/Fm (Table 2). In general, the maximum quantum yield of PSII (Fv/Fm) is about 0,80-0,83 (Drozdova et al., 2001) which means that treatment of seeds with monochromatic light did not affect the subsequent development of the plants.

Table 2. Maximum quantum yield of PSII (Fv/Fm) of plants grown from seeds of Mikrevo population.

Variant		Fv/Fm ± SD
Control	InfraRed	0.820 ± 0.002
GA_3	InfraRed	0.815 ± 0.001
	Red	0.826 ± 0.004
	Blue	0.835 ± 0.004

Conclusion

First trials of *ex situ* cultivation of *Alkanna tinctoria* by seeds, using hydroponic technologies, were successful and promising. The application of this method, together with the stimulation of the seed germination by gibberellic acid, shortened twice the time necessary for the vegetative period from seed germination to plant flowering. A pilot agriculture was established on the experimental field plot of IBER. It will be used for development of effective protocols for cultivation of the species under control conditions. This experiment could be also used as a model for initiation of agricultures of other medicinal plants from the Bulgarian flora, with resource deficit and conservation status.

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New and Conservationally Significant Small Mammals in the Diet of Two Wintering Groups of Long-eared Owls (Asio otus L.) from the Region of Silistra (NE Bulgaria)

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Abstract. The object of the study is the diet of two wintering groups of long-eared owls (*Asio otus* L.) from the town of Silistra. During the winter of 2013/2014, 511 pellets and other skeletal residues are collected. A total of 1538 specimens are established, of which 1500 skeletal parts of small mammals. A total of 23 species are identified: 5 species of Eulipotyphla, 3 species of Chiroptera and 15 species of Rodentia. Five species are recorded for the first time as prey of the long-eared owl in Bulgaria: *Sorex minutus* L., *Neomys anomalus* Cabr., *Barbastella barbastellus* Schr., *Nyctalus noctula* Schr. and *Apodemus uralensis* Pall. Six of the species are protected, two are included in the Red Data Book of Bulgaria and three are very rare. All identified small mammals, except synanthropic mice and rats, can be considered new to the research area. For the first time after almost 50 years, information about *A. uralensis* is given. All new species have very low abundance in owl's diet (0.07–0.3% of all preys), which could be resulting from their naturally low density or the randomness of catches as an atypical prey. These conservationally significant species show the exceptional possibilities of this method to establish rare species that are difficult to prove with other methods of research.

Key words: *Asio otus* pellets, Micromammalia, owls diet, Danube, rare and protected species.

Introduction

The dietary analyses of nocturnal birds of prey are one of the main methods of collecting scientific information on the distribution of small mammals and their quantitative significance (Simeonov, 1964; 1966; Simeonov & Petrov, 1986; Milchev, 2015; Milchev & Ivanov, 2016; Milchev et al., 2003; 2006; Birrer, 2009). Such research is extremely topical, given the multifaceted information we receive, both for birds' biological and ecological characteristics, and for a wide range of vertebrate animal preys, who are indicative for the state of the environment (Birrer, 2009).

Long-eared owl (*Asio otus* Linnaeus, 1758) is a specialised predator feeding mostly

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on small mammals. The owls spend the winter in groups of up to 30 – 40 birds, preferring coniferous trees and choosing areas with good food supply (Glutz von Blotzheim & Bauer, 1994; Marks et al., 1999; Mebs & Scherzinger 2000; Birrer, 2009). Long-eared owl often and long-ago is been an object of research in Bulgaria and worldwide. The analysis of its diet gives valuable data about the state of many rare or conservationally significant species, and also about the dominant species of small mammals of the particular region. Data from the winter months when owls gather at clusters of several dozen individuals, excreting their pellets in the same place, are particularly valuable. These roosting groups are very suitable for studying owls' diet, since the grouping allows analyses based on numerous pellets. This way an information about a large number of individuals, which could not be collected in any other way, is obtained. Such information for separate regions of Bulgaria is found in the works of Simeonov (1964, 1966), Simeonov & Petrov (1986), Milchev et al. (2003), Milchev & Ivanov (2016). Information on rare and protected species in owl's diet is particularly valuable, since these species are difficult to prove by other methods, given their low density or bio-ecological specificity. Very often this is the only information about the distribution of rare species in new localities, or for relatively common species which are difficult to prove by traditional catch methods (Popov & Milchev, 2001; Popov et al., 2004; Milchev, 2006; 2009; Milchev & Georgiev, 2012). The analysis of owls' food remains can prove or confirm the presence of new species, both for a particular region and for the national fauna (Popov & Milchev, 2001), and can provide a valuable craniometric information.

Another advantage of this method is that during the winter months there are no other suitable methods for catching small mammals, and the large number of "hunters" guarantee the accurate and complete representativeness of the small mammals preys available during this period of the year. The roosting wintering *Asio otus* groups usually gather in late October – early November and disperse in late February – early March (Simeonov & Petrov, 1986).

The aim of this study is to establish the prey species composition of two groups of overwintering long-eared owls living in one region, but hunting and feeding in different landscapes, and to emphasize the conservationally significant species. The objects of the survey are the food residues in owls' pellets from NE Bulgaria.

Material and Methods

The food remains from two wintering groups of long-eared owls during the winter months of 2013/2014 from the region of the town of Silistra (Fig. 1) were collected once on 18 – 21 April 2014. The first roosting group of birds was located in the Danube City Park (N 44°07'04'' E 27°15'21'', 19 m). Birds inhabited a group of old and tall false cypresses (*Chamaecyparis* sp.). The second group of wintering birds was on the ridge part of the Medzhidi Tabia Forest Park (N 44°06'08'' E 27°15'31'', 131 m), in 2 km distance from the first group. The pellets were collected under a group of black pines (*Pinus nigra* J.F.Arnold), around 50 – 60 years of age and about 10 – 12 m in height, bordering an asphalt road.

The collected material was cleaned and all bone remains, feathers and hairs from each pellet were separated individually. The materials from the disintegrated pellets were processed together, and bones and fragments of skulls and lower jaws were separated from the post-cranial bones. The cranial dimensions, craniometric parameters, their abbreviations, and their tabular orderliness are according to Peshev et al. (2004) for each relevant group of small mammals. The measurements are made with electronic caliper. The number of preys was calculated by number of cranial, mandibular and post-cranial bones. The determination of the mammals was according to Peshev et al. (2004), Popov et al. (2003), Görner & Hackethal (1987) and the collection of the University of Forestry, Sofia.

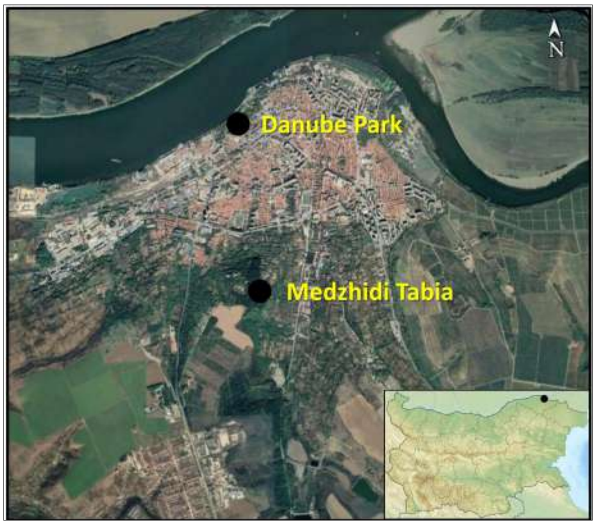

Fig. 1. Location of the two wintering *Asio otus* groups in the town of Silistra.

Results and Discussion

During the winter of 2013/2014, 511 pellets and other skeletal remains are collected from two wintering groups of *Asio otus* in Silistra. A total of 1538 prey specimens are found, of which 1500 skeletal parts of small mammals, 36 of birds and two pairs of Coleopteran elytra. Small mammals make up 97.5% of all prey specimens, and the established species represent 78% of all so far recorded mammal preys in Bulgaria. A total of 23 species of small mammals are identified: 5 insectivorous species (Eulipotyphla), 3 bat species (Chiroptera) and 15 species of Rodentia. Five species are recorded for the first time as prey of the long-eared owl in Bulgaria: *Sorex minutus* L., *Neomys anomalus* Cabr., *Barbastella barbastellus* Schr., *Nyctalus noctula* Schr. and *Apodemus uralensis* Pall. Six of the species are protected, two of them are included in the Red Data Book of Bulgaria (Golemanski, 2015). Three species can be considered very rare.

Further we discuss only the conservationally significant small mammals in the diet of the two wintering groups of long-eared owls from Silistra:

Eurasian pygmy shrew (Sorex minutus L.). It is reported for the first time in Bulgaria as a prey of the owls during the winter period. It is also new for the area of the town of Silistra. Information about the species is available from the region of the "Srebarna" Reserve (Genov, 1984; Sichanov et al., 2006; Popov et al., 2019). It has also been reported for Northern Dobrudzha (Laiu & Murariu, 1998; Murariu, 2005; Miu et al., 2018), as well as a prey of *Asio otus* in Romania (Sándor & Kiss, 2004, 2008). This species was not been established in fossil, subfossil and recent Eurasian eagle-owl (*Bubo bubo* (L.) preys in Rusenski Lom and Suha Reka on the Ludogorie Plateau (Mitev, 2004). In the same fossil deposits the author found numerous remains of other species belonging to the "mountain faunistic ensemble" (*Sorex araneus* L., *Microtus subterraneus* (S.-L.), *Myodes glareolus* Schr.). This complex has also been found in Northern Dobrudzha (Murariu & Stanciu, 2009; Miu et al., 2018) and probably represents a refugium preserved because of the microclimatic specificity of the Danube. Eurasian pygmy shrew is characteristic of the mountains, but it also occurs in mesophilic and mesohygrophilic habitats in the plains. It was found in both study sites (4 ex. in Danube Park and 2 ex. in Medzhidi Tabia). There are many suitable for the species wet and waterside habitats near the two areas, but regardless its natural density, it is a rare prey of the owl since it is a small and cryptic mammal. The material examined included fragments of skulls and lower jaws divided into left and right halves. The measurements taken of the skull fragments showed a complete overlap with those of Peshev et al. (2004) (Table 1).

Southern water shrew (Neomys anomalus Cabr.). This is the first record of this species as prey of *Asio otus* for Bulgaria. It is registered in Dobrudzha, both in Bulgaria (Genov, 1984; Sichanov et al., 2006; Popov et al., 2019) and Romania (Murariu, 2005; Miu et al., 2018). It was not established in Southern Dobrudzha (the vicinity of Dobrich)

(Simeonov, 1966; Milchev & Ivanov, 2016), as well as in late Pleistocene and Holocene eagle-owl deposits in NE Bulgaria (Mitev, 2004). The species is attached to water reservoirs and coastal territories. One individual from the Danube Park was identified. The sole sample is with a shattered brain lobe and a preserved facial lobe. The lower jaw is preserved with connected left and right halves. We did not measure this single specimen since its triviality in other regions of Bulgaria.

Bats. During the study we found three specimens of three species, of which two are reported as *Asio otus* prey for the first time in Bulgaria – western barbastelle bat (*Barbastella barbastellus* Schr.) and common noctule (*Nyctalus noctula* Schr.). The grey long-eared bat (*Plecotus austriacus* (Fischer) has been previously reported from Sofia (Milchev et al., 2003). All three specimens were determined by cranium and mandible. Some measurements are given in Table 2, Table 3 and Table 4. We found the common noctule in the Danube Park, and the western barbastelle and grey long-eared bat – in Medzhidi Tabia. Both localities offer suitable conditions for bats associated with old mesophilic canopy forests with sufficient dead, dying or living old wood. While the common noctule and grey long-eared bat are relatively widespread and their presence in the study area is traditional given the specifics of the landscape and habitats, the barbastelle bat is a typical mountain species. Its record in Silistra was quite unexpected, although it was found near the Danube Park once again in 1985, when the animal was weakly active, in hibernation (Kodzhabashev, pers. observ.). In Bulgaria, this species has been found in other plain territories near rivers: near the Vidbol River (Vidin district) (Ivan Pandurski, pers. comm.); near the Osam River (Pleven district) (Paunovic et al., 2003; Golemanski, 2015); near the mouth of the Kamchia River (Heinrich, 1936; Benda et al., 2003). There are two records of the species from the Danube Delta (Murariu et al., 2016). It is likely that the discovery of the barbastelle bat along relatively large rivers is related to its requirements for a cooler microclimate and a closed canopy of woody vegetation, such as are the natural riverine flooded forests.

All three species are relatively slow in flight, making them a potential prey. All three skulls were fully preserved, suggesting that probably the owls caught the bats in the air with their sharp claws. In capturing terrestrial mammals, owls almost always use their beak, most often aiming at the cranial area. That assures the killing of the prey and the absorbing of the caloric brain substance. The three bat species are strictly protected by Bulgarian and international legislation (see Appendix 1). The barbastelle bat is rare in the country, but its status for the region of Silistra is not clear. It is likely to be found year-round, inhabiting riparian mesohygrophilic and old mesophilic forests, where the conditions of the environment are relatively close to those in mountainous habitats. Further studies are needed to establish its status in the region of Silistra.

Table 1. Craniometric measures for *S. minutus* from Peshev et al. (2004) and our study (*here*).

	HPC		**L I_1-M_3**		**L I^1-M^3**		**LMd**		**LP^4-M^3**
	Peshev	***here***	**Peshev**	***here***	**Peshev**	***here***	**Peshev**	***here***	***here***
min.	3.05	3.09	5.7	5.9	4	6.15	6.8	6.71	3.53
max.	3.5	3.44	7.4	6.01	7.2	5.57	8.2	7.23	
aver.	3.26	3.19	6.25	6.26	5.8	6.41	7.9	6.97	
		n = 6		n = 6		n = 6		n = 2	

Table 2. Craniometric measures for *P. austriacus* from Peshev et al. (2004) and Silistra (*here*).

	HPC		L I^1-M^3		LMd		LP^4-M^3
	Peshev	***here***	**Peshev**	***here***	**Peshev**	***here***	***here***
min.	3.35	3.33		3.5	11.0	9.5	4.35
max.	3.65				11.7		

Table 3. Craniometric measures for *B. barbastellus* from Peshev et al. (2004) and Silistra.

	HPC	HMd/M_2	LMd
Silistra	3.1	1.2	9.6
Peshev et al., 2004	2.8	1.32	9.3

Table 4. Craniometric measures for *N. noctula* from Peshev et al. (2004) and our study (*here*).

	LMd		HPC		HMd/M_2		L I_1 - M_3	
	Peshev	***here***	**Peshev**	***here***	**Peshev**	***here***	**Peshev**	***here***
min.	14	14.2	4.4	4.5	2.1	2.2	8.65	8.65
max.	15.2		4.9		2.5		9.2	
	LC-M_3		**LP_4-M_3**		**LC.-P_4**			
	Peshev	***here***	**Peshev**	***here***	**Peshev**	***here***		
min.	7.7	7.45	5.95	5.8	2.6	2.63		
max.	8.15		6.4		2.85			

Dormice. Two species were established – hazel dormouse (*Muscardinus avellanarius* (L.) and forest dormouse (*Dryomys nitedula* Pall.). They were previously reported as *Asio otus* prey (Simeonov, 1964; 1966; Milchev & Ivanov, 2016), although during the winter they are in hibernation. The two species were registered, respectively, with three and two specimens, the first being found only in the Danube Park and the second – in the Medzhidi Tabia, which is probably due to the different preferences of these typical forest animals. Some measurements are given in Table 5. Unlike the more common forest dormouse, which mostly inhabits mesoxerophilic deciduous forests, the hazel dormouse is mostly a mountain species inhabiting old mesophilic forests with undergrowth well developed. The microclimatic conditions of the Danube Park, located on the banks of the Danube, the landscape, and the diverse forest canopy are a prerequisite for suitable living environment for mesophilous forest species, such as the hazel dormouse. The large current of the Danube moistens and cools the coastal air, which, combined with large-sized woody vegetation, creates conditions close to those in the low and medium mountain belts. This is an important fact, concerning the prolonged winter warmings. According to Popov & Sedefchev (2003), the awaking of the dormice in the winter months is detrimental to normal hibernation, and awakened animals die from exhaustion due to early consumption of spare fats.

Romanian hamster (Mesocricetus newtoni (Nehring). It is a protected species that has greatly reduced its range over the past 70 – 80 years as a result of the strong anthropogenic press, a consequence of the intensification in agriculture. For the region of Silistra, it has been firstly reported by Hristovich (1901). In 1925, Kovachev (1925) reported it for Silistra along with the common hamster (*Cricetus cricetus* (L.). Later, both species were also reported for the Srebarna Lake (Genov, 1984).

The Romanian hamster hibernates, but unlike the common hamster, in warmer winter days it is active (e.g. Markov, 1960). The species was found in *Asio otus* diet in the region of the towns of Dobrich (Simeonov, 1966) and Shumen (Simeonov & Petrov, 1986), too. According to Nedyalkov (2016), due to the strong anthropogenic press, the Romanian hamster has a very low density and highly fragmented range. Its main localities are in the Danube Plain and Dobrudzha, and those in southern Bulgaria are local and declining. According to Mitev (2004) and Peshev et al. (2004), the species has been dominant both throughout Bulgaria and the Balkan Peninsula during the late Pleistocene and almost throughout the early and middle Holocene, regardless the climatic changes associated with warming and humidification and the displacement of the xerophilic steppe and forest-steppe vegetation with mesophilic forests. The contemporary range of the species is greatly reduced not only by the changes in the land-use and management of agricultural land, but also by the recent climatic conditions. Coastal drained silts, loess banks of the Danube and thick chernozem soils are the refugia of the species in the region of Silistra. The Romanian hamster was found in the Danube Park, bordering with the high and drained banks of the Danube, which are suitable for the building of underground burrows. We found only one individual, which is indicative for its density. Still, this proof is important, given the high conservation value and the lack of information about the species for the whole Danubian Dobrudzha. During different studies of the small mammals in the Srebarna Reserve, the species has not been established, which makes the period since its last records more than 45 – 50 years. Romanian hamster is strictly protected (Golemanski, 2015). According to Murariu et al. (2009) it is very rare in Northern Dobrudzha and has a scattered distribution.

The specimen was determined by its lower jaws and fragments of the skull, as the brain capsule was shattered. According to the state of the teeth, the animal is old, but its measures are at the lower limits for Bulgaria, and even below the minimum (Table 6).

Ural field mouse (Apodemus uralensis Pall.). It is a species without national conservation status, but can be considered conservationally significant, given its modern status. The species has not been reported for Bulgarian fauna since 1972, when the latest studies on its bio-ecology were done (see Peshev et al., 2004). It was found in 1964 near Kostinbrod and in the Western Balkan Mountains (Peshev et al., 2004). There is no following information on the distribution of the species in Bulgaria ever since. Data on the species is available from Romanian Dobrudzha, where Murariu (2005) found it near the land border with Bulgaria, as well as in the interior of northern Dobrudzha. Mitev (2004) recorded the species in subfossil and more recent findings at a distance of less than 40 km from the city of Silistra, but without proof of the exact date of the biological material. The only other data about the past of the species are for the late Pleistocene from NW Bulgaria (Popov 2000).

Our research gives second certain location for the Ural field mouse at Medzhidi Tabia Park, after its records from the region of Sofia. Six individuals were determined confirming the existence of the species after a period of almost 50 years. Some measurements are given in Table 7. In the region of Medjidi Tabia the other two close and common species of mice occur – yellow-necked mouse (*Apodemus flavicollis* (Melch.) and wood mouse (*A. sylvaticus* L.), which is of interest from a scientific and practical point of view. The proportions of the three species in the diet of the owls from Silistra were approximately 30: 6: 1 (*A. flavicollis*: *A. sylvaticus*: *A. uralensis*). Similar proportions were established by Mitev (2004), too.

Table 5. Craniometric measures for *D. nitedula* from Peshev et al. (2004) and Silistra (*here*).

	LaP^4-M^3		LMd		La P_4-M_3	
	Peshev	***here***	**Peshev**	***here***	**Peshev**	***here***
min.	3.4	4.2	11.4	14.52	3.2	4.2
max.	4.6		17.1		4.6	

Table 6. Craniometric measures for *M. newtoni* from Peshev et al. (2004) and Silistra (*here*).

	IOW	LaM^1-M^3		DL		LMd		La M_1-M_3	
	here	**Peshev**	***here***	**Peshev**	***here***	**Peshev**	***here***	**Peshev**	***here***
min.	4.1	6.0	6.16	9.0	8.46	19.0	17.66	6.0	5.96
max.		8.8		11.0		23.0		8.8	
aver.		7.11		10.0		21.67		7.21	

Table 7. Craniometric measures for *A. uralensis* from Peshev et al. (2004) and Silistra (*here*).

	IOW		LaM^1-M^3		DL		LMd	La M_1-M_3	
	Peshev	***here***	**Peshev**	***here***	**Peshev**	***here***	***here***	**Peshev**	***here***
min.	3.90	3.75	3.45	3.23	5.40	4.95	7.92	3.32	2.74
max.	4.20	4.23	3.97	3.65	6.30	5.80	8.40	3.62	3.53
aver.	4.08	3.99	3.77	3.41	5.90	5.38	8.23	3.42	3.17
	n = 17	n = 2	n = 17	n = 3	n = 17	n = 2	n = 3	n = 17	n = 5

The five species recorded for the first time as prey of the long-eared owl in Bulgaria (*S. minutus*, *N. anomalus*, *B. barbastellus*, *N. noctula*, and *A. uralensis*) have very low abundance in owl's diet (0.07–0.3% of all preys), which could be resulting from their naturally low density or the randomness of catches as an atypical prey. These conservationally significant species show the exceptional possibilities of this method of studying owls' pellets to establish rare species that are difficult to prove with other methods of research.

According to the literary data available, the established small mammals in *Asio otus* diet belong to 25(29) species from four orders: Eulipotyphla (5 or 6 species), Chiroptera (1 species), Lagomorpha (1 species; in fact this is a domestic rabbit and we think that it should not be considered as prey), Rodentia (18 or 20 species) (Simeonov, 1964, 1966; Simeonov & Petrov, 1986; Milchev et al., 2003; Milchev & Ivanov, 2016). Six of these species are protected (Appendix 1). The difference in the number of species is due to the difficult for determination species, which are often determined by pairs of species or even only to genus level. Such taxa are *Microtus arvalis/rossiaemeridionalis*, *Apodemus flavicollis/sylvaticus/uralensis* and *Neomys anomalus/fodiens*.

In addition to already established, the possible potential small mammal preys of *Asio otus* for the region of Silistra, along with the less probable species (as southern birch mouse *Sicista subtilis* (Pall.) and grey dwarf hamster *Cricetulus migratorius* (Pall.), represent another 13 species, of which 9 are protected (see Appendix 1). In the list of the potential preys we do not include 5 species: the hedgehog (*Erinaceus roumanicus* B.-H.), the invasive rodents – muskrat (*Ondatra zibethicus* (L.) and coypu (*Myocastor coypus* (Mol.), cape hare (*Lepus capensis* L.), and from the predators – the weasel (*Mustela nivalis* L.). These species are relatively large, therefore, they are unlikely to become a prey. Furthermore, the weasel, regardless of its small size, is an extremely strong and aggressive predator, and it would become a prey only as an exception. Due to the lack of research on the bat fauna in the region of Silistra, a biological extrapolation was used according to surveys in the Srebarna Reserve, with a total of 8 established and supposed species, but it is probable more to be registered.

The full (according to literature and personal data) list of small mammals from the region of the town of Silistra includes 43 species from four orders: Eulipotiphla – 7 sp., Chiroptera – 10 sp., Lagomorpha – 2 sp., Rodentia – 24 sp. It is given in the Appendix 1, along with the conservation status of the animals. During our study we determined in owls' pellets 23 species of small mammals, which accounted 58% of the potential prey species and 77% of those established so far preys. The protected species, potential preys of *Asio otus* for the region, are 17 species, and 6 of them were found in our study. Another 4 species have been reported as owl's prey for other regions of Bulgaria.

These facts show the importance of such studies in registering terrestrial small mammals, among which some are extremely rare and difficult to prove by other methods. The registration of bats as potential preys of the owl can be carried out remotely with a detector, but the data from the diet analysis may complement the scientific information.

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Appendix 1. Species composition and conservation status of the small mammals from the region of the town of Silistra. *Legend:* BDA – Biological Diversity Act; RDB – Red Data Book; IUCN – IUCN Red List; Bern – Bern Convention; Bon – Bon Convention; EUR – EUROBATS; * - species from unpublished observations (N. Kodzabashev, pers. observ.).

№	Species	BDA	RDB	IUCN	Bern	Bon	HD	EUR
	Eulipotyphla							
1	**Erinaceus roumanicus* Barrett-Hamilton, 1906	III	LC	LC				
2	**Talpa europaea* Linnaeus, 1758		LC	LC	III			
3	*Sorex araneus* Linnaeus, 1758		LC	LC	III			
4	*Sorex minutus* Linnaeus, 1766		LC	LC	III			
5	*Neomys anomalus* Cabrera, 1907		LC	LC	III			
6	*Crocidura leucodon* (Hermann, 1780)		LC	LC	III			
7	*Crocidura suaveolens* (Pallas, 1811)		LC	LC	III			
	Chiroptera							
8	*Rhinolophus ferrumequinum* (Schreber, 1774)	II, III	LC	LC	II	II	II, IV	+
9	*Rhinolophus hipposideros* (Bechstein, 1800)	II, III	LC	LC	II	II	II, IV	+
10	*Plecotus austriacus* (Fischer, 1829)	II, III	LC	LC	II	II	IV	+
11	*Barbastella barbastellus* (Schreber, 1774)	II, III	VU	NT	II	II	II, IV	+
12	*Nyctalus noctula* (Schreber, 1774)	II, III	LC	LC	II	II	IV	+
13	*Pipistrellus pipistrellus* (Schreber, 1774)	II, III	LC	LC	II	II	IV	+
14	*Pipisterllus pygmaeus* (Leach, 1825)	II, III	LC	LC	II	II	IV	+
15	*Pipistrellus nathusii* (Keyserling, Blasius, 1839)	III	LC	LC	II	II	IV	+
16	*Eptesicus serotinus* (Schreber, 1774)	III	LC	LR	II	II	IV	+
17	*Vespertilio murinus* Linnaeus, 1758	II, III	LC	LC	II	II	IV	+
	Lagomorpha							
18	**Lepus capensis* Linnaeus, 1758							
19	**Oryctolaguus cuniculus* (Linnaeus, 1758) d.f.							
	Rodentia							
20	**Sciurus vulgaris* Linnaeus, 1758		LC	LC	III			
21	**Spermophilus citellus* (Linnaeus, 1766)	II	VU	VU	II		II, IV	
22	**Glis glis* (Linnaeus, 1766)		LC	LC	III			
23	*Muscardinus avellanarius* (Linnaeus, 1758)	II, III	LC	NT	III		IV	
24	*Dryomys nitedula* (Pallas, 1778)	II	LC	NT	III		IV	
25	*Sicista subtilis* (Pallas,1773)	III	CR	VU	II			
26	**Nannospalax leucodon* (Nordmann, 1840)		LC	DD				
27	*Micromys minutus* (Pallas, 1771)		NT	NT				
28	*Apodemus sylvaticus* (Linnaeus, 1758)		LC	LC				
29	*Apodemus flavicollis* (Melchior, 1834)		LC	LC				
30	*Apodemus uralensis* (Pallas, 1811)		LC	LC				
31	*Apodemus agrarius* (Pallas, 1771)		LC	LC				
32	*Mus specilegus* Petenyi, 1882		LC	LC				
33	*Mus musculus musculus* (Linnaeus, 1758)							
34	*Rattus norvegicus* (Berkenhout, 1769)							
35	*Rattus rattus* (Linnaeus, 1758)							
36	*Cricetulus migratorius* (Pallas, 1773)	II, III	VU	LC				
37	*Cricetus cricetus* (Linnaeus, 1758)	II, III	VU	LC	II		IV	
38	*Mesocricetus newtoni* (Nehring,1898)	II, III	VU	NT	II			
39	**Ondatra zibethicus* (Linnaeus, 1758)							
40	*Arvicola amphibius* (Linnaeus, 1758)		LC	LC				
41	*Microtus arvalis* (Pallas, 1778)		LC	LC				
42	*Microtus rossiaemeridionalis* (Ognev, 1924)		LC	LC				
43	*Microtus subterraneus* (de Selys-Longchamps, 1836)		LC	LC				

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Landscape Structure Impacts the Small Mammals as Prey of Two Wintering Groups of Long-eared Owls (Asio otus L.) from the Region of Silistra (NE Bulgaria)

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Abstract. We studied the diet of two wintering groups of long-eared owls (*Asio otus* L.) from the town of Silistra. During the winter months of 2013/2014, significant differences in the species composition and proportions of the main small mammals in owls' diet were recorded, due to differences in living conditions. A total of 1538 specimens were established, of which 1500 (97,5%) skeletal parts of small mammals, 36 bird specimens (2,3%) and two Coleoptera specimens. We collected 511 whole pellets in which 1183 specimens were identified; the other specimens were found in scattered pellets' parts. A total of 23 species of small mammals were identified: 5 species of Eulipotyphla, 3 species of Chiroptera and 15 species of Rodentia. Twelve of them were common for the two groups of wintering birds. Significant for the owls' diet were 8 species from the genera *Microtus* (3 species), *Apodemus* (3 species) and *Mus* (2 species). In the first territory (Danube Park), with primary importance were the openly living species – voles ($N_{(number\ of\ species)}$ = 54%, $B_{(biomass)}$ = 59%), and in the second (Forest Park Medzhidi Tabia) – forest, field and domestic species of mice (N = 56%, B = 48%). The recorded differences in the small mammal species composition and their percentage share were resulting from the features in landscape structure of the two regions studied, which showed a determining role for the diet of long-eared owls.

Key words: *Asio otus* pellets, Micromammalia, owls diet, Danube River coast.

Introduction

Long-eared owl (*Asio otus* Linnaeus, 1758) is a specialised predator feeding mostly on small mammals. The owls spend the winter in groups of up to 30–40 birds, preferring coniferous trees and choosing areas with good food supply (Glutz von Blotzheim & Bauer, 1994; Marks et al., 1999; Birrer, 2009; Mebs & Scherzinger, 2000). Researches on the long-eared owls' diet are extremely topical, given the multifaceted information we receive, both for its biological and ecological characteristics, and for a wide range of vertebrate animal preys, who are indicative for the state of the environment (Wijnandts, 1984; Korpimaki & Norrdah, 1991; Birrer, 2009). Through the analysis of the diet of the long-eared owl we obtain valuable data about the state of many rare or conservationally significant species of mammals and birds on the one hand (Simeonov, 1964; Simeonov & Petrov, 1986), and about the dominant species of small mammals of the particular region (Birrer, 2009) – on the other. Through dietary analyses, the regularities in the gradients of the environment and the importance of individual components determining the specificities in the communities of the preys can be established (Marti, 1973; 1976; Tome, 1994; 2000; 2003; Sharikov & Makarova, 2014; Tulis et al., 2015). Data from the winter months when owls gather at clusters of several dozen individuals, who excrete their pellets in the same place, are particularly valuable (Dziemian et al., 2012; Cecere et al., 2013). Such information for separate regions of Bulgaria is found in the works of Simeonov (1964; 1966), Simeonov & Petrov (1986), Milchev et al. (2003), Milchev & Ivanov (2016).

Landscape structure and microclimatic features have a determining importance for the living environment on which the species composition and distribution depend (Korpimaki & Norrdah, 1991; Tome, 2003; Aschwanden et al., 2005; Romanowski & Zmiehersky, 2008; Mori & Bertolino, 2015). In order to establish these patterns, it is necessary to compare different groups of owls at the same time, in the same region, but in areas with a different landscape structure. The aim of this study was to establish and compare the prey species composition of two groups of overwintering long-eared owls living in one region, but hunting and feeding in different landscapes. Such a research is carried out for the first time in Bulgaria. The objects of the survey are the food remains in owls’ pellets from the region of the town of Silistra (NE Bulgaria).

Material and Methods

The food remains from two wintering groups of long-eared owls during the winter months of 2013/2014 from the region of the town of Silistra (Fig. 1) were collected once on 18–21 April 2014. The first roosting group of birds was located in the Danube City Park (N 44°07'04'' E 27°15'21'', 19 m). Birds inhabited several microhabitats with different tree species (cypresses, pines, ivy-covered large trees). The second group of wintering birds was on the ridge part of the Medzhidi Tabia Forest Park (N 44°06'08'' E 27°15'31'', 131 m), at 2 km distance from the first group. The pellets there were collected under a group of black pines (*Pinus nigra* J.F.Arnold), around 50–60 years of age and about 10–12 m in height, bordering an asphalt road.

Fig. 1. Location of the two wintering *Asio otus* groups in the town of Silistra.

The collected material was cleaned and all bone remains, feathers and hairs from each pellet were separated individually. The materials from the disintegrated pellets were processed together, and bones and fragments of skulls and mandibles were separated from the post-cranial bones. The determination of the mammals was according to Peshev et al. (2004), Popov & Sedefchev (2003), Görner & Hackethal (1987) and the collection of the University of Forestry, Sofia. Young specimens of *Apodemus flavicollis* (Melchior, 1834) and *A. sylvaticus* (Linnaeus, 1758) were identified to a genus level, and in analysis of the abundance and biomass of both species they were distributed proportionally, as the proportions of the adults. According to the latest information from Nedyalkov et al. (2019) and Nedyalkov (pers. comm.), in the region of Silistra both the common vole (*Microtus arvalis* (Pallas, 1778) and the southern vole (*M. rossiaemeridionalis* Ognev, 1924) occur. Since they are very hard to determine without chromosomal analyses, they were united in the analysis and discussion as *Microtus arvalis/ rossiaemeridionalis*. The determination of the house mouse (*Mus musculus musculus* Linnaeus, 1758) and steppe mouse (*M. specilegus* Petényi, 1882) for part of the individuals was also impossible, given the heavily broken skulls and the lack of completion between the skull and the mandible. Non-determinable individuals are proportionally distributed between the identified to species level ones.

Birds from the pellets were divided into four size-weight groups according to Prof. Z. Boev (pers. comm.): first size-weight class, with average biomass 8,5 g: Wren (*Troglodytes*) – Robin (*Erithacus*); second, with average biomass 41,2 g: Sparrow (*Paser*) – Hawfinch (*Coccothraustes*); third, with average biomass 97,5 g: Starling (*Sturnus*) – Thrush (*Turdus*), and fourth, with average biomass 210 g: Jay (*Garrulus*) – Magpie (*Pica*). Their number was determined by the remains of skulls, thoracic bones and limb bones, and the size class – by model skeletons corresponding to a given size class.

The abundances and biomass of the small mammal species and genera were calculated as a percentage of the total number of preys or biomass per region. Information about the biomass of the mass species comes from measurements of individuals from different regions of Bulgaria. For the indeterminable species (some voles and mice), the weight was averaged. Information from the literature (Popov & Sedefchev, 2003; Görner et al., 1987) has been used for the rare species. The individual species biomasses used are included in Table 1.

Results and Discussion

During the study of the two wintering groups of *Asio otus* in Silistra, a total of 1538 prey specimens were established, of which 1500 skeletal parts of small mammals from 23 species, 36 of birds and two pairs of Coleopteran elytra (Table 1). Small mammals made up 97,5% of all prey specimens, and 96% of the total biomass in the diet of the wintering owls. The established small mammals were: 5 insectivorous species (Eulipotyphla), 3 bat species (Chiroptera) and 15 species of Rodentia. They made up 58% of the potential prey species for the region, and 77% of the established prey species so far for the whole country (see Kodzhabashev et al., 2020). Five species were recorded for the first time as prey of the long-eared owl in Bulgaria (*Sorex minutus*, *Neomys anomalus*, *Barbastella barbastellus*, *Nyctalus noctula* and *Apodemus uralensis*), six species are protected, three species can be considered very rare (see Kodzhabashev et al., 2020). Rare and protected species were represented by a small number of specimens (from 1 to 3–6), making up 0,07–0,3% of all small mammal preys identified. With the same low ratios were all species newly established as preys of *Asio otus*. We collected 511 whole pellets, and the 1183 preys in them had a total biomass of 35393,5 g. The other specimens were found in scattered pellets' parts. The average number of preys per pellet was 2,32. The average biomass was 69 g, and the average weight per one prey was 29,9 g. These results are similar to those of Marti (1976).

In total, for the region of Silistra, the significant preys in owl diet were representatives of three genera of rodents: *Microtus* ssp., with 47% of the abundance and 51% of the biomass; *Apodemus* ssp., with 27% of the abundance and 26% of the biomass; *Mus* ssp., with 20% of the abundance and 15% of the biomass. United, these three genera had an abundance of 94% of all preys

Table 1. Content of the pellets collected in two hunting territories of *Asio otus* L. in Silistra, NE Bulgaria. Av.w. – average weight; B – biomass.

Prey species	Av.w.(g)	Silistra total (2016)			Danube Park			Medzhidi Tabia		
		No ex.	No ex. %	B %	No ex.	No ex. %	B %	No ex.	No ex. %	B %
Mammalia										
Sorex araneus Linnaeus, 1758	10	8	0.52	0.17	4	0.53	0.17	4	0.51	0.18
Sorex minutus Linnaeus, 1766	5	6	0.39	0.06	4	0.53	0.08	2	0.26	0.04
Neomys anomalus Cabrera,1907	12	1	0.07	0.03	1	0.13	0.05			
Crocidura suaveolens (Pallas, 1811)	5	9	0.59	0.1	5	0.66	0.11	4	0.51	0.09
Crocidura leucodon (Hermann, 1780)	10.5	3	0.2	0.07	1	0.13	0.04	2	0.26	0.09
Plecotus austiacus (Fischer, 1829)	11.5	1	0.07	0.02				1	0.13	0.05
Barbastella barbastellus (Schreber, 1774)	9.5	1	0.07	0.02				1	0.13	0.04
Nyctalus noctula (Schreber, 1774)	29	1	0.07	0.06	1	0.13	0.12			
Muscardinus avellanarius (Linnaeus, 1758)	27	3	0.2	0.17	3	0.39	0.34			
Dryomys nitedula (Pallas, 1778)	28.5	2	0.13	0.12				2	0.26	0.25
Micromys minutus (Pallas, 1771)	7	11	0.72	0.17	1	0.13	0.03	10	1.29	0.31
Apodemus agrarius (Pallas, 1771)	20	104	6.76	4.5	17	2.24	1.44	87	11.2	7.61
Apodemus uralensis (Pallas, 1811)	16.5	6	0.39	0.2				6	0.78	0.43
Apodemus sylvaticus (Linnaeus, 1758)	25	54	3.51	2.9	20	2.63	2.12	34	4.37	3.72
Apodemus flavicollis (Melchior, 1834)	35	221	14.4	16.7	69	9.08	10.3	152	19.5	23.3
Apodemus flavicollis/ sylvaticus	30	35	2.28	2.3	21	2.76	2.68	14	1.8	1.84
Mus spicilegus Petényi, 1882	23	149	9.69	7.4	67	8.82	6.55	82	10.5	8.25
Mus musculus (Linnaeus, 1758)	23	7	0.46	0.35	7	0.92	0.68			
Mus musculus/spicilegus	23	152	9.88	7.5	91	12.0	8.9	61	7.84	6.14
Rattus norvegicus (Berkenhout, 1769)	375	1	0.07	0.8				1	0.13	1.64
Rattus rattus (Linnaeus, 1758)	200	1	0.07	0.4	1	0.13	0.85			
Mesocricetus newtoni (Nehring, 1896)	100	1	0.07	0.2	1	0.13	0.42			
Arvicola amphibius (Linnaeus, 1758)	200	2	0.13	0.9	2	0.26	1.7			
Microtus arvalis/rossiaemeridionalis	36	570	37.1	44.2	317	41.7	48.5	253	32.5	39.8
Microtus subterraneus (Selys, 1836)	20	151	9.82	6.5	96	12.6	8.16	55	7.07	4.81
Total (mammals):		*1500*	*97.5*	*95.8*	*729*	*95.9*	*93.2*	*770*	*99.1*	*98.59*
Aves (size classes)										
Wren–Robin	12.5	6	0.39	0.16	5	0.66	0.27	1	0.13	0.06
Sparrow–Hawfinch	42.5	18	1.17	1.65	15	1.97	2.71	3	0.39	0.56
Starling–Thrush	90	12	0.78	2.33	10	1.32	3.82	2	0.26	0.79
Total (birds):		*36*	*2.34*	*4.16*	*30*	*3.95*	*6.8*	*6*	*0.78*	*1.4*
Coleoptera										
Dytiscus sp.	2	1	0.065	0.004	1	0.13	0.01			
Coleoptera *sp. g.*	2	1	0.065	0.004				1	0.13	0.01
Total (beetles):		*2*	*0.13*	*0.008*	*1*	*0.13*	*0.01*	*1*	*0.13*	*0.01*
Total:		**1538**			**760**			**778**		

and 92% of the total biomass. Similar results and distribution of the main groups of preys established Milchev & Ivanov (2016) for the region of Dobrich.

Our results showed that preys with biomass between 21 and 50 g predominated, both in relation to their abundance, and each species' biomass (Fig. 2). They were followed by those weighing between 11 and 20 g. The share of the very small and very large preys was negligible. This analysis confirmed the data about the diet of the long-eared owl in south-eastern Europe (Birrer, 2009), as well as the results obtained by Tome (1994), on the optimal dimensions of priority and significant small mammal preys.

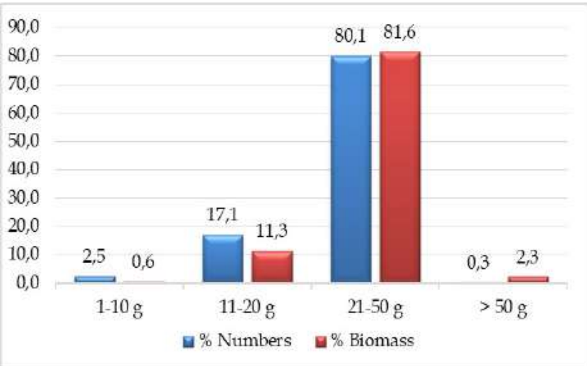

Fig. 2. Share of the different size-weight classes of the mammal prey in owls' diet.

We found 13 mammal species common to the two regions, including the 8 main for the owl's diet species (*M. arvalis*, *M. rossiaemeridionalis*, *M. subterraneus*, *A. flavicollis*, *A. sylvaticus*, *A. agraius*, *M. specilegus*, *M. musculus*), and the remaining 5 species (*S. araneus*, *S. minutus*, *C. suaveolens*, *C. leucodon*, *M. minutus*) had low abundance and biomass, i.e. they were of secondary importance. The other mammal species found had a small number of specimens, due to two main reasons – they are either difficult to capture, inaccessible and non-traditional (accidental) preys, or they are rare. The first group includes the rats (*R. norvegicus* and *R. rattus*), and the water vole (*A. amphibius*) always associated with the presence of water reservoirs. They are large, fast, strong and heavy, which is why they are most often presented with a small number of specimens and abundance between 0,1–0,3% and 3-4% (Simeonov & Petrov, 1986; Milchev et al., 2003; Milchev & Ivanov, 2016); in our research it was 0,2%. However, due to their relatively large mass, their share in the biomass in the owl's diet is significant (Simeonov & Petrov, 1986). The weight and size of these large preys are commensurate and even exceed those of the owl, which adults weigh from 227 to 326 g (Simeonov, 1990), and those of the brown rat are from 250 to 500 g (Popov & Sedefchev, 2003). The inclusion of these "heavy" preys in the diet biomass in their full mass is highly speculative and not entirely correct, given the ability of the owls to swallow the entire prey or to consume all its biomass. There are also no evidences if the predators return to the prey's corpse. Probably, a large proportion of the eaten specimens of these species are young, not reached the optimal size and weight for their species (Marti, 1976).

Accidental preys include amphiboitic and fossorial mammals. In our study, we found one water shrew (*N. anomalus*), whose presence is associated with the bordering Danube River. During the examination of the territory, fresh molehills and piles of dug-out soil were found, suggesting the activity of moles and mole-rats in the winter season. The European mole (*Talpa europea* L., 1758) and the lesser mole-rat (*Nannospalax leucodon* Nordmann, 1840) are potential owls' prey, but they are not traditional, given their hidden lifestyle and rare appearance on the surface, especially in the winter months. Another active in winter species is the squirrel (*Squirus vulgaris* L., 1758), which is dendrophilous and active during the day, hence it is an accidental owls' prey. It has been found as a prey just once in Bulgaria (Milchev et al., 2003). Near the Forest Park Medzhidi Tabia, within range of the owls' hunting territory, there was an active colony of ground squirrels (*Spermophilus citellis* L., 1766). This species is also a potential prey, registered once for Bulgaria (Milchev et al., 2003), but in our study it was not found, probably due to its daily activity and winter hibernation. Similar is the case with the edible dormouse (*Glis glis* L., 1766), who has winter hibernation, but has been found repeatedly in owls' diet, although its abundance was only 0,7% (Simeonov, 1964, Simeonov & Petrov 1986). In our study, we found the hazel dormouse (*M. avellanarius*) (3 ex.) and forest dormouse (*D. nitedula*) (2 ex.), which can be taken as an exception and atypical behavior, given the biological characteristics of these animals during the winter. According to Peshev et al. (2004), all three species are in hibernation by the end of April, but the initial period is not firmly established. Given our knowledge of the periods of formation of the wintering groups of owls, we can assume either that dormice fall into hibernation after mid-October, or during the winter, when prolonged uncharacteristic warmings occur, they temporarily come out from a state of hibernation and fall in wintering birds' menu. As an exception, the forest dormouse might be pointed, because it is known it can interrupt its hibernation for short periods (Peshev et al., 2004).

The identified three bat species can also be considered accidental prey of wintering owl groups. So far, only the gray long-eared bat (*P. austriacus*) has been registered as a prey of wintering owls in Bulgaria (Milchev et al., 2003). The other two species, the common noctule (*N. noctula*) and the western barbastelle bat (*B. barbastellus*), were here established as prey for the first time in Bulgaria. Six bat species have been reported as owls' prey for the territory of northern Eurasia (Sharikov & Makarova, 2014) – two species for Slovakia (Obuch, 1998), and for the Mediterranean zone two species and *Pipistrellus* spp. are reported (Garcia et al., 2005). These long-time studies on wintering groups of owls showed a dependence of the number of bats caught from the specific climatic conditions. When prolonged warmings in winter occur, bats are activated and fall into the owl menu, and in the absence of temperature fluctuations (if such warmings are missing), bats disappear from owl's diet. According the authors, owls do not have certain preferences for specific bat species, but hunt those available in the airspace.

To the category of the rare species found in owls' pellets, we can name the western barbastelle bat, Romanian hamster (*M. newtoni*) and the Ural field mouse (*A. uralensis*). The first two species are protected by the Biological Diversity Act and included in the Red Data Book of Bulgaria, and the third species has no national conservation status, but it has not been confirmed for our fauna since 1972, when the last studies on its bio-ecology were carried out (see Kodzhabashev et al., 2020).

The differentiated analysis of the hunting areas of the two groups of wintering owls showed significant differences in the proportions of the main food components. There were also differences found in the species composition of the preys. The food remains collected from the first group of owls from the Danube Park included 760 preys: 729 mammal specimens of 19 species, 30 specimens of birds of three size classes, and one beetle (Table 1). The average number of preys in one pellet was 2,22 and the average biomass was 67,7 g. The average weight of one mammal prey was 30,93 g. From the second group of wintering owls found in the Medzhidi Tabia Forest Park, remains from 778 preys were found: 771 mammals of 17 species, 6 birds of the same three classes and one diving beetle of the family Dytiscidae. The average weight of one mammal prey was 29,24 g.

Literary data about the daily dose of food needed to satisfy the energy needs of one owl individual are highly contradictory, the daily ratios varying from 80 to 120 g (Simeonov & Petrov, 1986). Our results showed that the daily ration limits vary between about 70 g (contained in one pellet) and 140 g (in two pellets). Given the average biomass of the preferred preys, 21–50 g, followed by these of 11–20 g. (Fig. 2), we can make different combinations of the eight main small mammal species preys. It is very likely that the preys in one pellet (2–4 specimens on average, depending on the individual mass), are the nutritional norm of one owl for a day. According to Marti (1976), *Asio otus* in Europe consumes 60 g per day at an average prey weight of 32,2 g. However, the diet is highly dependent on climatic conditions (temperature, snow cover, wind, etc.) and energy balance, which also determines the necessary calories for the normal functioning of all life processes (Wijnandts, 1984; Sharikov & Makarova, 2014).

In addition to the differences found in the average biomass and the number of preys in pellets from the two regions, we also observed differences in the species composition (Table 1), as well as in the abundance of the different prey species (Fig. 3, 4).

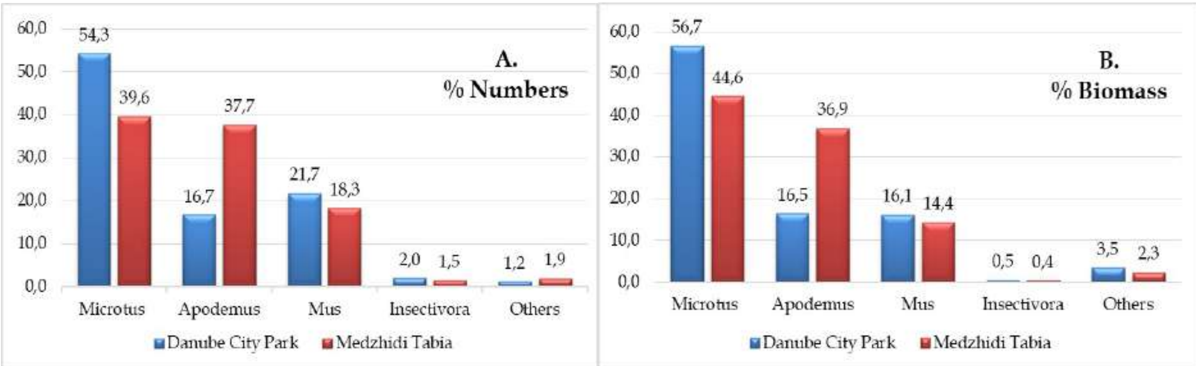

Fig. 3. Share of the most significant groups of preys:
A. Share in the total abundance; B. Share in the total biomass.

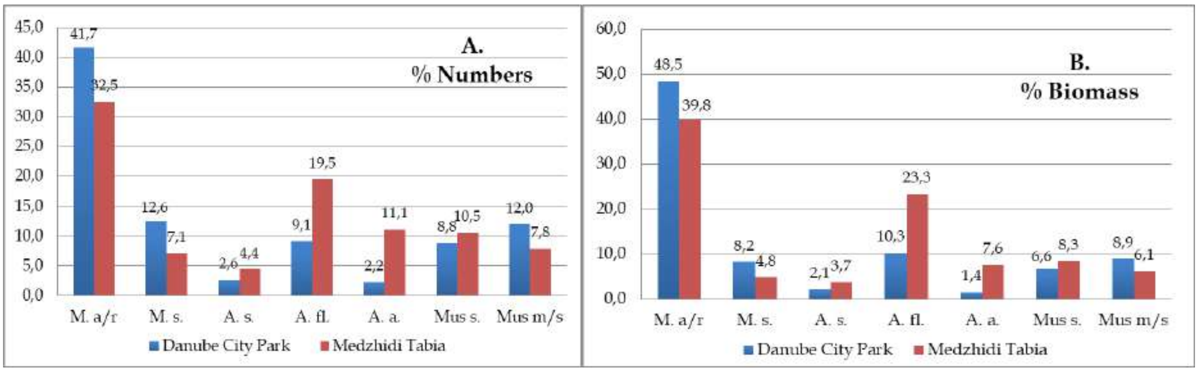

Fig. 4. Share of the most significant species of preys: M. a/r – *Microtus arvalis/rossiaemeridionalis*, M. s. – *M. subterraneus*, A. s. – *Apodemus sylvaticus*, A. fl. – *A. flavicollis*, A. a. – *A. agrarius*, Mus s. – *Mus specilegus*, Mus m/s – *M. musculus/specilegus*;
A. Share in the total abundance; B. Share in the total biomass.

In the hunting territories of the owls in the Danube Park, the share of the voles made up 54% of the total numbers and 57% of the total biomass, and for the Forest Park Medzhidi Tabia these values were 40% and 45%, respectively (Fig. 3, 4). Just the opposite was the share of forest and field mice (Fig. 3, 4). In the Danube Park, genus *Apodemus* had 17% of the total abundance and 17% of the total biomass; in Medzhidi Tabia the abundance was 38% and the share in the total biomass was 37%. The share of domestic mice of the genus *Mus* was similar for the two hunting regions, respectively: 22% of the numbers and 16% of the biomass in the Danube Park, and 19% of the numbers and 15% of the biomass in Medzhidi Tabia. The most significant difference in the diet of the two groups of wintering owls resulted from the main preys – voles and forest mice, which have different requirements for the specific environmental conditions and different behavior during the winter period. The results presented on Fig. 4 showed the significance of the main prey species. In both regions, dominant were the voles from the *Microtus arvalis*/*rossiaemeridionalis* group.

Habitats in the Danube Park, located next to the Danube River coast and bordering the urban environment, combined with compositions of watered lawns and groups of wood-shrub vegetation, can be divided into synanthropic (urban), semisynanthropic (forest park) and natural (coastal). The synanthropic landscape is extremely heterogeneous with a wide variety of micro-habitats, predetermining the rich species composition of small mammals. The landscape structure offers suitable conditions for open-living mesophilous and mesohygrophilous species. Regular mowing and presence of predominantly ornamental shrub and tree species with atypical (unknown to the native animals) seeds, are probably shaping an unsuitable environment for some of the forest dwellers, including *A. flavicollis* and *A. agrarius*. The presence of mesohygrophilous species, characteristic mainly of mountain habitats, is registered for both the Northern Dobrudzha (Murariu, 2005; Miu et al., 2018), and some plain regions of Bulgaria (Peshev et al., 2004) and Southern Dobrudzha (Simeonov, 1966; Milchev & Ivanov, 2016). As such species, *S. minutus*, *N. anomalus*, *M. subterraneus*, *B. barbastellus* can be classified. According to Miu et al. (2018), other typical mountain species such as *Myodes glareolus* (Schreber, 1780) and *Neomys fodiens* (Pennant, 1771) are also registered near the Danube Delta. In Bulgaria, *M. glareolus* is found at the sea level around the mouth of the Kamchchia River (Peshev et al., 2004), and *N. fodiens* – near Plovdiv (Markov, 1957) and Yambol (Simeonov & Petrov, 1986). Only in the Danube Park we found *N. anomalus*, *A. amphibius*, *M. avellanarius*, *R. rattus*, *M. musculus*, *M. newtoni*, and *N. noctula*. Their requirements are very different and can be grouped into four habitat complexes – synanthropic and eusynanthropic (for the domestic mouse and black rat), water and hygrophilic (for *N. anomalus* and *A, amphibius*), forest mesophilic with rich undergrowth (for *M. avellanarius*), and natural and cultural open, steppe-like areas with thick and deeply drained soil horizon (for *M. newtoni*). The main preys in the Danube Park were of the genus *Microtus*, representatives of the open habitats, but *M. subterraneus* is mesophilous, and the other two are xeromesophiles, preferring cultivated areas and agrocoenoses.

The hunting territories of the owls from the Forest Park Medzhidi Tabia were extremely diverse. Next to the hill where the park is located, lay many agricultural lands, pastures and small livestock farms, two micro dams and two small but permanent creeks powered by the micro dams. The forest habitats are old black pine and black locust plantations and semi-natural forests with numerous hollow trees, mostly walnuts. In this region only, we found *P. austriacus*, *B. barbastellus*, *D. nitedula*, *A. uralensis*, and *R. norvegicus*. Here we found the three forest mice species – *A. flavicollis*, *A. sylvaticus*, and *A. uralensis*, and the ratio between them was, respectively, 26: 6: 1. The predominance of forest and field mice (Fig. 4) is in accordance with the dominating forest habitats, fragmented by numerous mesophilic gullies, meadows and agricultural areas. The main preys with greatest importance in the owls' diet in this area were *A. flavicollis* and *A. agrarius*, followed by the steppe mouse (*M. specilegus*).

The established in the Danube Park places for hiding during the day were groups of several tall false cypresses (*Chamaecyparis* sp.) or black pines with a height of 8–10 m, located next to very lively, roadside places. In the specific year 2013, the total number of owls counted on 26 December was 56. In the area of the Medzhidi Tabia, such places, established on 26 and 27 December, were different from where we collected the biological material and the counted birds were about 40, found amongst an old black-pine plantation.

During our visit, at 19 April 2014, when we collected the biological material, we found a shell of a freshly hatched owl egg, which indicated that the breeding period probably started at the beginning of March or even in the end of February. These data differ significantly from those given by Simeonov & Petrov (1986) and Simeonov (1990), who stated that the breeding period begins after mid-March. It is likely that the beginning of the breeding period depends on the specific climatic conditions of the current year. The observed trends of global climatic changes, with shortening of the periods with cold and snowy days during the winter, are likely to cause atypical biological reactions, disrupting the natural rhythm of the biological clock of small mammal species and their natural cyclic hibernation in the winter season.

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Characterization of High Light Inducible (Hli) Proteins of Chlorophyll-protein Complexes of Cyanobacteria

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Abstract. Stress Hli proteins (Hlips) of cyanobacterium *Synechocystis* sp. are necessary for the survival of cells and their adaptation to high light. Nonetheless, the whole picture of Hlips functioning and distribution in these cells is not completely understood and the data for other cyanobacteria such as *Arthrospira platensis* is missing. Studies of *Synechocystis* sp. have shown that HliA protein is associated with the main chlorophyll-protein complexes: with trimers and monomers of photosystem I and photosystem II complexes. According to the NCBI database, there are three *Hli* genes in *A. platensis* genome that encode proteins of 47, 64, and 69 amino acids long. In current study MALDI-TOF mass spectrometry analysis of *A. platensis* revealed presence of Hli 47 amino acids long only. Identified Hli protein is associated with photosystem I and photosystem II and is a homologue of HliC *Synechocystis* sp. Bioinformatics analysis of the amino acid sequence of the identified Hli protein of *A. platensis* revealed a high degree of homology with the amino acid sequences of proteins of a number of other multicellular cyanobacteria and a lesser degree with the Hli amino acid sequence of unicellular cyanobacteria. Results of current study confirms the importance of Hlips for the photoprotection of the photosynthetic apparatus of cyanobacteria.

Key words: high light inducible proteins, stress proteins, abiotic stress.

Introduction

Cyanobacteria are widely used as a model organism either in applied or basic research. Cyanobacterium *Synechocystis* and its transformed strains are used in biotechnology to produce various industrial chemical compounds (Gale et al., 2019; Pope et al., 2020). The photosynthetic apparatus of cyanobacteria and higher plants has a high similarity, which makes cyanobacteria a convenient model for studying plant photosynthesis. Light is essential for photosynthesis, but excess of absorbed light energy can disrupt photosynthetic apparatus and lead to photoinhibition and photodestruction. When absorbed energy cannot be fully used in electron transport reactions the content of singlet oxygen and other reactive oxygen species are increasing. Thus, photosynthesis and photoprotection are two interrelated processes.

Cyanobacteria must have the ability to acclimate to changing of light conditions. Protection mechanisms of photosynthetic apparatus of cyanobacteria under light stress can be divided into short-term and long-term processes. Short-term processes are associated with quenching of excited chlorophyll molecules and energy migration in photosynthetic apparatus. These responses occur rapidly and are usually completed within several minutes. The long-term processes are associated with changes in gene expression of proteins that play an important role in cell defense responses, in particular against photo-oxidative damage. The long-term reactions are much slower and may take up to several days to complete. These processes involve changes in photosystem I (PSI) / photosystem II (PSII) ratio during adaptation to high light, synthesis of antioxidant enzymes and biosynthesis of light stress proteins: Elip (Early Light Induced Protein) in plants and Hlip (High Light Inducible Protein) in cyanobacteria.

Cyanobacterial Hli proteins are necessary for maintaining normal cell activity. Mutants with inactivated *hli* genes die from high light (Xu et al., 2004; Yurina et al., 2013). Intended functions of Hli proteins are the next: regulation of chlorophyll biosynthesis, transport and binding of free chlorophyll molecules, quenching of singlet oxygen, assembly and repair of PSII, non-photochemical quenching of absorbed light energy (Havaux et al., 2003; Yao et al., 2012). However, the main function of these proteins is still unclear.

Most studies of Hli proteins were performed on cyanobacterium *Synechocystis* sp. PCC 6803. Four *hli* genes were identified in the genome of *Synechocystis* sp. - *hliA, hliB, hliC* and *hliD* (Kilian et al., 2008; Yurina et al., 2013). It is known that: the synthesis of these proteins is increased by 3-4 times under light stress, Hli proteins are localized in the thylakoid membrane, they belong to the family of chlorophyll a/b binding proteins (LHC) and have a molecular weights about 10 kDa (Kilian et al., 2008). It was shown that stress Hli proteins of cyanobacterium *Synechocystis* are necessary for cell survival and adaptation in high light. However a lot of question about their structure, localization, functioning and role in wide range of cyanobacteria are remained unsolved. In current research the characteristics of high light inducible proteins of chlorophyll-protein complexes of cyanobacteria unicellular *Synechocystis* sp. and multicellular *Arthrospira platensis* were performed.

Material and Methods

Cells and growth conditions. The object of the study were the cyanobacterium *Synechocystis* sp. PCC 6803: wild-type cells, PSII-less mutant (ΔpsbDI, ΔpsbDII, ΔpsbC) and *Arthrospira platensis* IPPASB-256. Cells were cultivated in BG-11 medium (Akulinkina et al., 2015) at 28°C. The culture was bubbled with air under normal light conditions (40 µmol photons/m2 • s) and under stress conditions (150 µmol photons/m2 • s, 1 h). PSII-less mutant cells were grown at low light intensity (5 µmol photons/m2 • s) and supplemented with 5 mM glucose, and antibiotics (chloramphenicol 20 mg/ml, erythromycin 15 mg/ml, spectinomycin 20 mg/ml).

Isolation of thylakoid membranes. Thylakoid membranes of *Synechocystis* sp. and *A. plathensis* were isolated as described by (Shubin et al. 1993) with modification. Briefly, the cells were disrupted using a French-press (three times for each sample) in a medium A (50 mM MOPS, pH 7.0; 0.4 mM sucrose; 10 mM NaCl; 1 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged consistently at 5000 g for 10 min and at 50 000g at 4°C for 60 min. The membranes were resuspended in thylakoid buffer A to a chlorophyll a concentration of 1 mg/ml. To fractionate the chlorophyll-protein complexes, 10% dodecyl maltoside was added to the thylakoid membranes to achieve a detergent to chlorophyll ratio of 15:1. The membrane was solubilized at 4°C for 30 min. After incubation the lysate was centrifuged at 18 000 g for 10 min. The supernatant was subjected to Clear Native PAGE (CN PAGE) and SDS-PAGE.

SDS-PAGE. Proteins were fractionated by 12,5% SDS-PAGE. Protein concertation in the sample was determined by the method of Bradford (1976). Before applying the samples heated at 95^0C for 10 min and then centrifuged at 18 000 g for 10 min. SDS-PAGE was performed as described (Akulinkina et al., 2015).

Two-dimensional electrophoresis and MALDI-TOF protein identification. Proteins were fractionated using two-dimensional electrophoresis: in the first direction CN PAGE was used, in the second direction - 12.5% SDS-PAGE (Wang et al., 2008). Mass spectrometry MALDI-TOF was used for proteins identification. Protein identification was performed using the Mascot program (www.matrixscience.com). The mass spectra were processed using the FlexAnalysis 3.3 software package (Bruker Daltonics, Germany). MALDI-TOF analysis was performed in the center for collective use "Industrial biotechnologies" Research Center of Biotechnology of the Russian Academy of Sciences.

The photochemical activity of PSI was determined as described earlier (Wang et al., 2008). The data presented are mean values from at least three independently grown cultures and experiments. Error bars represent standard errors of mean values. The statistical assessment of the results was performed using the Student's t-test.

Results and Discussion

One of the nowadays-discussed questions of *Synechocystis* biology is which of the chlorophyll-protein complexes in thylakoid membranes is associated with HliA protein? Conflicting data of localization of Hli proteins in the chlorophyll-protein complexes photosystem I and photosystem II were published (Wang et al., 2008; Komenda, Sobotka, 2016). The fractionation of thylakoid membrane of wild-type *Synechocystis* cells using CN PAGE/SDS PAGE was performed. It revealed the next fraction: PSI trimers, and monomers, PSII dimers, and monomers. Western blot analysis revealed the presence of HliA protein in fractions containing PSI trimers, PSI monomers, and PSII complexes that is consistent with previously obtained by us data (Akulinkina et al., 2015). To check the conflicting published data on the association of Hli proteins with PSI, a mutant lacking PSII complex was used. CN PAGE/SDS PAGE with Western blotting of mutant cells reveled that HliA protein was associated with the fraction of PSI complex.

For understanding the role of HliA in functioning of PSI *Synechocystis* the wild-type and ΔPSII mutant cells were used. Grown under low light ΔPSII mutant cells accumulates HliA protein meanwhile in wild-type cells Hli A is absent under these conditions. Both cell types were cultivated in low light and methylviologen-dependent oxygen absorption was measured. It was detected that the electron transport rate through the PSI in ΔPSII mutant cells was significantly higher than in wild-type cells grown under the same conditions (Fig. 1). The photochemical activity of the PSI complex in the membranes of a ΔPSII mutant with HliA protein was three to four times higher than in the membranes of wild-type cells without HliA protein. This data indicates that HliA protein is necessary for optimal PSI activity. The results on the low rate of photochemical absorption of PSI oxygen in the membranes of wild-type cells that contain almost none of them were consistent with previously published (Wang et al., 2008).

Numerous protective mechanisms have been developed in photosynthetic organisms in evolution for adaptation under light stress. Transition from single to multicellular organization cells could develop additional approaches of adaptation to light conditions. As compared to unicellular *Synechosystis* the multicellular *A. platensis* has another mechanism of photoprotection due to presence of long-wave chlorophyll (Karapetyan et al., 2014). It is also interesting that the spectral characteristics of chlorophyll-protein complexes of trimers and monomers of PS I *A. platensis* differ from

Synechocystis. Long-wave forms of chlorophyll were found in the thylakoid membranes of multicellular cyanobacterium *A. platensis* (Karapetyan et al., 2014). Probably, this may indicate the presence of specific features in the mechanism of protection of *A. platensis* from light stress. Long-wave chlorophyll with a fluorescence band at 760 nm was also found in other multicellular cyanobacteria - *Pseudoanabaena* sp., *Phormidium uncinatum* and *Nostoc muscorum*, but not in unicellular cyanobacteria *Synechocystis* sp. and *Synechococcus elongatus*, often used for research in the physiology and biochemistry of photosynthesis (Gobets et al., 2001). Apparently, this may indicate the presence of features in the mechanism of protection *of A. platensis* and other filamentous cyanobacteria from light stress.

In current study association of chlorophyll-protein complexes of thylakoid membranes with Hli proteins *Arthrospira platensis* were investigated. According to the NCBI database, there are three Hli genes in *A. platensis* genome that encode proteins of 47, 64, and 69 aa (amino acids) long. Clear Native PAGE and two-dimensional electrophoresis followed by mass spectrometry MALDI-TOF were used to determine the association of Hli proteins with chlorophyll protein complexes of thylakoid membranes. The identification of all colored protein spots on 2D-electrophoregram was performed. On Fig. 2 identified low-molecular-weight protein are shown. In our study MALDI-TOF mass spectrometry analysis of *A. platensis* proteins fractionated by two-dimensional PAGE revealed presence of Hli 47 aa long only (Fig. 2). It was observed that the identified Hli protein is associated with PSI and PSII complexes.

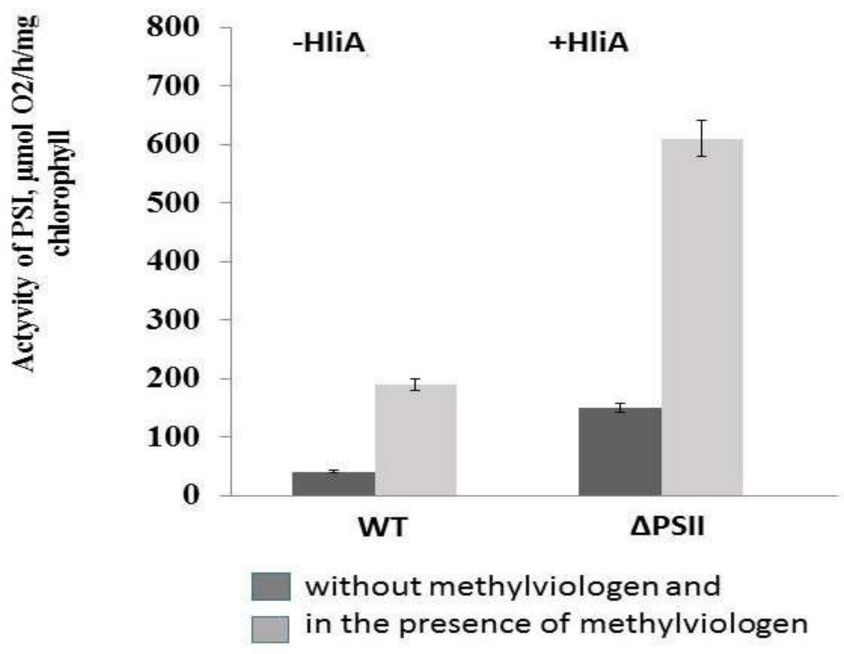

Fig. 1. Photochemical activity of PSI complex of thylakoid membranes of *Synechocystis* sp. wild-type (WT) cells and mutant without PSII in the absence and in presence of methylviologen.

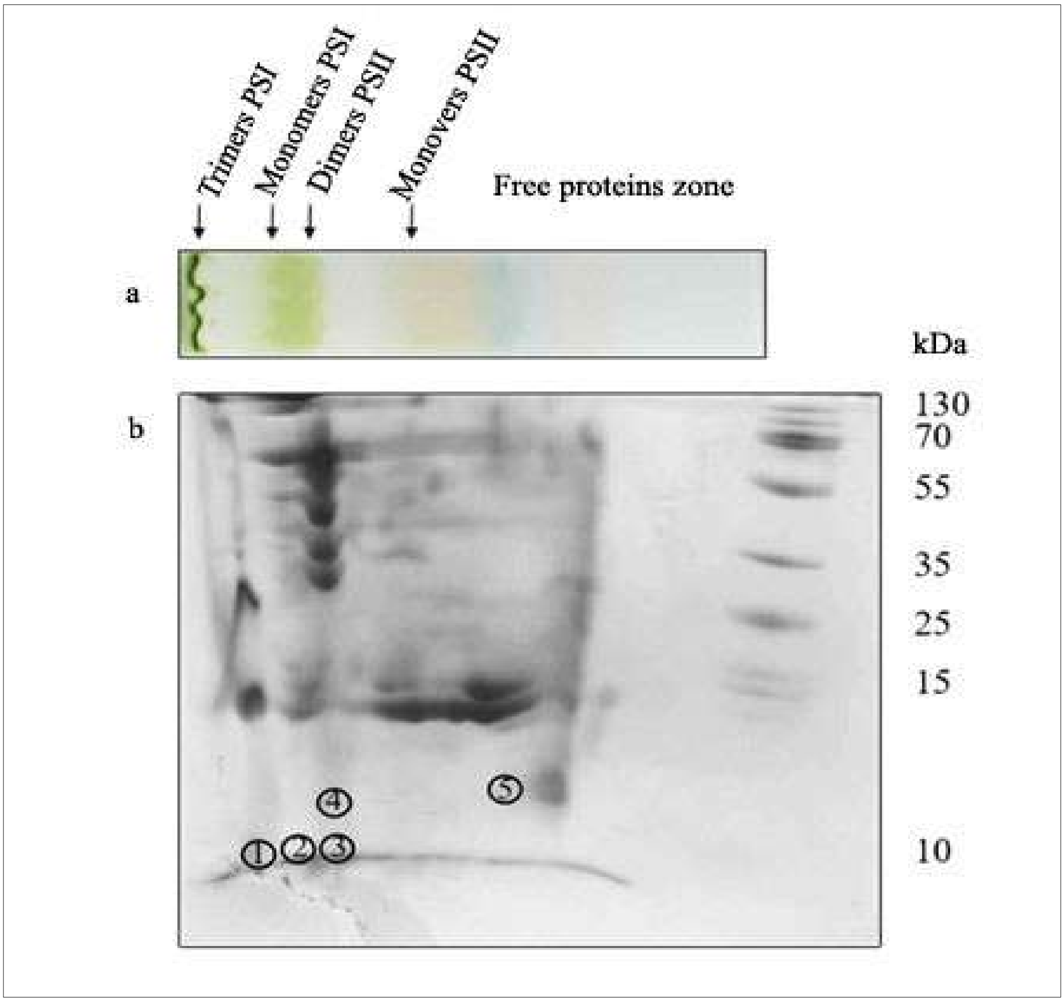

Fig. 2. 2D-electrophoresis of thylakoid membrane proteins of *A. platensis* after light stress: (a) PAAG under native conditions of chlorophyll-protein complexes; (b) SDS-PAGE of thylakoid membrane proteins (1-5 – protein spots identified by MALDI-TOF mass spectrometry): 1-3- Hli 47 aa proteins; 4 – Psb27 protein of PSII; 5 – alpha subunit of phycocyanin.

Bioinformatics analysis of amino acid sequence of identified Hli 47 aa protein of *A. platensis* revealed a high degree of homology with amino acid sequences of proteins of a number of other multicellular cyanobacteria and a lesser degree with the Hli amino acid sequence of unicellular cyanobacteria. Alignment of amino acid sequences of Hli proteins of different species revealed a conservative region corresponding to transmembrane helix (Fig. 3). The aa (Glu, Asn, and Arg) presumed to be involved in chlorophyll binding (Funk et al., 2011) were located. This allows as to conclude that identified Hli 47 aa protein *A. platensis* is homologue of HliC protein *Synechocystis* sp. (Fig. 3).

Structural model of low-molecular light-induced protein Hli 47 aa *A. platensis* was calculated basing on the crystal structure of photoprotective protein PsbS of spinach (Fig. 4). The structural data pointing out that at least four chlorophyll molecules and two β-carotene molecules can bind to the HliC protein and to its homologue Hli 47 aa. Hydrophobicity profile shows that identified Hli 47 aa protein is a membrane protein.

Fig. 3. Alignment of amino acid sequences of Hli proteins of *Synechocystis* sp. and *A. platensis* using the UniProt program.

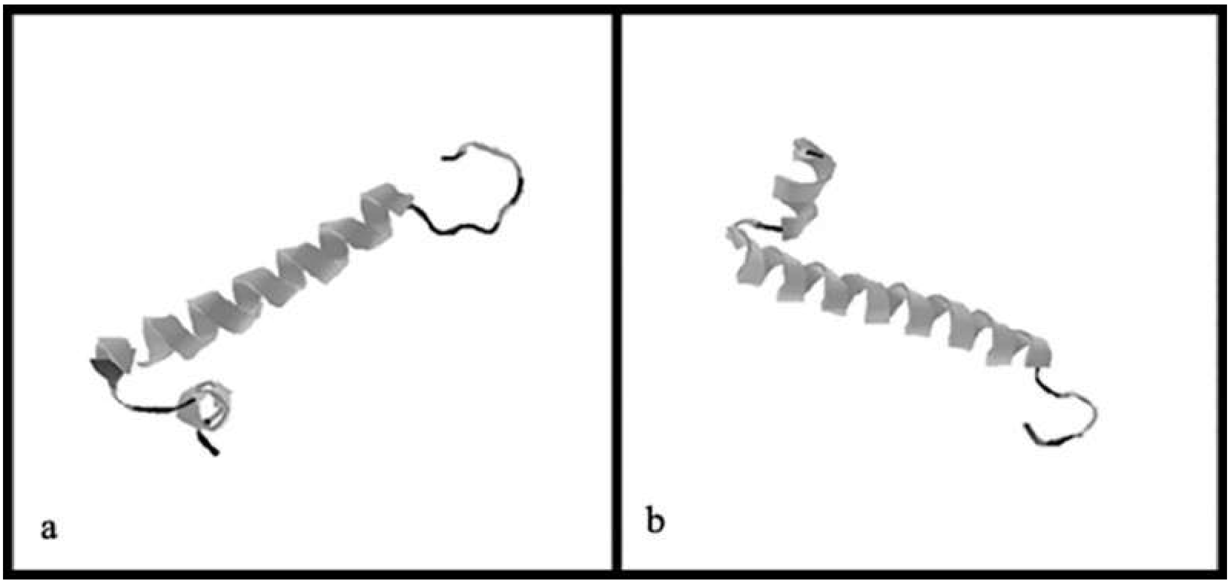

Fig. 4. Structural model of low-molecular light-induced protein Hli 47 a.a. of *A. platensis* in different projections. The model is calculated basing on the crystal structure of the photoprotective protein PsbS of spinach (*Spinacia oleracea*). (a) side view along the plane of the thylakoid membrane, (b) view from the side of the stroma. RCSB PDB database website.

Conclusions

It is shown that light-induced protein HliA *Synechocystis* sp. PCC 6803 is associated with photosystem I complex, as well as with photosystem II complex and is necessary for optimal photochemical activity of PSI cells of cyanobacterium.

High light-induced protein Hli 47 a.a. *Arthrospira platensis* was first identified. This protein was found to be associated with photosystem I and II under light stress.

Comparative analysis of Hli proteins of cyanobacteria *A. platensis* and *Synechocystis* sp. revealed the structural and functional homology of Hli 47 a.a. *A. platensis* and HliC *Synechocystis* sp.

Results of current study involving data of association of Hli proteins with complexes of photosystems I and II, their necessity for optimal photochemical activity, as well as the presence of chlorophyll-binding domains, indicate the importance of these proteins for the photoprotection of the photosynthetic apparatus.

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Effects of Salt Stress on the Photosynthesis of Maize and Sorghum

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Abstract. In this study, the effects of salt stress on the photosynthetic processes in sorghum (*Sorghum bicolor* L. *Albanus concep*) and maize (*Zea mays* L. *Mayflower*) were compared. The plants were grown in half-strength Hoagland solutions containing different NaCl concentrations (0, 50, 150 and 250 mM NaCl) for 6 days. Pulse Amplitude Modulated chlorophyll fluorescence, photooxidation of P_{700} and pigment analysis were used for characterization of the salinity effects on the studied plants. The treatment of plants with the high concentrations of NaCl led to an inhibition of the chlorophyll fluorescence parameters like the photochemical quenching, the rate of photosynthesis and the linear electron transport in sorghum and maize. All these changes corresponded to decrease in the pigment content and changes in chlorophyll *a*/*b* ratio. The analysis of the P_{700} photooxidation revealed that the photosystem I photochemistry was inhibited at the highest NaCl concentration in both studied plants. Data also revealed that sorghum is more sensitive to salt stress than maize. The reasons for different effects of salt stress on the maize and sorghum are described.

Key words: chlorophyll fluorescence, pigment content, salt stress, sorghum, maize.

Introduction

Salt stress is one of the major abiotic stress factors that restricts growth and yields of many crop plants (Acosta-Motos et al., 2017; Ashraf & Harris, 2013). Over the past years the climate changes have led to increase of soil salts (especially sodium salts), making much of the cultivated land unsuitable for agricultural purposes. This has become a major environmental problem worldwide, as the soil salinity affects about 30% of irrigated and 6% of the total area (Daliakopoulos et al., 2016). In Bulgaria, saline soils occupy approximately 33 310 ha (Shishkov & Kolev, 2014), as they are most common in the Plovdiv region - along the Maritza River. There are also affected areas around Burgas, Varna, Veliko Turnovo, Pleven, Sliven, Stara Zagora and Yambol. Depending on their electrical conductivity (EC), saline soils are divided into some classes: non-saline (< 2 mS/cm), slightly saline (2 – 4 mS/cm), moderately saline (4 - 8 mS/cm), very saline (8 - 16 mS/cm) and highly saline (> 16 mS/cm) (Ivushkin et al., 2019). The extent of salt-induced negative effects in plants is determined by the salt concentrations in the soil, the duration of stress and the genotype of plants species (Acosta-Motos et al., 2017; Dulai et al., 2019; Munns, 2005).

The subjects of this study were two important crops from family *Poaceae*: maize (*Zea mays* L. *Mayflower*) and sorghum (*Sorghum bicolor* L. *Albanus concep*). Sorghum is a herbaceous plant that is used for food, feed, alcoholic beverages and biofuel. Moreover, sorghum is the fifth most spread crop in the world (Mundia et al., 2019). Areas occupied by sorghum are not as widespread as other cereals in Bulgaria (about 6 000 ha), but they have increased in recent years, especially due to the recurrent of droughts and high temperatures of the south part of the country (Slanev, 2018). Maize is also representative of the cereal family and is used for grain, silage and green fodder. Today, it is the world's second-largest cereal crop (Gong et al., 2015). In recent years, the arable land with maize in Bulgaria are about 418,000 ha (Tanev, 2017). Unlike sorghum, it is not dry-resistant crop and high yields cannot be obtained without irrigation.

Further a positive relationship between the photosynthetic capability and crop production has been well documented (Long et al., 2006). At the same time, it has been found that the photosynthesis is very sensitive to soil salinity (Ashraf & Harris, 2013; Dulai et al., 2019; Kalaji et al., 2018). Previous studies revealed that salt stress affects differently the amount of photosynthetic pigments in plants, as in salt-tolerant species, the content of chlorophylls and carotenoids increases, while in salt-sensitive species, it decreases (Akram & Ashraf, 2011; Lu et al., 2003; Santos, 2004; Sudhir & Murthy, 2004).

One of the sensitive sites of photosynthesis under salt stress is the photosynthetic apparatus (Jusovic et al., 2018; Stefanov et al., 2019), as the photosystem II (PSII) complex is considered to be the most sensitive site (Baker, 1991; Hakala et al., 2005; Lu et al., 2003; Vass, 2012). The previous studies have demonstrated that the sensitivity to salt stress of this complex depend on the type of the plants (Abdeshahian et al., 2010; Al-Taweel et al., 2007; Jajoo, 2014; Loreto et al., 2003; Saleem et al., 2011). The study of Mehta et al. (2010) has shown that salt stress affects the heterogeneity of PSII with respect to the antenna size. The changes in both the donor and the acceptor side of PSII have been also reported (Jajoo, 2014). In recent years, the studies with wheat plants have shown that the changes in the functions of the photosynthetic apparatus have been accompanied by an increase in the energy transfer to the photosystem I (PSI), as a result of unstacking of the thylakoid membranes and/or a change in the size of PSI antenna (Jusovic et al., 2018; Meng et al., 2016).

The aim of this study was to compare the impact of salt stress on the photosynthetic processes of two economically important crops: the maize (*Zea mays* L. *Mayflower*) and the sorghum (*Sorghum bicolor* L. *Albanus concep*). For this purpose, we examined the influence of three concentrations of NaCl (50 mM, 150 mM and 250 mM NaCl) on the pigment content, chlorophyll fluorescence and PSI photochemistry (photooxidation of P_{700}). The experimental results evaluated the degree of soil salinization at which the investigated plant species can be grown.

Materials and Methods

Plant growth conditions and treatments

The experiments were carried out with hybrids of maize (*Zea mays* L. *Mayflower*) and sorghum (*Sorghum bicolor* L. *Albanus concep*). The plants were grown in half-strength Hoagland solutions. The plants were grown in a photothermostat with controlled conditions: a 12 h light/dark photoperiod, a light intensity of 150 μmol/m^2.s, 28°C (daily)/25°C (night) temperature and a 55% relative humidity. The effects of different concentrations of NaCl (50 mM, 150 mM and 250 mM) for 6 days were investigated. The treatments of plants with NaCl were performed on 15 days old plants. Electrical conductivity (EC) of nutrient solutions with different amounts of NaCl were experimentally established (Table 1).

Table 1. Electric conductivity (EC) of half-strength Hoagland solutions with different amount of NaCl. The values marked with different letters have statistically significant differences at $p < 0.05$.

NaCl (mM)	0	50	150	250
EC (mS/cm)	1.01 ± 0.07^{d}	5.99 ± 0.82^{c}	14.70 ± 1.40^{b}	23.20 ± 1.23^{a}

The pigments were extracted from leaves with an ice-cold 80% (v/v) acetone in the dark. Then the homogenates were centrifuged at 2500xg for 8 min at 4 °C. The total chlorophyll content and total carotenoids were determined spectrophotometrically at 470 nm, 646.8 nm, and 663.2 nm. For determination of the pigment amount, the equations of Lichtenthaler (1987) were used. The pigment ratios chlorophyll *a/b* *(chl a/b)* and carotenoids/chlorophylls (*car/chl*) were also determined. The measurements were made on spectrophotometer (SPECORD 210 PLUS, Edition 2010, Analytik-Jena AG, Germany).

Room temperature chlorophyll fluorescence

The chlorophyll fluorescence at room temperature of dark-adapted leaves was measured using a pulse amplitude modulated fluorometer (model 101/103, Walz GmbH, Effeltrich, Germany). The dark adaptation of leaves was 20 min. The maximum fluorescence levels in the light-adapted state (Fm′) were determined by a 0.8 s saturated pulse at 2800 μmol/m^{2}s PPFD. The actinic light was 150 μmol/m^{2}s PPFD. The following parameters were determined: the coefficient of photochemical quenching – $q_P = (Fm' - F')/Fv'$; the linear electron transport rate – $ETR = (Fm' - F')/Fm' \times 150 \times 0.5$. The determination of the parameters was made as in Stefanov et al. (2016). The chlorophyll fluorescence decay ratio $R_{Fd} = Fd/Fs$ was determined according to Lichtenthaler et al. (2005), where Fd is fluorescence decrease from Fm to a steady state chlorophyll fluorescence (Fs) after continuous saturated illumination (2800 μmol/m^2.s PPFD). This ratio (R_{Fd}) correlates with the net assimilation of CO_2 (Lichtenthaler et al., 2005).

P_{700} photooxidation

The measurements of PSI photochemistry by the oxidation-reduction properties of P_{700} were made using a PAM-101/103 fluorometer (Walz, Effeltrich, Germany) equipped with an ED-800T emitter-detector system. The oxidation–reduction kinetics of P_{700} were determined by the illumination of dark-adapted (for 20 min at room temperature) leaf discs with a far-red light supplied by a photodiode (102-FR, Walz, Effeltrich, Germany). The relative changes of P_{700}^{+} ($\Delta A/A$) was assessed by measuring of the far-red light induced absorbance changes around 830 nm as described in Dankov et al. (2009).

Statistical analysis

Mean values ± SE were calculated from the data of at least two independent experiments with four replicates of each variant. Statistically significant differences between variants of studied parameters were identified by analysis of variance (ANOVA) followed by a Tukey's post-hoc tests for each parameter. Prior to the tests, the assumptions for the normality of raw data and the homogeneity of the variances were checked. The homogeneity of variance test was used to verify the parametric distribution of data. Values of $p < 0.05$ were considered as significantly different.

Results and Discussion

Photosynthetic pigment content

The influence of different NaCl concentrations on the pigment content of studied hybrids of maize and sorghum is shown on Table 2. Data revealed that the treatment with the lowest studied NaCl concentration (50 mM) led to a slightly increase of photosynthetic pigments. In opposite, the pigment content decreased after the treatment with higher NaCl concentrations, as the effect on the carotenoids was smaller than the chlorophylls. The experiments with sunflower callus cultures indicated that the impact of salt stress on chlorophyll amount is a result from more stronger influence on the chlorophyll biosynthesis rather than the chlorophyll degradation (Akram & Ashraf, 2011; Santos, 2004**)**. In addition, it has been also shown that the decrease in the total chlorophyll content in salt-stressed leaves is mainly due to the degradation of chlorophyll *a*, which is much more sensitive to salinization than the chlorophyll *b* (Misra et al., 1990).

The variation in the pigment composition after salt treatment influenced the ratio *chl a/b*. Similar increase of the *chl a/b* ratio under salinization was registered in wheat (Shahbaz et al., 2008). In previous investigations have been shown that changes in membrane stacking and amount of light harvesting complex of PSII can also influence on this ratio (Apostolova et al., 2006; Stoichkova et al., 2006). Having in main this statement, it could be suggested that NaCl treatment of maize and sorghum can influence the membrane stacking. Previous investigations also revealed that the high salt level reduces the number and the thickness of stacked granal thylakoid membranes as well as the disintegration of grana **(**Lee et al., 2013; Meng et al., 2016; Stefanov et al., 2019).

Influence of the salt stress on the photosynthetic apparatus

The influence of different NaCl concentrations on the function of photosynthetic apparatus is shown on Fig. 1. The reduction in the pigment composition at the highest NaCl concentration (250 mM) corresponds with a strongly inhibition of the functions of photosynthetic apparatus in both studied crop plants. Data revealed an inhibition of the photochemical quenching (q_P) with 31% for the maize and 68% for the sorghum, which is a result from a decrease of the open PSII centers, and corresponds with an inhibition of the electron transport rate (ETR). At the same time, a decrease of the parameter R_{Fd} and the amount of P_{700}^+ were also registered. The inhibition of all studied parameters (R_{Fd}, q_P, ETR and ΔA/A) was stronger in the sorghum than in the maize (Fig. 1). The salt-induced effects on the functions of photosynthetic apparatus could be a result from changes in the structure and the composition of the thylakoid membranes (Stefanov et al., 2019). In addition, experimental results showed smaller salt-induced changes after the treatment with 150 mM NaCl, while after the treatment with 50 mM NaCl the values of studied parameters were similar to those of untreated plants.

Table 2. Effect of different NaCl concentrations on the pigment content in leaves of maize (*Zea mays* L. *Mayflower*) and sorghum (*Sorghum bicolor* L. *Albanus concep*). The plants were treated for six days. The values in the same column are marked with different letters have statistically significant differences at $p < 0.05$.

	Chl (mg/g DW)	Car (mg/g DW)	Chl *a/b*	Car/Chl
Mayflower				
Control	21.36 ± 2.00 ^{bc}	4.48 ± 0.30 ^{bc}	4.40 ± 0.09 ^{d}	0.21 ± 0.01 ^{bc}
50 mM NaCl	24.73± 1.31 ^{ab}	4.98± 0.28 ^{ab}	4.88 ± 0.09 ^{bc}	0.20 ± 0.02 ^{bc}
150 mM NaCl	17.26 ± 1.25 ^{c}	3.91 ± 0.18 ^{c}	5.05 ± 0.12 ^{ab}	0.23 ± 0.02 ^{bc}
250 mM NaCl	7.78 ± 0.28 ^{d}	2.74 ± 0.03 ^{d}	4.06 ± 0.03 ^{e}	0.35 ± 0.01 ^{a}
Albanus				
Control	25.55 ± 1.94 ^{ab}	5.60 ± 0.39 ^{a}	4.73 ± 0.04 ^{c}	0.22 ± 0.01 ^{bc}
50 mM NaCl	27.57 ± 1.64 ^{a}	5.50 ± 0.30 ^{a}	5.09 ± 0.06 ^{ab}	0.20 ± 0.01 ^{c}
150 mM NaCl	19.12 ± 1.67 ^{c}	4.21 ± 0.37 ^{bc}	5.14 ± 0.06 ^{a}	0.22 ± 0.01 ^{bc}
250 mM NaCl	7.98 ± 0.73 ^{d}	1.96 ± 0.15 ^{e}	3.78 ± 0.13 ^{e}	0.24 ± 0.01 ^{b}

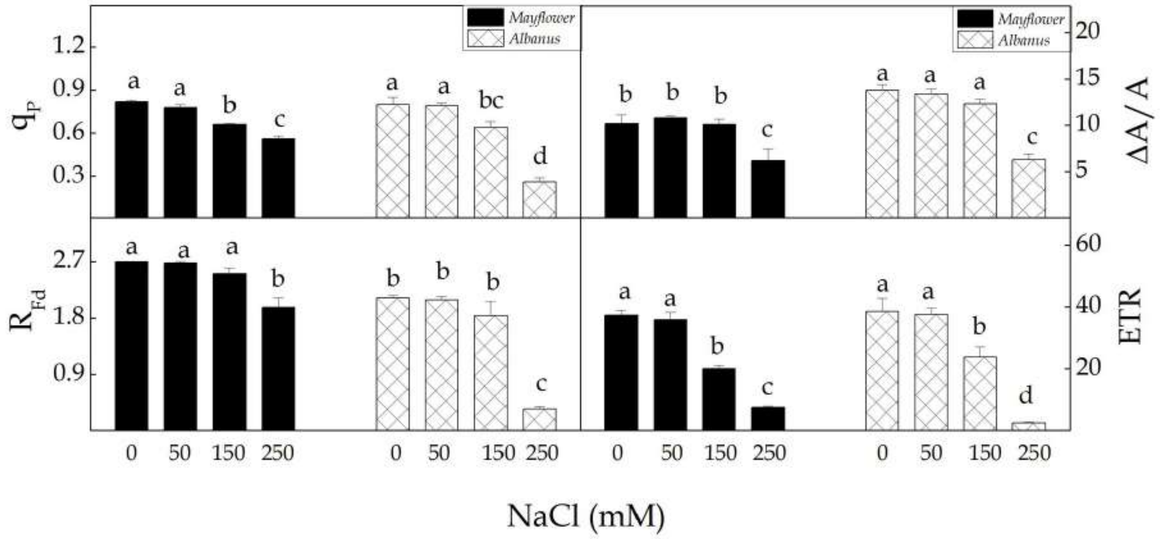

Fig. 1. Effect of different NaCl concentrations on the functions of photosynthetic apparatus of maize (*Zea mays* L. *Mayflower*) and sorghum (*Sorghum bicolor* L. *Albanus concep*). R_{Fd} - ratio of chlorophyll fluorescence decay, ETR - linear electron transport rate, q_P - photochemical quenching coefficient and ΔA/A - relative changes in the amount of P_{700}^+. The plants were treated for six days. The values marked with different letters have statistically significant differences for respectively parameter at $p < 0.05$.

Conclusions

Data revealed that the treatment of maize and sorghum plants with the highest concentration of NaCl (250 mM) lead to a decrease of chlorophylls and carotenoids, an inhibition of the rate of photosynthesis (R_{Fd}), the linear electron transport rate (ETR) and the photochemical quenching (q_P). At the same time, the photochemistry of PSI is also influenced at the highest NaCl concentration. The results indicated that the applied middle concentration (150 mM NaCl) decrease the investigated photosynthetic parameters to a smaller extent. In addition, results also showed that the lowest NaCl concentration (50 mM NaCl) does not influence the pigment content and the functions of photosynthetic apparatus of both studied crops.

On the base of the results in this study, it could be concluded that *Sorghum bicolor* L., *Albanus concep* and *Zea mays* L. *Mayflower* can grow at very saline soil (EC from 8 to 16 mS/cm) but in highly saline soil (EC more than 16 mS/cm) the maize is more salt tolerant than sorghum.

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Assessment of Flood Regulation Capacity of Different Land Cover Types in Krumovitsa River Basin (Eastern Rhodopes)

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Abstract. The area of interest is located in the Eastern Rhodopes, where the winter precipitation maximum often causes floods and material damages. The aim of the presented research is to perform an assessment of flood regulation capacity of different land cover types in the basin of Krumovitsa River, one of the main tributaries of Arda River. Therefore, a drainage network and different land cover types are analyzed and discussed. Due to the size of the area of interest (673 km^2) and its variety the entire study is based on analysis of drainage network and available CORINE land cover classes from different years. High resolution imagery and digital elevation model is used for the visual interpretation of the derived maps. The changes in land cover and its spatial pattern in different years are evaluated. The area of each land cover type is calculated both in absolute in relative units (% of the whole study area). An attempt is made to relate the drainage density with different land cover types. Presented results show relevant information for land cover and flood regulation management. Land cover maps could be used in further evaluation of regulating ecosystem services.

Key words: ecosystem services, floods, Eastern Rhodopes, land cover change.

Introduction

Floods are common phenomena that are restricted by climate, landforms, geological settings, vegetation and land use. Different land cover types have various capacity for flood regulation and flow retention as generally areas with dense vegetation canopy prevent the transformation of overland flow into a channelized flow, which is related to inundations and floods. The area of interest is located in the Eastern Rhodopes, where the winter precipitation is often intensive and torrential, which leads to the rapid melting of snow cover and increase of water levels in rivers. According to Panayotov (1981), the average percentage distribution of precipitation and runoff by hydrological seasons for the Krumovitsa River near the town of Krumovgrad (fig. 1) is as following: precipitation – winter 40.1%, spring 28.9%, summer-autumn 31.0% and runoff - winter 61.5%, spring 32.7%, summer-autumn - only 5.8%. Therefore, the risk of flooding is mainly in the winter months, whereas in the summer and early autumn the discharge is minimal.

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The aim of the presented paper is to perform an assessment of flood regulation capacity of different land cover types in the basin of Krumovitsa River (fig. 1) one of the main tributaries of Arda River. The area of interest covers about 673 km^2 which includes different land cover types and landscapes with various degree of anthropogenic influence. The length of Krumovitsa River is 58.5 km as of from source area near Makaza Pass between Gyumyurdzhinski Snezhnik and Maglenik Ridges to the confluence in Arda River. The mean annual discharge of the Krumovitsa River is 7.32 m^3/s, while the discharge of Arda River near the village of Vehtino is 16.2 m^3/s (Hristova, 2012).

Material and Methods

Land cover and landscapes change over time as land use pattern shifts in both rural and urbanized areas. Thus, detecting land cover changes in a given period is important for understanding the current pattern of the land cover. Land cover data is actively used in mapping and assessment of ecosystems and their services in the European Union (Maes et al., 2014).

The following spatial data sets were used to achieve the purpose of the study: CORINE Land Cover Classes for years 1990, 2000, 2006, 2012 and 2018 available by Copernicus Programme in shapefile format; a digital elevation model (DEM) from Shuttle Radar Topography Mission (SRTM1N42E025V3) with cell size 30 m in GeoTiff format (http://earthexplorer.usgs.gov/) and scanned topographic maps in scale 1:50 000. All datasets are analyzed with the GIS software product of ESRI ArcMap 10.1 (ESRI, 2012).

Secondly, drainage network is extracted from DEM using several different tools, provided by the selected software. The workflow is presented in different papers (Band, 1986; Morris, Heerdegen, 1988; Tarboton et al., 1991; Gurnell, Montgomery, 2000) and can be summarized as a series of successive actions: Fill → Flow Direction → Flow Accumulation. The output from the Flow Accumulation tool is reclassified (Reclassify tool) and converted into a vector layer (Stream to Feature Tool). The derived layer, representing drainage channels is used to calculate drainage density. A polygon grid with a cell size of 1 km^2 was created (Grid Index Feature tool) to calculate the drainage density per each cell (Identify, Summary Statistics). The acquired data was converted to centroids (Feature to Point) and interpolated using Natural Neighbor method to produce a map of drainage density per square kilometer. An attempt is made to relate the channel density with different land cover types by exacting the values of drainage density within each land cover class (Zonal Statistics). The higher the density in a given land cover, the higher the potential of channelizing overland flow and flooding.

Obtained results were used to assess flood regulation capacity of the different land cover types (up to 2018) based on mean drainage density. Thus, all land cover classes were grouped into three categories – areas with low, moderate, and high flood regulation capacity. Furthermore, this approach allows mapping of flood regulation capacity and estimation of risk areas.

Results and Discussion

The basin of Krumovitsa River consists of 17 CORINE Land Cover Classes at level 3 (Table 1). Their nomenclature is based on unique code identification and individual name of each class. Due to changes in land use and landscape pattern the territorial extent of each class changes over time (Table 1). However, the most common land cover types during the studied period (1990 – 2018) are broad-leaved forest (CLC code 311) followed by transitional woodland-shrub (CLC 324) both covering between 252.12 (in 2006) and 277.57 km^2 (in 1990) of the total basin area. Mixed forests (CLC 313) cover less than 10% of the study area and remain relatively sustainable during the last 30 years

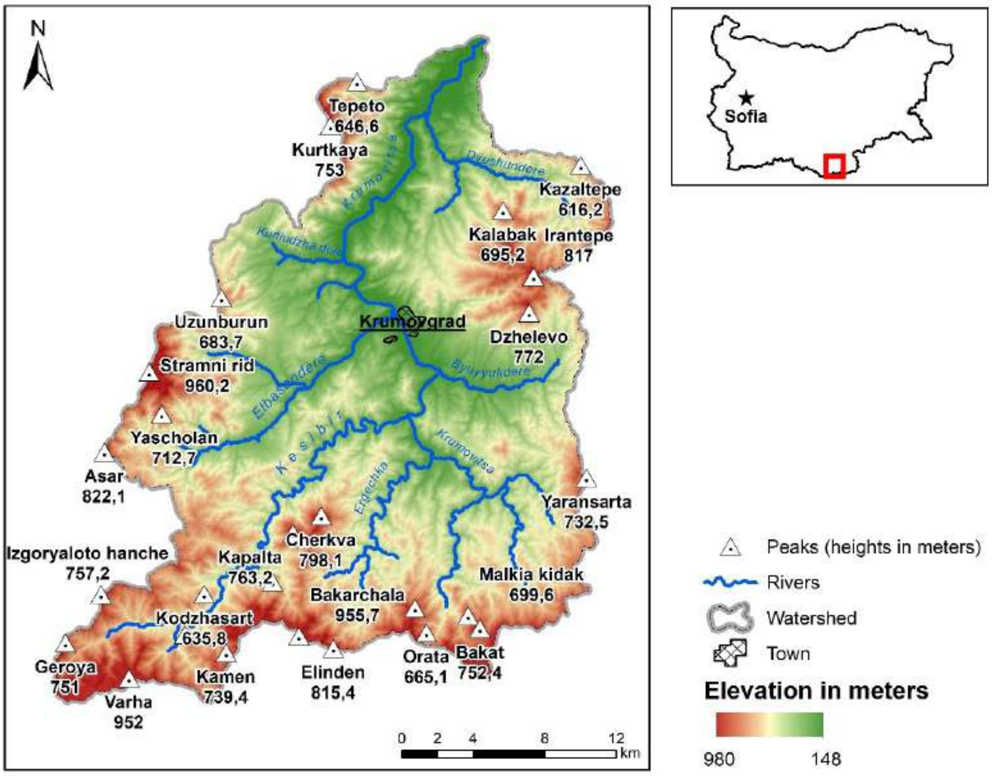

Fig. 1. Map of Krumovitsa River Basin.

Table 1. Territorial distribution (%) of CORINE Land Cover (CLC) classes in Krumovitsa Basin.

CLC/ Year	1990	2000	2006	2012	2018
112 Discontinuous urban fabric	1.38	1.45	1.21	1.21	1.21
121 Industrial or commercial units	-	-	0.0655	0.0653	0.0653
131 Mineral extraction sites	0.24	0.24	0.08	0.08	0.17
211 Non-irrigated arable land	13.29	12.88	11.55	11.53	11.18
221 Vineyards	0.04	0.04	0.04	0.04	-
231 Pastures	7.39	7.75	2.23	2.23	2.34
242 Complex cultivation patterns	4.72	4.74	5.63	5.63	5.69
243 Land principally…	14.61	14.75	18.29	18.26	18.47
311 Broad-leaved forest	20.82	20.37	20.44	20.56	20.59
312 Coniferous forest	6.9	6.99	7.01	7	6.83
313 Mixed forest	9	9.63	9.69	9.67	9.38
321 Natural grasslands	0.78	0.82	5.82	5.81	5.83
323 Sclerophyllous vegetation	-	-	-	0.0003	0.0019
324 Transitional woodland-shrub	20.4	19.88	17.07	17.04	17.37
331 Beaches. dunes. sands	0.05	0.06	0.06	0.06	0.07
333 Sparsely vegetated areas	0.31	0.31	0.72	0.72	0.72
511 Water courses	0.08	0.08	0.08	0.08	0.08
Total %	100	100	100	100	100

mainly as a result of their locations in the low populated upstream areas. Forrest canopy plays a significant role in flood management as trees intercept water from precipitation, therefore decreasing the surface flow. Moreover, Non-irrigated arable land (CLC 211) and Land principally occupied by agriculture, with significant areas of natural vegetation (CLC 243) are also predominant landscapes, which controls the overland flow and the development of erosional processes.

During the study period is observer an increase of CLC 234 as the areas with such land cover increments by 3,86 %, although significant differences in territorial distribution occurs between year 2000 and 2006 (table 1). At the same time natural grasslands (CLC 321) also expanding with about 5%, while other types of land cover types remain practically unchanged in terms of absolute or relative area. They are as follows: water courses (CLC 511), vineyards (CLC 221), coniferous forests (CLC 312) and beaches, dunes, sands (CLC 331) (Table 1).

Sclerophyllous vegetation (ClC 323), previously unobserved before 2012, tends to increase its areas in 2018 (Table 1). It covers small patches of land near the south border of the basin, east of Orata peak (Fig.1). This could be explained by climate change or simply by the insignificant size of these patches as the minimum cartographic unit of CORINE is considered to be 25 ha (Kosztra et al., 2019). Thus, this vegetation might be present in the study area even before 2012. Nevertheless, the exact reason of the expansion of sclerophyllous vegetation is out of the scope of the presented paper.

Simultaneously, between year 2000 and 2006 the areas of pastures (CLC 231) decline with 5,52% as well as territory covered with transitional woodland-shrub, which reduce their areas by 2.81%. These changes in landscape could be related with the processes of depopulation in these parts of the country, which lead to rapid changes in land use and associated adjustment of natural landscapes and environmental processes.

Different types of land cover have various flood regulation capacity since they act as a factor for transformation of precipitation and overland flow. The presence of fluvial landforms such as rills, gullies and valleys is the main indication of predominant channelized flow which often is associated with floods. High drainage density indicates high flood risk (Pallard et al., 2009), especially in areas with sparse vegetation cover. The drainage density of Krumovitsa Basin is 2.24 km/km^2, which is an expected value considering the mountainous territory. However, this parameter has great spatial variability on a local level. The drainage density per square kilometer varies between 0.112 and 5.43 km/km^2 as it tends to be higher along Krumovitsa River and its main tributaries (Ergechka, Kesibir, Elbasandere, Byuyyukdere, Dyushundere) and lower than average near the watersheds (Fig. 2). The areas nearby river beds ant terraces are highly prone to flooding during heavy rainfall events.

Each land cover class has a specific values of drainage density per square kilometer, hence a distinctive flood regulation capacity (Table 2). As it can be expected, stream density varies within each type of land cover, represented of different number of areals (table 2). In this regard, the mean drainage density values can be used as a quantitative measurement of flood regulating services of each land cover class. The lowest mean densities are observed in areas with predominant vegetation canopy while the industrial areas and riparian features (CLC 331) have highest drainage densities (Table 2).

Using the mean drainage density within each type of land cover a simple classification of flood regulation capacity is proposed. The areas with high flood regulation capacity encompass all land cover classes with mean drainage density bellow 2 km/km^2. They are as follows: Sclerophyllous vegetation (CLC 323), broad-lived forests (CLC 311) and natural grasslands (CLC 321), covering about 26.28% of the total basin area located in the peripheral regions near the watershed. Land cover pattern can be easily compared with the map of flood regulation capacity (Fig. 3). The majority of the basin is distinguished by moderate flood regulation capacity (Fig. 3) with is prerequisite for a sustainable landscape development. This group is defined by having drainage density values between 2,1 and 3 km/km^2, and it is comprised of 11 CORINE land cover classes. Less than 1% of the area of interest is considered to be of great risk to flooding. Those sites are represented by industrial and commercial units (CLC 121) near the town of Krumovgrad and river terraces, and sandbanks (CLC 331) along Krumovitsa River in its downstream (Fig. 3). Although areas with low regulation capacity are very small in size, they are located in or nearby settlements or arable land, which is predisposed to damage in case of a flood event.

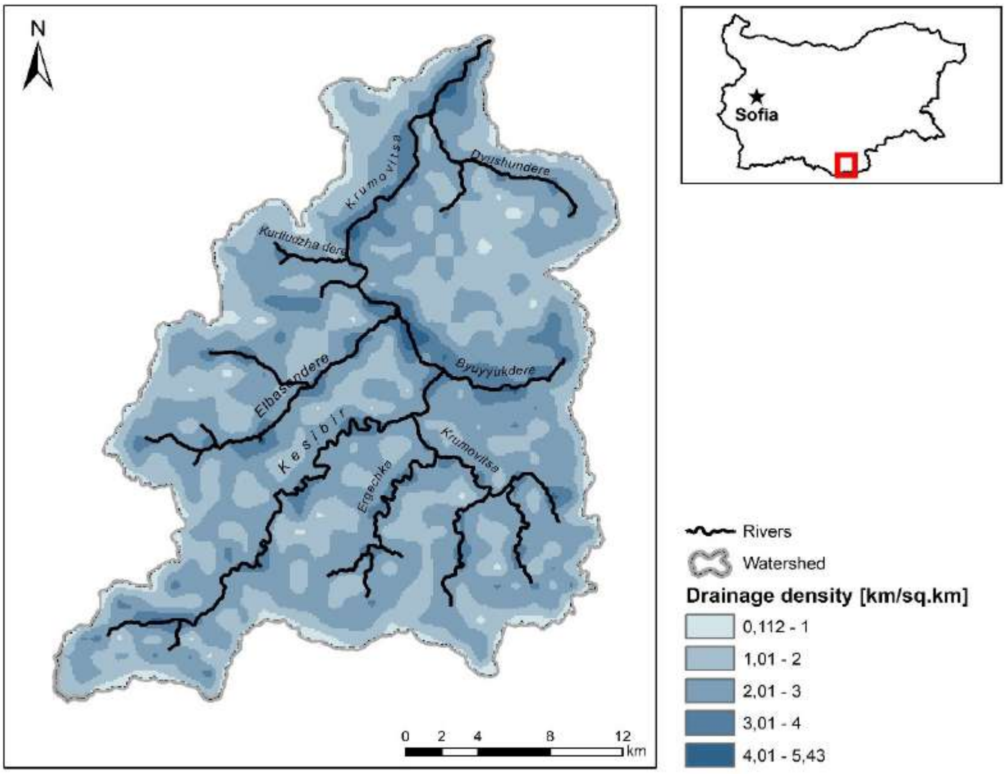

Fig. 2. Drainage density per square kilometer in the Krumovitsa Basin.

Table 2. Drainage network density within different CORINE Land Cover (CLC) classes in Krumovitsa Basin.

CLC code	Level 3 nomenclature	Drainage Network [km/km^2]				
		min	max	range	mean	st. dev.
112	Discontinuous urban fabric	0.380	5.200	4.820	2.415	1.017
121	Industrial or commercial units	3.182	4.360	1.178	3.912	0.246

131	Mineral extraction sites	0.993	2.912	1.920	2.276	0.510
211	Non-irrigated arable land	0.304	5.433	5.129	2.581	0.827
231	Pastures	0.233	3.579	3.346	2.090	0.606
242	Complex cultivation patterns	0.335	4.008	3.673	2.368	0.628
243	Land principally occupied by agriculture, with significant areas of natural vegetation	0.313	4.540	4.228	2.233	0.608
311	Broad-leaved forest	0.112	3.840	3.728	1.897	0.599
312	Coniferous forest	0.339	4.017	3.678	2.243	0.584
313	Mixed forest	0.192	4.303	4.111	2.171	0.592
321	Natural grasslands	0.522	3.988	3.466	1.994	0.523
323	Sclerophyllous vegetation	0.608	0.670	0.062	0.639	0.031
324	Transitional woodland-shrub	0.130	4.350	4.220	2.173	0.646
331	Beaches, dunes, sands	3.079	4.546	1.467	3.889	0.384
333	Sparsely vegetated areas	0.886	3.651	2.765	2.300	0.541
511	Water courses	0.559	3.477	2.918	2.506	0.828

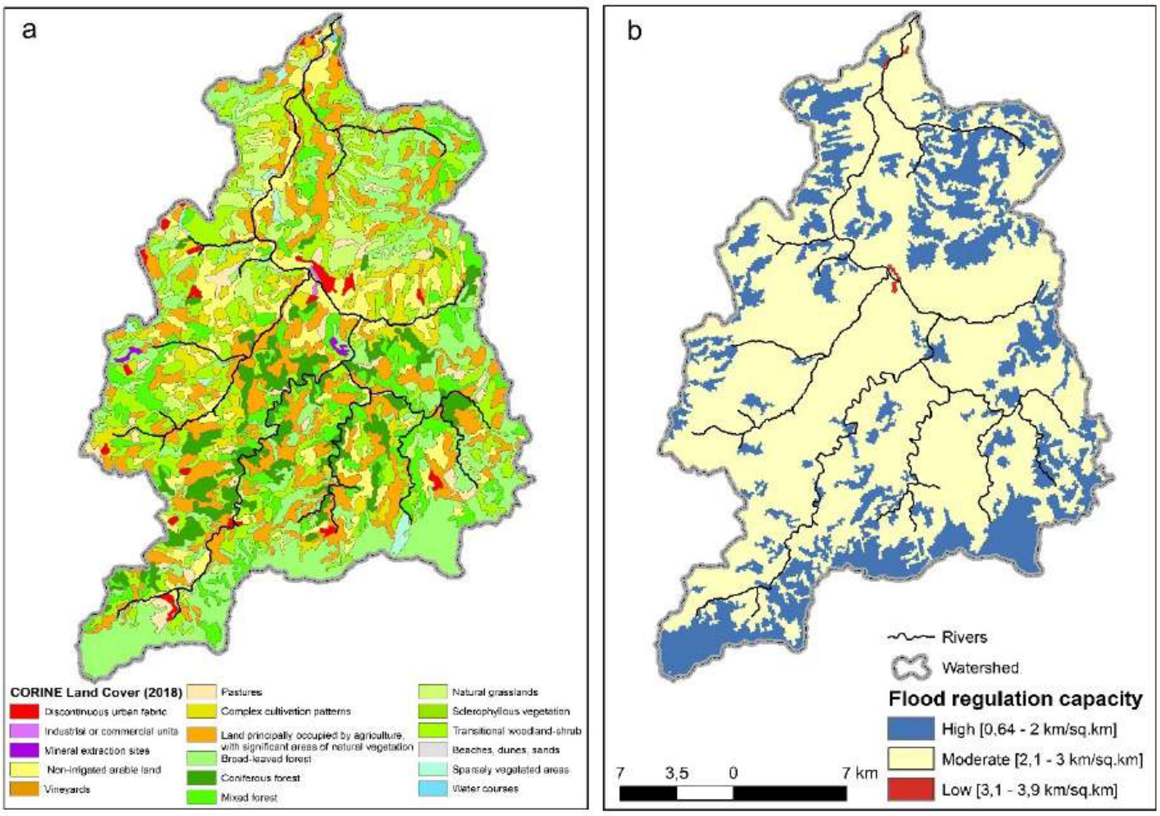

Fig. 3. Land cover pattern in 2018 (a) and flood regulation capacity (b) in the Krumovitsa River Basin.

Conclusions

The land cover in the studied area remains relatively persistent during the last 28 years (1990 - 2018). No significant changes are observed, except the expansion of Sclerophyllous vegetation (CLC code 323) since 2012. The lands principally occupied by agriculture with significant areas of natural vegetation (CLC code 234) increase their area in the study period, which is an important factor for retrieval of primary landscapes and their structure. Simultaneously, the areas of pastures (CLC 231) decreases while natural grasslands (CLC 321) expand their territories with similar rate. Thus, the changes in land use are related with changes in land cover.

The majority of the Krumovitsa Basin (about 37%) is covered with forests (deciduous, coniferous and mixed) which are acting as an erosion and flood limiting factor. The presence of both forest and shrub/grass vegetation reduces the transformation of overland flow into channel flow.

Drainage density is a reliable indicator of landscape susceptibility to flooding. Therefore, the mean drainage density of different land cover types can be used for the assessment of their flood regulation capacity. Using this indicator, the studied river basin is divided into three parts. However, the areas with low flood regulation capacity, e.g. prone to flooding, are urbanized territories and riparian features (floodplains and river terraces). About 73.6% of the Krumovitsa Basin is assessed as an area with moderate flood regulation capacity. Broad-leaved forests and natural grasslands are the most common land cover types with high flood regulation capacity. Therefore, their areas should be preserved for the conservation of their ecosystem functions and services.

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Observed Reduction by a Factor of 10 in the Whole-body Total β-activity of Small Mammals from Alpine Ecosystems in Rila Mountain, Bulgaria

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Abstract. Radio-ecological studies were conducted on small mammal species at two sampling locations in Rila Mountain, Bulgaria. As monitor species, the snow vole (*Chionomys nivalis* Martins, 1842) and wood mouse (*Apodemus sp.* – *Apodemus flavicollis* Melchior, 1834, and *Apodemus sylvaticus* Linnaeus, 1758) were sampled in the vicinity of Musala Peak (2925 m a. s. l.), and wood mouse (*Apodemus sp.*) and bank vole (*Myodes glareolus* Schreber, 1780) were sampled from the area of Skakavtsite (1450 m a. s. l.). Total β-activity was measured by use of a low-background beta counter (LAS 3A level activity system with 30% efficiency on ^{40}K). The values in the investigated rodents show high variation: from 144 up to 1081 Bq/kg without strong region and species dependence. These data fall within the normal range for non-polluted environments. The results were compared to data obtained on Rila Mountain in the period 1993-1996. A reduction by a factor of 10 was observed over the two-decade time period, attributable in part to the decay of deposited anthropogenic β-emitters after the Chernobyl accident in 1986. The results are consistent with models and projections for the reduction of the specific activity of radionuclides in Europe as a function of time.

Key words: radioecology, β-activity, biomonitoring, small mammals.

Introduction

Anthropogenic deposition of radionuclides is a critical problem, particularly in heterogeneous landscapes, such as mountainous regions. Recent studies demonstrate demonstrated that high alpine environments are negatively influenced by environmental pollution. Atmospheric deposition of contaminants at high elevations is greater than those of low elevation regions, because of orographic effects, cloud deposition, wind speed etc. (Lovett &

Kinsman, 1990). The use of living organisms as sentinel species in monitoring environmental pollution has been extensively developed during the last decades. Small mammals such as rodents were selected for use as bioindicators due to their positions in the food chain, rapid maturation, large population numbers, wide geographical distribution, and biological reaction to environmental changes. They have been used for this purpose for a long time, with body burdens of toxicants being determined in either the whole body or in specific target organs and tissues (Martin & Coughtry, 1982; Wern, 1986; Talmage & Walton, 1991; Pascoe et al., 1995). Bulgarian studies have been conducted, focusing specifically on the whole-body total β-activity of several monitor species, including *Apodemus* species, and, especially in Rila Mountain, the snow vole (*Chionomys nivalis* Martins, 1842) (Iovtchev et al., 1995; 1996; Metcheva et al., 1995; 2008). The isotopes Cs-137, Cs-134 and Sr-90 present in the biosphere are the basic anthropogenic β-emitters (Thorn & Vennard, 1976, Metcheva et al., 1995). Additionally, I-131 and I-133 also emit β-particles but are very short-lived; an important natural source is K-40, but it is a relatively weak emitter, responsible for only a small percentage of the dose, as well as a small percentage of measurable β-activity. The total β-activity in mammals has to be less than 4.8 Bq/g. (Thorn & Vennard, 1976). If it is higher, it is obligatory to measure concentrations of each radionuclide.

The primary sources of residual α and β-activity are the naturally occurring radioisotopes of uranium and thorium, and any residual fallout from anthropogenic weapons testing and the Chernobyl reactor accident in 1986. Nowadays the main source of anthropogenic β-activity in Europe is fallout due to the Chernobyl accident in 1986 (Chesser et al., 2000; 2006). The immediate aftermath of the reactor accident created unique conditions of uneven deposition of radionuclides over Central and Eastern Europe, with the most affected areas being adjacent to the power plant itself (Ukraine, Belarus, Russia) and areas in Central Europe (Salzburg, Austria) and Scandinavia (Northern Sweden) being contaminated to a different degree due to changing weather paterns. There is a scarcity of data regarding radionuclide deposition in Bulgaria, with most of the available values coming from routine radiation monitoring in the area around Kozloduy NPP and the measurements contucted during the French-Bulgarian joint project "Musala OM2", which took place during the 1990s (Iovtchev et al., 1995; 1996; Metcheva et al., 1995; Tsibranski, 2014). The current study is a contemporary re-assessment of the radiological situation in Rila Mountain with respect to small mammal species, and its main aim is to compare data recorded more than 20 years ago to presently obtained results.

Materials and Methods

Monitor species of small mammals were caught in the summer seasons of 1993-1996 and 2017 and 2018 using live-bait and snap traps on the territory of Rila Mountain at two different altitudes – the peak of Musala 2925 m a.s.l., and the locality Skakavtsite (between Beli Iskar Village and Beli Iskar Reservoir), 1450 m a.s.l (Fig. 1).

The following rodent species were captured during the investigated periods: wood mouse (*Apodemus sp.*) and bank vole (*Myodes glareolus* Schreber, 1780) from Skakavtsite, and snow vole (*Chionomys nivalis*) and wood mouse (*Apodemus sp.*) from the peak of Musala. The number of the caught animals is presented in Table 1. Animals were terminated in a humane manner according to the National and European laws on animal welfare.

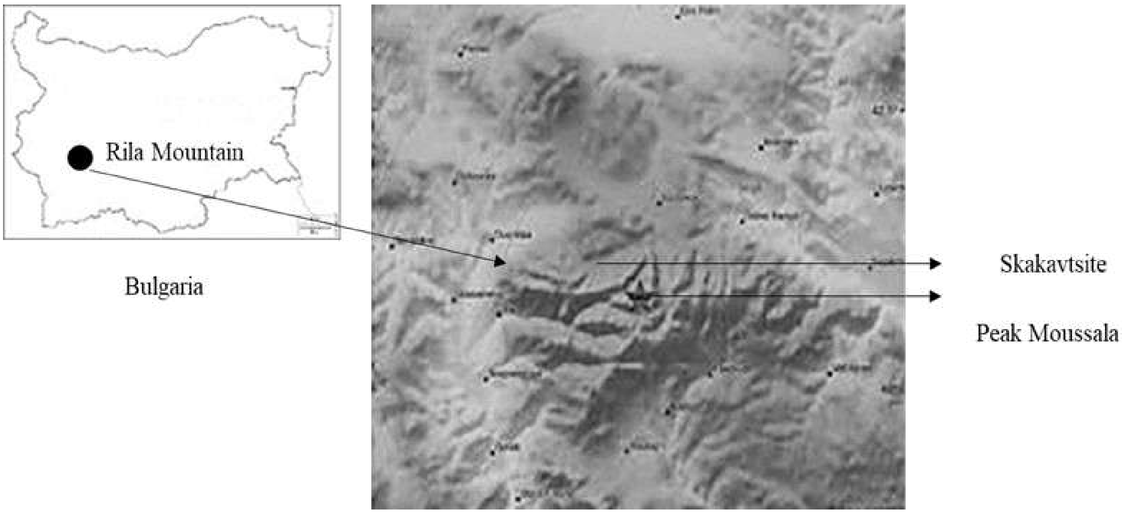

Fig. 1. Trapping points in Rila Mountain during the investigated period.

All collected animals were dissected to remove the intestinal tract and its contents, as well as most internal organs and tissues — liver, kidneys, spleen, and bones. The samples were oven-dried at 60°C for approximately three days to obtain dry weight and analyzed for total β-activity (Iovtchev et al., 1996). All collected samples were treated according to the standard procedure: mineralization at 400°C for 4 hours and measurements of material (13.26 mg/cm) by means of a LAS 3A low level activity system (30% efficiency on ^{40}K and background 1 cpm-1) (Iovtchev et al., 1996). Total β-activity was measured using a low-background beta counter (LAS 3A level activity system with 30% efficiency on ^{40}K and background 1 cpm, INRNE) and was compared with measurements of a ^{40}K standard prepared and measured in the same manner. The background of the detector was determined by counting an empty planchette for 900 min (Iovtchev et al., 1996). Data were standardized and one-way analysis of variance (ANOVA) was used to determine any significant differences among independent groups of parameters. Results are expressed as mean ± standard deviation. p -values less than 0.05 were considered to be statistically significant. Presently obtained results were compared to data, obtained in the same manner more than 20 years ago.

Results

The results for total β-activity are presented in comparison with those obtained in the 1993-1996 investigation period (Table 2).

Table 1. Number of the caught individuals of the investigated small rodent species during the two studied periods.

Location	Skakavtsite		Musala	
	1993-1996	2017- 2018	1993-1996	2017- 2018
Species	n	n	n	n
Apodemus sp.	16	13	14	12
Myodes glareolus	15	22	-	-
Chionomys nivalis	-	-	20	12

Table 2. Total β-activity in Bq/kg in the whole body of monitor species of small rodents from the two investigated areas in Rila Mountain in the beginning and the end of the twenty-year investigation period.

Skakavtsite	β activity mean ± SD		Musala	β activity mean ± SD	
	1993-1996	2017- 2018		1993-1996	2017- 2018
Apodemus sp.	3000.1±160	366.3 ± 8.1	*Apodemus sp.*	2520.3±160	230.3 ± 7.2
Myodes glareolus	2710.8±130	524.2 ± 5.3	*Chionomys nivalis*	3270.5±150	382.0 ± 8.3

The results for total β-activity in the investigated internal organs and tissues in all the monitor species show higher activity in muscles -about 5000 Bq/kg (1993-1996) and 420 Bq/kg (2017- 2018). Similar but lower were these values for the liver – about 4000 Bq/kg and 310 Bq/kg respectively. Few species-specific differences in the quantities were found among investigated animals. The lowest β-activity was found in the bones - about 1000 Bq/kg and 120 Bq/kg respectively. In all cases it is possible to conclude that the accumulation of the total β-activity in the internal organs follows the trend: muscles > liver >bones.

Discussion

The obtained data are consistent with models for the dynamics of radionuclide deposition, mobility and decay within terrestrial ecosystems (Beresford et al., 2008).

When investigating wild rodents from the Chernobyl exclusion zone, an international team obtained similar patterns of results during the late 1990s-early 2000s (Chesser et al., 2000; 2006). Investigations in areas with different degrees of contamination showed that *M. glareolus* is particularly prone to bioaccumulation of cesium radionuclides (^{134}Cs and ^{137}Cs) which explains the high absorbed dose in these animals (Chesser et al., 2000; 2006; Beresford et al, 2008). *M. glareolus* is a predominantly herbivorous species. Since it feeds on the green parts of plants, which preferentially accumulate cesium, it is expected to exhibit higher uptake of ^{134}Cs and ^{137}Cs than, for example, *Ap. flavicollis*. This can help explain why the observed reductions in total β-activity are more significant for *Apodemus* species (about tenfold) than they are for *M. glareolus* (about 5 times).

What can be expected for the future? The main residual radionuclides from Chernobyl at present are ^{137}Cs (half-life of about 30 years) and ^{90}Sr (half-life of about 29 years). The majority of other radionuclides, released into the atmosphere during the accident, have either decayed, or are not biologically significant (for instance, Krypton-85 has a half-life of about 10 years, but is a noble gas, hence cannot be expected to bioaccumulate). This means that cesium and strontium are the most significant anthropogenic β-emitters radionuclides in the environment Metallic strontium is only weakly soluble in water, leaving ^{137}Cs as the main circulating anthropogenic β-emitter in terrestrial ecosystems. The importance of other long-lived radionuclides, such as ^{241}Am, and plutonium isotopes to internal β-paricle dose will remain insignificant (Labunska et al., 2016). This means that in the future, due to the leading role of ^{137}Cs as anthropogenic β-emitter, researchers are likely to see progressive decrease in β-activity in the European terrestrial small rodents, which will be more pronounced in *Apodemus* species and less pronounced in *M. Glareolus*.

Conclusions

The current investigation reveals a decrease in measured whole-body total β-activity in small rodents from alpine ecosystems in Rila Mountain, Bulgaria. This decrease is more pronounced in

Apodemus species and *Ch. nivalis* and less pronounced in *M. glareolus*, a phenomenon attributable to the higher significance of ^{137}Cs as a component of an overall decreasing contamination by anthropogenic β-emitters.

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Population of the Flathead Grey Mullet (Mugil cephalus, L. 1758) from the Bay of Burgas, Bulgarian Black Sea Coast

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Abstract. The study is aimed to investigate the dynamics of age distribution, size structure and annual growth rates of *Mugil cephalus* from the Bay of Burgas. The species inhabits inshore marine waters, as well as estuaries, lagoons and rivers and has high economic importance. Only four age groups were determined for the recent period (2015-2018), compared to the previous period (2010 - 2013) when the flathead grey mullet population has eight age groups. For the period 2010-2013, the size classes ranged from 3.1 cm to 49 cm. For the period 2015-2018, the maximum total length registered was 27 cm. The equation describing the relationship between the length of the body and the radius of the scale is $L=0.4177S + 0.376$, $r^2=0.9769$. The von Bertalanffy's equation for length growth of the flathead mullet is $L_t=139.8[1-e^{-0.04(t+0.07)}]$, $r^2=0.9996$ and for the weight growth is $W_t = 3139[1-e^{-0.1723\ (t-0.372)}]^{3.0654}$, $r^2=0.9328$. The equation describing the length-weight relationship of *Mugil cephalus* is $W=0.0122*L^{3.0961}$, $r^2=0.991$. Values of Fulton's condition index for the age groups from 1 to 4 varied between 1.54 and 1.82. Comparison between the results obtained for the periods 2010-2013 and 2015-2018 shows deterioration of the population of the flathead grey mullet in the surveyed area.

Key words: population parameters, von Bertalanffy's equation, Fulton's condition index, Black Sea coast, *Mugil cephalus.*

Introduction

Environmental changes due to anthropogenic factors affect all parts of the plant and animal world in inland waters, seas and oceans. The Black Sea is close to the so-called "red line" beyond which ecosystem degradation processes may become irreversible. Commercial fishing is the most unfavorable factor as it directly destroys a significant part of the populations of certain species, which in terms affects all other species that are in strictly specific relationships with the intensely exploited ones. The mullets as species with rapid growth rate are sensitive to various anthropogenic impacts and changes. The dynamics of the stocks of these species is highly dependent on both the size of catches and the changing ecological status of the Black Sea over the years (Prodanov et al., 1997).

The flathead grey mullet (*Mugil cephalus* L.) is cosmopolitan species occurring in tropical, subtropical and temperate coastal

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waters in all major oceans (Quignard & Farrugio, 1981). This species occupies a wide variety of marine, estuarine and freshwater environments, but spawning occurs in the sea (Karapetkova & Zhivkov, 2006; Ghaninejad et al., 2010). As expected with the above distribution pattern, *M. cephalus* is a strongly euryhaline species capable of living in waters ranging from fresh to hyperhaline (Koutrakis, 2011; Minos et al., 1994). The flathead grey mullet is also found in both clear and turbid areas, sandy and muddy habitats, and can survive in waters with a wide range of dissolved oxygen levels (Minos et al. 1994; 1995).

The dynamics of stocks of *M. cephalus* is highly dependent on both the size of catches and the changing ecological status of the Black Sea over the years. The only published data on the grey mullet are its catches, growth and migrations of fishes from the Bulgarian Black Sea coast before 40-50 years (Alexandrova, 1957a; b; 1961; 1964; 1967; 1973). These peculiarities of the species and the lack of regular data on the status of its populations require specialized studies on its population parameters and stocks.

The current study is aimed to compare data for two periods in the age-size range of *M. cephalus*, as well as to evaluate new data on the Length-Weight growth of the species in the Bay of Burgas, as well as data on the Condition factor.

Material and Methods

M. cephalus specimens were collected during the period May 2010 – July 2018 from different areas of the Burgas Bay, Bulgarian Black Sea coast (Fig. 1). The fishing unit was a trammel net with a length of 50 m, a depth of 1,5 m and a stretched mesh size of 25 mm, set for 24 h. Trammel nets are less selective than gill nets (Perrow et al., 1996) and because of this they were used.

The length-weight growth, condition factor (K) was studied in 1359 specimens. Standard length (SL±1mm), total weight (TW±1g), gutted (somatic) weight (W±1g) were measured. The age was determined by the scales at a magnification of 17.5x with Projector Dokumator, Lasergeret (Carl Zeiss, Jena).

The mean lengths and weights were used to calculate the von Bertalanffy growth equations: L_∞, W_∞, k and t_o (Growth II program, version 2.1.0.48).

The condition factor (K) was calculated by four ways:

I. Directly - as a coefficient *k* of the equation $W = aL^n$, as the parameters are determined separately for each age group and the whole population (Dikov & Zhivkov, 1985; Hyslop, 1987; Zhivkov & Raikova-Petrova, 1988), were W is a weight of the fishes, L is the length of fishes, *a* and *n* are coefficients;

II. By Fulton’s classical formula: $K_f = (W/L^3)*100$, were W is the weight of the fishes, L – length of fishes;

III. According to Fulton's formula, using the exponent *n* of the formula $W = aL^n$, derived for the whole population or the respective age group (Dikov & Zhivkov, 1985; Zhivkov & Raikova-Petrova, 1988);

IV. By the values of the fish weights (W) at the same, randomly selected lengths, calculated by the equation $W = aL^n$. The population that has a higher value of W at the same value of L is considered to be fatter. (Basami & Grove, 1985; De Silva, 1985; Goldspink, 1979).

Results and Discussion

The main objective was to extend and deepen the knowledge of the population-biological characteristics of *M. cephalus*.

An analysis of the age composition of the caught ichthyologic material for the two periods 2010 – 2013 and 2015-2018 was made. (Fig. 2).

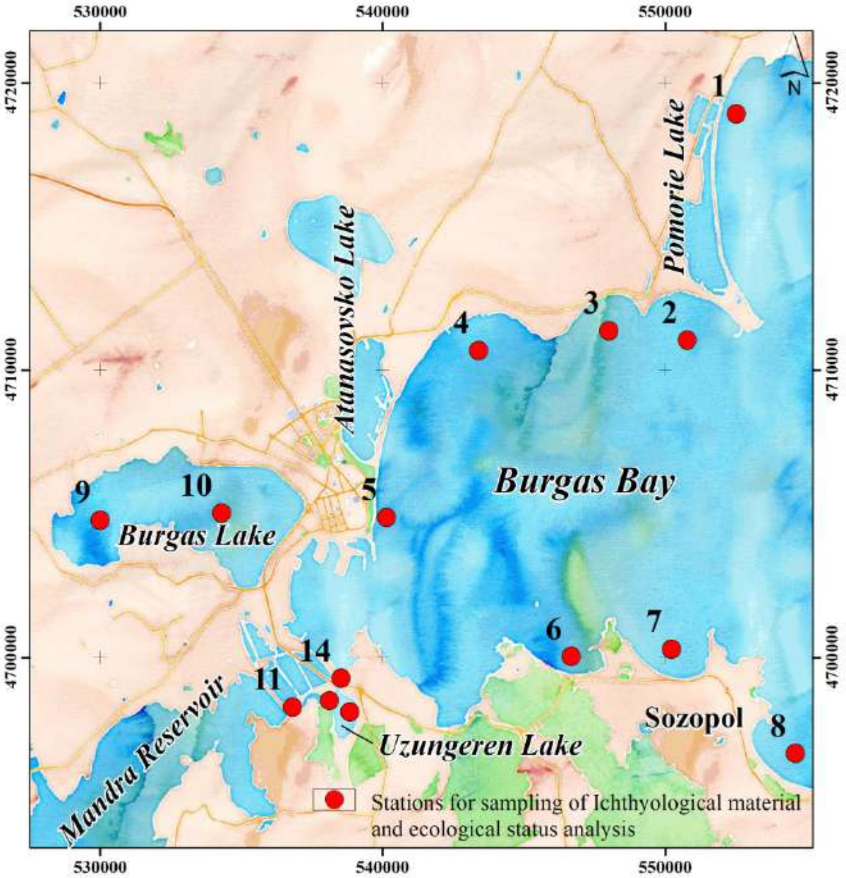

Fig. 1. Location of sampling sites along the Bay of Burgas, Bulgarian Black Sea coast.

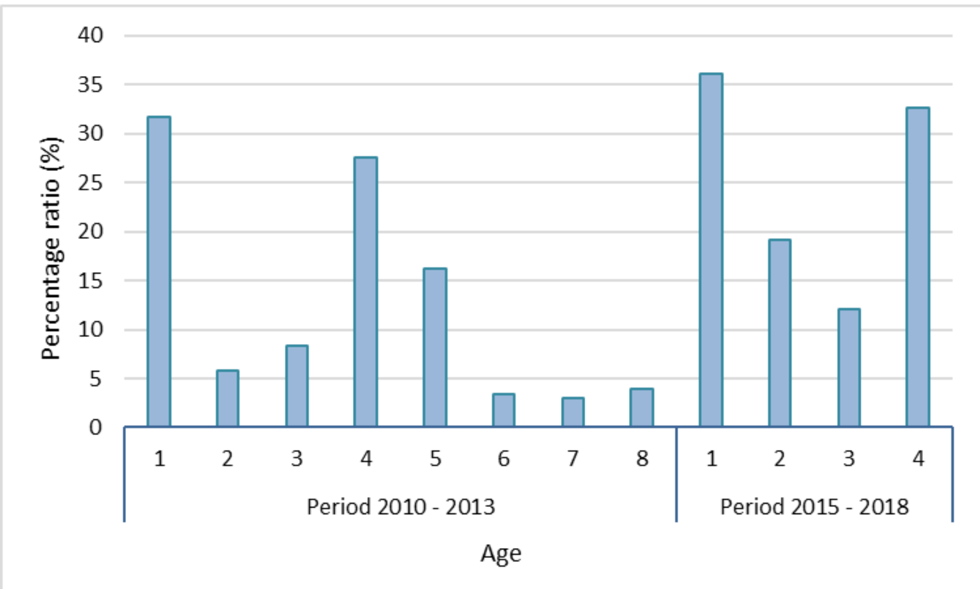

Fig. 2. Age distribution of *Mugil cephalus* from Burgas Bay for two periods.

Only four age groups were determined for the recent period (2015-2018), compared to the previous period (2010-2013) when the flathead grey mullet population has eight age groups.

The established age structure ranks the *M. cephalus* population among the populations with an average life cycle (with a maximum age of 8 to 12 years) (Quignard & Farrugio, 1981). The lower number of fish from large age groups, as well as the weighted average age, indicate the maintenance of a young population. For the period 2010-2013, the size classes ranged from 3.1 cm to 49 cm. For the period 2015-2018, the maximum total length registered was 27 cm (Fig. 3).

The reason for this is most likely the intensive fishing, which is aimed mainly at larger specimens (Bekova, 2020). Another factor that affects the population structure is the deteriorating food base (Bekova et al., 2013). The heavy pollution of some of the coastal areas, and in particular of our southern coast, should not be underestimated (Bekova et al., 2019).

According to von Bertalanffy's model, the asymptomatic length (L_∞) is 139.8 cm (Fig. 4). The reason for this result is the low value of the growth factor k - 0.04, indicating a meager rate of growth of the individuals in the population. Low values we get for k explain the higher values we get for $L\infty$ since two parameters are inversely related - the smaller the value of the integer k, the greater are the values of L_∞ (Ricker, 1975). For the *M. cephalus* Alexandrova (1957a) received values for L_∞ - 69.1 cm, at k = 0.416 and t_0 = 0.0843. In comparing asymptotic to the maximum observed in our capture length (Lmax = 48,7 cm) Lmax:L_∞ gives little value 0.24. This indicates that according to the methodology of Hohendorf (1966) the population of *M. cephalus* in the region not used enough growth potential.

Obtaining higher levels of L_∞ and accordingly, lower values of k are usually observed in populations with a low initial length and slower growth rate.

The asymptotic average weight (W_∞) that we get to flathead mullet from the Bulgarian Black Sea coast is 3139 g (Fig. 5). By comparing this value to the maximum set in our catches (Wmax=1800 g), we obtain 0.6 by Wmax:W_∞ of Hohendorf (1966). Again here, it is confirmed that the population does not fully exploit its growth potential.

As with linear growth, high values for W_∞ and shallow k values are most likely due to the low weight of fish in the first year and the process of self-regulation of growth.

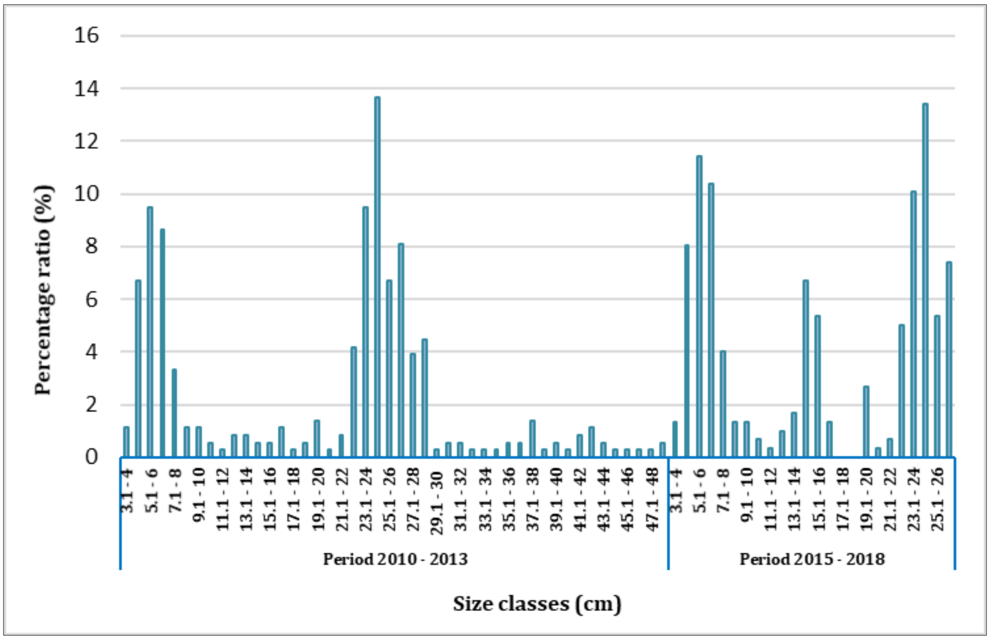

Fig. 3. The size structure of *Mugil cephalus* from the Bay of Burgas for two periods.

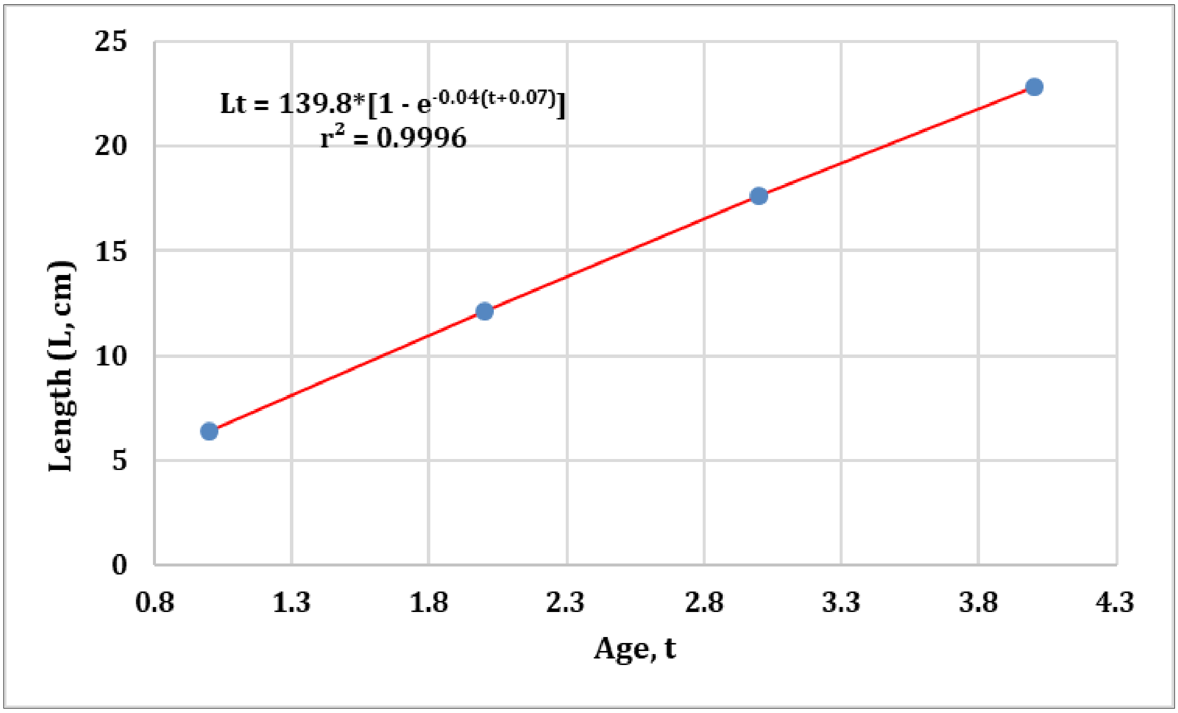

Fig. 4. The von Bertalanffy's equation for length growth of the *M. cephalus* from the Bay of Burgas for the period 2015 – 2018.

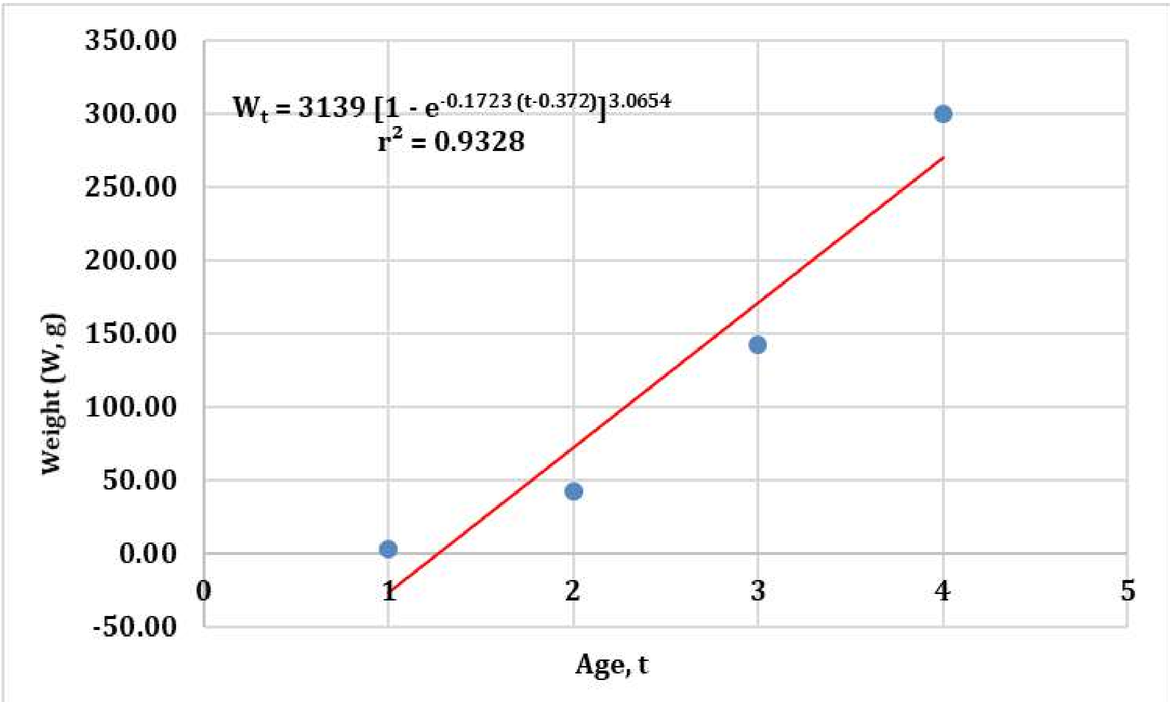

Fig. 5. The von Bertalanffy's equation for weight growth of the *M. cephalus* from the Burgas Bay for the period 2015 – 2018.

It was established that 90% of the habitat of mullet fish in the Usungeren Lake is already located at depths below 1 m (Fig. 6). These conditions are no longer suitable for wintering of these species but are suitable for their conservation. The results are based on the first bathymetric model of the lake and analysis of structural changes that have occurred, resulting in a decrease in depths and areas compared to the 1960s. Juveniles spend their first year in conditions varying from fresh water to seawater, and, over a period of 48 hours, fish of 20-30 mm standard length can survive transfer from seawater to fresh water, and, by 40-69 mm, salinity tolerances and osmotic regulatory capacities are fully developed (Harison, 2003; Prodanov et al., 1997). Adults tolerate wide ranges in salinity, from freshwater to hypersaline conditions (0-126‰) and temperature from 5-37°C (Harison, 2003). This in our climate zone shows that shallow waters are not suitable for wintering species such as mullets, which do not tolerate low water temperatures during the winter.

Table 1 represents the conditional factor of *M. cephalus* from the Burgas Bay: by the classical equation of Fulton $K_f=W/L^3$, by its modification $K_n=W/L^n$, by $K_a=a$ from the equation $W=aL^n$ and by the mass calculated by the same equation at randomly chosen equal lengths. The last method is the most accurate because it takes into account the differences in the growth rate during different periods of life. The values of Fulton's condition index for the age groups from 1 to 4 varied between 1.54 and 1.82. The results show that the studied population does\`t optimal conditions to growth.

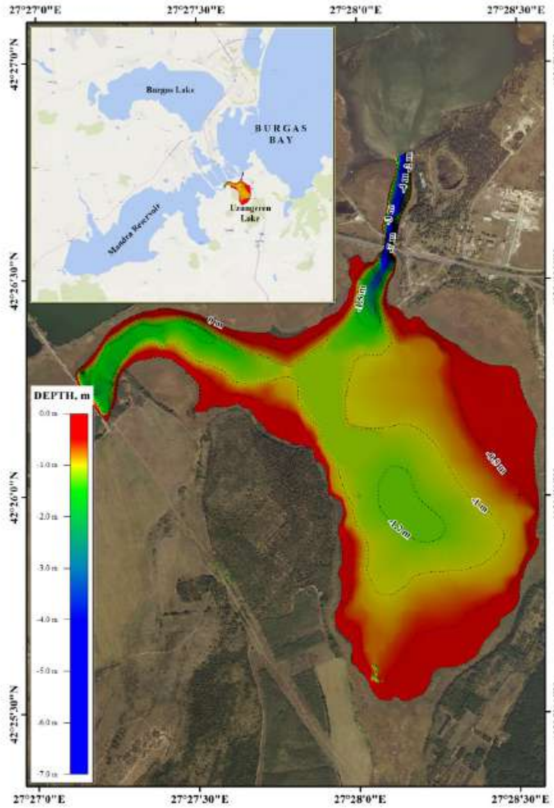

Fig. 6. Bathymetric map of the Uzungeren Lake (Burgas Bay).

Table 1. The conditional factor of *M. cephalus* by age groups from the Bay of Burgas.

Condition factor (K)			
$W=aL^b$	K_a	K_n	K_f
$W_1=0.0238L_1^{2.7544}$	2.4	2.9	1.81
$W_2=0.0423L_2^{2.5885}$	4.2	4.7	1.54
$W_3=0.0117L_3^{3.1371}$	1.2	1.2	1.80
$W_4=0.0143L^{3.0752}$	1.4	1.4	1.82

Conclusions

Comparison between the results obtained for the periods 2010-2013 and 2015-2018 shows the deterioration of the population of the flathead grey mullet. For the recent period (2015-2018) only four age groups were determined, compared to the previous period (2010 - 2013) when the flathead grey mullet population has eight age groups. For the period 2010-2013, the size classes ranged from 3.1 cm to 49 cm. For the period 2015-2018, the maximum total length registered was 27 cm. The lack of certain age and size classes for the period 2015-2018 compared to 2010-2013 speaks for worsening environmental conditions and the intensive fishing press, leading to a sharp decline in the populations of mullets. Our results show that the population of grey mullet is characterized by slow growth, most likely due to the deteriorating living conditions and the early age of maturation. The results showed that the species not used enough growth potential for their populations.

Acknowledgments

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Relationships between Size, Weight, Age and Fecundity of the Chelon auratus and Chelon saliens (Mugilidae) from the Bulgarian Black Sea Coast

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Abstract. The study is aimed to demonstrate the relationships between the fecundity, length, weight and age of *Chelon auratus* and *Chelon saliens* from the Bulgarian Black Sea coast. Absolute fecundity of *Ch. auratus* varied from 327126 oocytes for age 3 (L-16.0 cm, EW-55 g) to a maximum of 4103879 for age 9 (L-32.4 cm, EW-321g). For *Ch. saliens*, absolute fecundity varied from 162890 oocytes for age 2 (L-16.9 cm, EW-52 g) to a maximum of 892441 oocytes for age 6 (L-33.7 cm, EW-315 g). The mean estimated fecundity was 213642 eggs for *Ch. auratus* and 54882 eggs for *Ch. saliens*. Relationship between absolute fecundity-weight, fecundity-length was best fitted by the following equations: *Ch. auratus* - F=9476+10241*W, r2=0.921 and F=-2384206+152333*L, r2=0.906; *Ch. saliens*: F=215473+1698.7*W, r2=0.916 and F=-457777+36837*L, r2=0.924. The relationship between fecundity and age was best described by the exponential equation: *Ch. auratus* - F=106354e1.5475, r2=0.969 and *Ch. saliens* - F=103041e1.1264, r2=0.994. The average weighted relative fecundity was calculated as 492 for *Ch. auratus* and 272 for *Ch. saliens*. A more accurate indicator to describe the relative fecundity is the coefficient b from the equation F=a+b*W. For *Ch. auratus* the coefficient b was 10241, and for *Ch. saliens* was 1698.

Key words: absolute fecundity, relative fecundity, Black Sea, *Chelon auratus, Chelon saliens*.

Introduction

Fecundity is a significant biological indicator characterizing both - the condition of individuals and the reproductive capacity of the population. This is a species attribute which values change in the course of ontogenesis and depending on the environmental conditions. Knowledge of fecundity makes it possible to judge the effectiveness of natural fish reproduction.

The golden grey mullet (*Chelon auratus*) is the most common among mullet fish along our Black Sea Coast (Stoyanov et al., 1963; Zashev, 1961). Like grey mullet (*Mugil cephalus*), it is a pelagic, shoaling and highly mobile fish. The leaping mullet (*Chelon saliens*), like the golden grey mullet, is a pelagic, shoaling and extremely agile fish. In summer, they make significant leaps over the water. They slightly tolerate changes in water salinity and are somewhat sensitive to temperature decrease (Minos et al., 1995). The maximum length of the golden grey mullet is around 60 cm and a weight of 1.5 kg (Harrison, 2003; Stoyanov et al., 1963). The maximum sizes of the golden grey mullet in the Black Sea reach up to 43 cm, but the commercial ones usually range from 18 cm to 25 cm (Harrison, 2003). The leaping mullet reaches a length of up to 35 cm, but here it is caught in sizes of 16-18 cm and weight up to 0.8 kg (Harrison, 2003). Spawning starts at 2 - 3 years of age (Stoyanov et al., 1963). The golden grey mullet reaches sexual maturity at 2-3 years of age (Harrison, 2003; Stoyanov et al., 1963). It spawns from August to October in both the nearby coastal zone and the high seas at a water temperature of 18.4 - 22.2°C (Stoyanov et al., 1963; Zashev, 1961). The juveniles, after reaching the length of 2.5-3.5 cm, move to the shore for feeding (Stoyanov et al., 1963). Its fecundity is very high. According to Stoyanov et al. (1963), the fecundity of the golden grey mullet is from 0.16 to 4 million, and according to Pavlovskaya (1969) from 0.86 to 2.41 million. The leaping mullet reaches sexual maturity at the age of 2-3 years (Stoyanov et al., 1963; Zashev, 1961). It spawns from June to the end of September in both the near-coastal zone and the high seas (Stoyanov et al., 1963; Zashev, 1961). At a temperature of 24-27°C, the spawning takes place in the open sea. Its fecundity, according to Stoyanov et al. (1963), ranges from 500,000 to 2,100,000 eggs and according to Pavlovskaya (1969) from 0.97 to 3.71 million eggs. The larvae hatch within 24 hours (Stoyanov et al., 1963).

The economic importance of the golden grey mullet is significant (Zashev, 1961). Of all mullets, it is the most caught one. Its annual catch in the Azov-Black Sea basin and the Caspian Sea amounts to about 4 500 tons (Zashev, 1961). In Bulgaria, its average annual catch, along with that of the leaping mullet, amounts to about 100 tons. In some years it reaches 300-400 tons (Zashev, 1961). The catch of Leaping mullet is not reported separately from that of Golden grey mullet. In Bulgaria, they go under the name leaping mullet. In fact, the main share belongs to the golden grey mullet, and the leaping mullet is only a co-catch (in NAFA statistics).

Given the environmental changes and the anthropogenic pressure affecting the Black Sea ecosystems and the constant fluctuation in population numbers of these fish species, it is necessary to shed more light on their reproductive biology.

Material and Methods

Ch. auratus and *Ch. saliens* specimens were collected during the period May 2010 – July 2018 from different areas of the Bulgarian Black Sea Coast (Fig. 1 - sampling sites in red points). The samples were taken by cast nets fishing - mesh size 22-38 mm, length 50 m and height of the nets between 1.5 m and 2 m. Fish fecundity was studied on 582 specimens. Standard length (SL ± 1mm), total weight (TW ± 1g), gutted (somatic) weight (W ± 1g) and gonad weight (g ± 0.1 g) were measured. The age was determined by counting the number of annuli (rings) on a scale at a magnification of 17.5x using Projector Dokumator, Lasergeret (Carl Zeiss, Jena).

As input parameters for the analysis of fecundity the total individual fecundity (the number of mature eggs, ready to be spawned from one individual during one spawning season) and the total fecundity (the average number of eggs of the individuals from one egg, size or weight group) were used (Anohina, 1969; Spanovskaya & Grigorash, 1976).

The relative fecundity was measured in two ways: 1. As a relation between the total fecundity and the gutted weight of the fish RF=F/W (Spanovskaya & Grigorash, 1976); 2. By the coefficient b from the equation F = a+b*W (Zotin, 1961).

Data were statistically processed in XLSTAT, version 2019.1.1.56334.

Results and Discussion

The absolute fecundity of *Ch. auratus* and *Ch. saliens* populations varied between

327 126 – 3 627 876 and between 198 421 – 778 930 eggs, respectively. The averagely weighted fecundity of the golden grey mullet was 213 642 and 54 882 of the leaping mullet. The specimen of *Ch. auratus* with the lowest individual fecundity had a size of 16.0 cm and mass without guts of 55 g, and the one of *Ch. saliens* with the lowest individual fecundity was 16.9 cm with mass without guts 52 g. The fishes with the highest fecundity (3 627 876 - *Ch. auratus* and 778 930 - *Ch. saliens*) were 32.4 cm and 33.7 cm, respectively and 321 g and 315 g weight without guts but were not the largest examined specimens. The youngest specimens of *Ch. auratus* and *Ch. saliens* tested for this indicator were respectively 3 and 2 years old. In these age groups, the average fecundity was 534 981 eggs for *Ch. auratus* and 199 809 eggs for *Ch. saliens*.

The absolute fecundity of both mullet species by size groups is presented in Table 1. The fecundity range in the smaller size groups was smaller and increased with increasing fish sizes.

Increasing the length of the fish increased fecundity values (Table 1). Graphically this relation is shown in Fig. 2 - A, B. The most appropriate function for its description is the linear one. The degree of correlation between the average values of F and L was very high ($r2 = 0.906$ – *Ch. auratus*; $r2 = 0.924$ - *Ch. saliens*).

The absolute fecundity of mullet fish by weight classes is presented in Table 2. There was a tendency to increase fecundity by increasing fish mass with the highest average values observed in the last weight group (251-500 g for *Ch. auratus* and 301-320 g for *Ch. saliens*). The dependence of fecundity on the mass of mullets is best described with a linear function (Fig. 3 - A, B). The degree of correlation between the average values of F and W was very high ($r2 = 0.92$ - *Ch. auratus*; $r2 = 0.92$ - *Ch. Saliens*).

The change in absolute fecundity with the age of the fish is shown in Table 3. The fecundity range of *Ch. auratus* was the highest at four and five years of age specimens (2.2 times), and at three, seven and nine-year-olds were significantly lower (1.3 times). For *Ch. saliens* the highest fecundity range was at four and five-year-olds (1.5 times).

The relation F-t in the population of mullet fish along the Bulgarian Black Sea Coast is shown in Fig. 4 - A, B. Here also the degree of correlation between the studied parameters was very high ($r2=0.97$ - *Ch. auratus*; $r2 = 0.99$ - *Ch. saliens*).

Apart from the size and age of the fish, fecundity depends also on how many times the female has participated in the breeding process. Typically, the specimens which spawn for the first time are characterized by lower values of fertility. This is probably one of the reasons for the high variability of the indicator in some of the studied size, weight and age groups. Table 3 shows the distribution of absolute fecundity by age groups. In *Ch. auratus* fecundity values of three and seven-year-old fish were nearly the same (1.3 and 1.5); in *Ch. saliens* the differences in three and six years olds were minimal. In the third age group, in both species, females that spawn for the first or second time were united. In older age groups, all individuals had already participated in the breeding process more than twice. The greater fecundity range at 4 and 5 years old mullets was probably because there were included both fishes spawning for the first time and those spawning for the third or fourth time.

In the Caspian Sea, the absolute fecundity of the golden grey mullet in a 25-30 cm size group varied from 500-600 thousand to 2-3 million eggs with a length of 45-50 cm (Askerov et al., 2003). Belyaeva et al. (1989) has published an absolute fecundity range from 740 thousand to 4.82 million eggs. The absolute fecundity of the golden grey mullet with an average size of 33.8 cm was in the field from 254,700 to 2,925,600 eggs (Khoroshko, 1981). Belyaeva et al. (1989) have reported fecundity ranging from 500 thousand to 3 million eggs. These values are close to those reported by Abdolmalaki et al. (1998), namely from 270 811 to 2 699 590 eggs. For *Ch. saliens* along the northeastern coast of the Caspian Sea, Patimar (2008) has reported absolute fecundity ranging between 135 014 (for 3+) and 389 790 (for 7+) eggs. For the same species, but in the Aegean Sea, Koutrakis (2011) has reported absolute fecundity ranging from 245 000 to 555 000 eggs.

Our values for the absolute fecundity of mullets are close to those reported by Ghaninejad et al. (2010) for the golden grey mullet and Koutrakis (2011) for the leaping mullet.

Table 1. Change of absolute fecundity in different length classes of *Ch. auratus* and *Ch. saliens* from the Bulgarian Black Sea Coast.

Species	Length class (L, cm)	Average length (L, cm)	Absolute fecundity (F, eggs) Range	Absolute fecundity (F, eggs) Mean	n
Chelon auratus	20.1 - 21	20.84	327126 - 578642	499362	29
	21.1 - 22	21.46	428290 - 902441	711543	39
	22.1 - 23	22.8	810002 - 1409007	1210483	45
	23.1 - 26	25.31	949998 - 2009875	1597256	66
	26.1 - 30	28.11	1423877 - 2884955	2372642	72
	30.1 - 38	36.92	2799483 - 3627876	2982170	51
Chelon saliens	19.1 - 20	19.71	198421 - 284002	203640	32
	20.1 - 25	21.89	319942 - 500 144	410958	74
	25.1 - 30	26.32	349933 - 601472	541222	98
	30.1 - 35	31.88	572941 - 778930	689433	76

Fig. 1. Location of sampling sites along the Bulgarian Black Sea Coast.

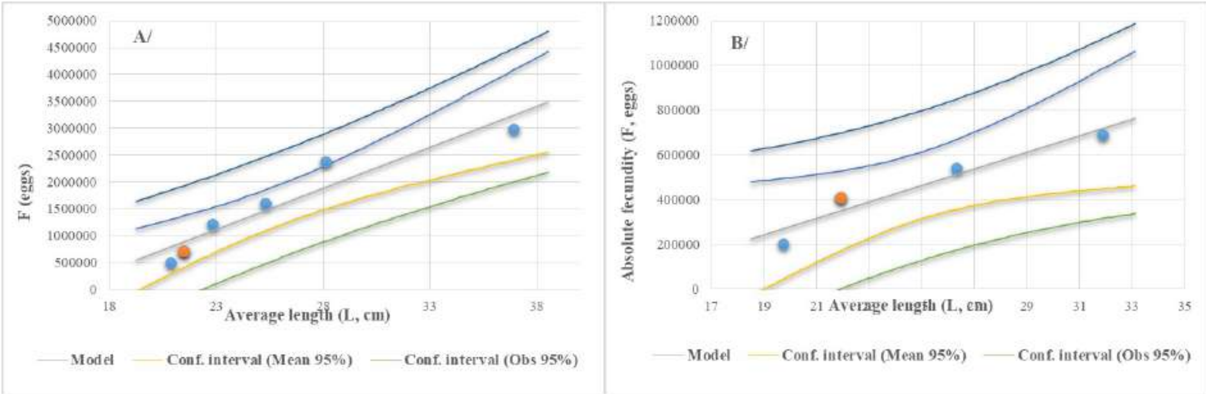

Fig. 2. Relationship between the absolute fecundity (F, eggs) and the average length (L, cm) of A/ *Ch. auratus* and B/ *Ch. saliens* from the Bulgarian Black Sea Coast.

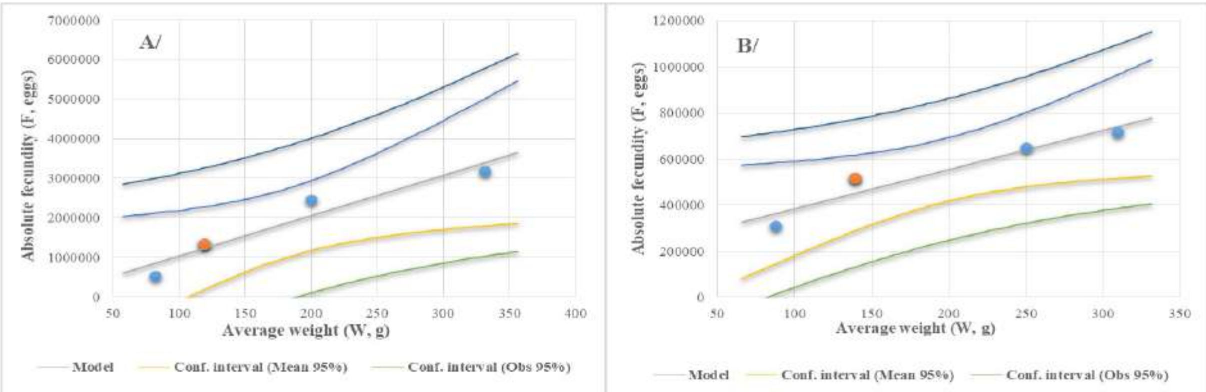

Fig. 3. Relationship between the absolute fecundity (F, eggs) and the average weight (W, g) of A/ *Ch. auratus* and B/ *Ch. saliens* from the Bulgarian Black Sea Coast.

Table 3. Change of absolute fecundity (F) in different age classes (t) of *Ch. auratus* and *Ch. saliens* from the Bulgarian Black Sea Coast.

Species	Age (t)	Absolute fecundity (F, eggs)		n
		Range	Mean	
	3	488290 - 622733	534981	39
	4	612982 - 1300443	8999793	64
	5	821712 - 1787563	1246874	95
Ch. auratus	6	1468557 - 2324902	1991981	55
	7	1676354 - 2422937	2000189	20
	8	1525634 - 3233409	2412860	12
	9	2199346 -3490371	3201002	17
	2	162890 - 228439	199809	36
	3	331982 - 402138	319081	69
Ch. saliens	4	397452 - 598218	490933	88
	5	498176 - 721982	601985	59
	6	690768 - 892441	729946	28

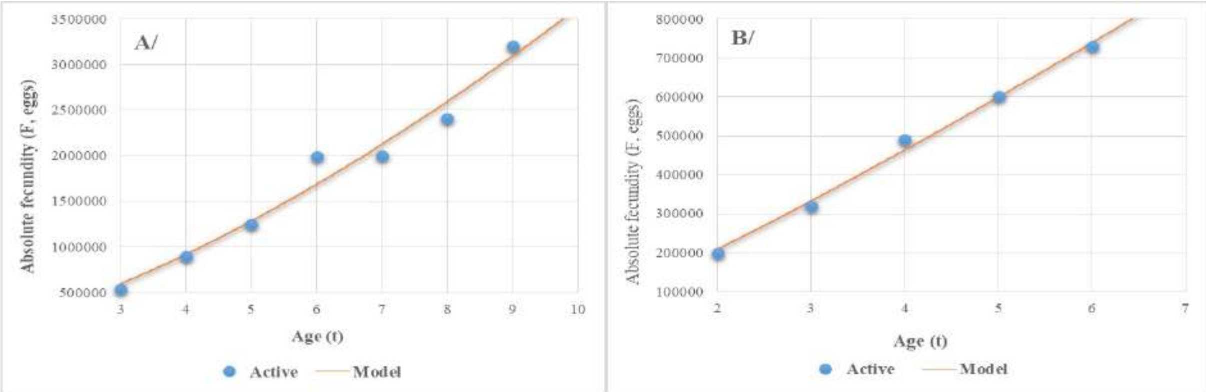

Fig. 4. Relationship between the absolute fecundity (F, eggs) and the fish age (t) of A/ *Ch. auratus* and B/ *Ch. saliens* from the Bulgarian Black Sea Coast.

The average weighted relative fecundity of both mullet species populations from the Bulgarian Black Sea Coast was as follows for *Ch. auratus* - 492 and *Ch. saliens* - 272. The average values of the relative fecundity in different weight groups are shown in Table 4. The regression line between F and W passes over the origin of the coordinate system. It is for this reason that Živkov et al. (1999) has suggested using the coefficient b (10241 - *Ch. auratus*, 1698 - *Ch. saliens*) from the equation F = a + bW (Fig. 2) as an indicator of relative fecundity. This coefficient indicates the average relative rate of increase of the absolute fecundity depending on the weight of fish and describes with sufficient mathematical accuracy the relation between the values F and W.

Table 4. Changes in the relative fecundity by weight classes for *Ch. auratus* and *Ch. saliens* from the Bulgarian Black Sea Coast.

Species	Weight class (g)	Mean weight (g)	Relative fecundity	n
Ch. auratus	61 - 100	82.2	6614.61	29
	101 - 150	119.2	11324.94	98
	151 - 250	199.9	12295.57	122
	251 - 500	331.2	9634.14	53
Ch. saliens	71 - 100	87.8	3557.53	37
	101 - 200	138.9	3750.01	67
	201 - 300	250.1	2604.81	108
	301 - 320	309.5	2326.77	68

The data on the relative fecundity of the populations studied by us could not be compared with data of other populations of the same species as such data were not available in the literature we knew. Only Koutrakis (2011) has reported data on the relative fecundity of *Ch. saliens* from the northern part of the Aegean Sea, an average value of 1822 in the range 1507-2501, which is lower than that obtained by us.

Conclusions

The absolute fecundity of *Ch. auratus* and *Ch. saliens* populations varied between 327 hurat126 – 4 103 879 and between 162 890 – 892 441 eggs, respectively. The averagely weighted fecundity of the golden grey mullet was 213 642 and 54 882 of the leaping mullet. The values of the fecundity rose with increasing the fish length, weight and age. The average weighted relative fecundity of both mullet species populations from the Bulgarian Black Sea Coast was as follows for *Ch. auratus* - 492 and *Ch. saliens* - 272. The regression line between F and W passes over the origin of the coordinate system. It is for this reason the coefficient b is an indicator of relative fecundity. This coefficient indicates the average relative rate of increase of the absolute fecundity depending on the weight of fish and describes with sufficient mathematical accuracy the relation between the values F and W.

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The Resistance and Resilience of Soil Enzymes after the Application of Fungicide Azoxystrobin to Loamy Sand Soil

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Abstract. The use of fungicides in crop protection effectively eliminates fungal pathogens of plants. However, they may cause changes in soil microorganisms concerning microbial ability to mediate soil functions. The aim of the study was to evaluate the changes in soil environment, and soil enzyme resistance and resilience (beta-glucosidase, urease, acid and alkaline phosphatases and arylsulphatase) in a response to the increasing concentrations of azoxystrobin (Az), applied under the trade form Quadris[R]. A laboratory study was carried out for 120 days on soil mesocosms, amended with Az in concentrations from 0.00 mg kg^{-1} to 35.00 mg kg^{-1}. Az soil amendment caused changes in soil physico-chemical properties and microbial activity. Microbial responses immediately (day 1) after Az application, showed that more resistant to the fungicide were urease, beta-glucosidase and arylsulphatase in the opposite to the acid phosphatase, which demonstrated high sensitivity to the chemical stress. One month later, the resistance of beta-glucosidase, urease and acid phosphatase decreased even more compared to day 1, the resistance of alkaline phosphatase remained unchangeable, whereas the resistance of arylsulphatase slightly increased. The calculated resilience on day 120 manifested that enzymes were not able to recover within four months after fungicide application to soils. Pearson correlation analysis demonstrated significant linear relationships between Az soil residues and enzyme resistance/resilience. Our results highlighted that the application of Quadris[R] altered soil enzyme system for more than four months, which might reflect the speed of organic matter turnover in soil, especially that of organophosphates.

Key words: fungicide azoxystrobin, Quadris[R], soil, chemical stress, enzyme resistance, enzyme resilience.

Introduction

Fungicides are chemical compounds used in crop protection to control the development of fungal pathogens. Strong worldwide demand owing to increase in agricultural activities is expected to be a key driver for the growth of the fungicide market in future. Primarily, the use of fungicides is aimed at controlling target organisms. Despite this fact, it is not possible to predict their environmental fate. The highest quantities of fungicides are accumulated in soil, which may cause changes in the terrestrial environments, often manifested by decreasing soil fertility.

Fungicides are classified by chemical type such as triazoles, benzimidazoles, chloronitriles, dithiocarbamates, phenylamides and strobilurins. Among the fungicides with a natural origin, strobilurins accounts for the largest share of the global fungicide market. Strobilurins form part of the group of quinone-outside inhibitors (QoI), inhibiting mitochondrial respiration at the Qo site of cytochrome b, part of the cytochrome bc1 complex (Complex III), and thus preventing spore germination and mycelial growth in fungal pathogens (Bartlett et al., 2002). Azoxystrobin, (methyl (E)-2-{2-[6-(2-cyanophenoxy) pyrimidin-4-yloxy] phenyl}-3-methoxyacrylate), is one of the strobilurin members, and can be used to control diseases caused by pathogenic fungi such as ascomycetes, basidiomycetes, oomycete, and imperfect fungi (Bartlett et al., 2002). Because of its broad-spectrum activity, this fungicide has become the leader in the world fungicide market (Bai, 2007). However, many authors evidenced that azoxystrobin (Az) negatively affected soil microorganisms, disturbing the structure of soil fungal communities (Bending et al., 2007; Sopeña & Bending, 2013), inhibiting soil respiration (Guo et al., 2015; Wang et al., 2018), and changing the activity of soil enzymes (Baćmaga et al., 2015; Guo et al., 2015; Sopeña & Bending, 2013; Wang et al., 2018). Soil enzymes catalyze an extensive number of biological processes in soil and provide a unique assessment of soil function mediated mainly by soil microbiota (Yang et al., 2013). The major authors' interest was referred to the Az influence on the activity of soil dehydrogenase, and less to the activity of soil urease (Alvarez-Martin et al., 2016; Baćmaga et al., 2015; Bending et al., 2007; Guo et al., 2015; Sopeña & Bending, 2013; Wang et al., 2018). In some cases the information is contradictory as most of the authors reported slight to strong negative effects of Az applied at low and high concentrations, respectively. In both cases, the effects were manifested at a later stage of Az exposure. Unlike the other authors, Alvarez-Martin et al. (2016) reported no significant effect of Az on dehydrogenase activity at both low (0.2 mg kg^{-1}) and high (25 mg kg^{-1}) fungicide concentrations.

Considering this fact, the aim of our study was to evaluate the effect of Az on soil health by determining the resistance and resilience of soil enzymes (beta-glucosidase, urease, phosphatase acid phosphatase, alkaline phosphatase, and arylsulphatase) under chemical stress.

Material and Methods

Soil sampling and soil properties

Soil was collected from a grassland located near Gabra village (Sofia region, Bulgaria) - 42°31´48.36´´N and 23°37´28.20´´E. Five subsamples were pooled randomly from a 0 – 20 cm soil depth, sieved through a 2 mm mesh, and mixed in aliquots after determining the dry weights of 1 g samples at 105 °C in an oven for 24 h. Soil was classified as loamy sand with texture of 83 % sand, 2 % clay, and 15 % silt. Total organic carbon was 15.7 g kg^{-1}, and nitrogen Kjeldahl - 1.67 g kg^{-1}. Soil pH (H_2O) was acidic with original value of 5.67 (Executive Environment Agency, personal communication). During the experiment, values of soil pH, inorganic nitrogen (NO_3 and NH_4), inorganic phosphates (HPO_4) and Az soil residues were followed. Soil pH was measured potentiometrically (HANNA Instruments) after mixing soil in 0.01 mol l^{-1} $CaCl_2$ solution (1:5; weight : volume), and shaking it for 30 min. Soil bioavailable forms of inorganic nitrogen (NO_3-N and NH_4-N) and phosphates (HPO_4) were determined spectrophotometrically according to the methods of Keeney and Nelson (1982), and Olsen (1982), respectively. A gas chromatography was used to assess the Az soil residues extracted by methanol: ethylacetate (75 : 25, v/v) solution after 1 h sonification of samples in an ultrasonic bath (35 kHz and 285 W) (Aleksova, 2019).

Design of mesocosm experiment

Soil mesocosms were prepared as each of them contained 2000 g of dry weight equivalent soil amended with Az under the form of commercial substance Quadris[R] (Syngenta). Quadris[R] was applied in concentrations of 2.90 mg kg^{-1} (Az1), 14.65 mg kg^{-1} (Az2) and 35.00 mg kg^{-1} (Az3) calculated towards the active ingredient methyl(E)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate, and indicated by gas-chromatography method as Az soil residues a day after soil treatments. A mesocosm without fungicide was used as a control (Az0). In the study was used the name of the active principle (Az) although it was tested the commercial formulation containing multiple (active principle plus adjuvants) ingredients. Each mesocosm was prepared in triplicates. Soil water content was adjusted to 40% of the maximum water holding capacity. The soil moisture was maintained by weighting soils every 3 days using sterile distilled water in order to compensate for any moisture loss. The mesocosms were incubated at 22 ± 1 °C in dark to prevent physical degradation of Az by light. Soil samples were collected randomly in triplicates from each mesocosm on the 1st (D1), 30th (D30), and 120th (D120) day after Az application.

Soil enzyme activities

The method of enzyme activity determination was based on 1 g soil cultivation with the respective enzyme substratum, extraction and colorimetric determination of the enzyme products. Beta-glucosidase (BGl), urease (Ur), alkaline phosphatase (AlP), acid phosphatase (AcP) and arylsulphatase (Ars) activities were determined following the method of Eivazi & Tabatabi (1988), Kandeler & Gerber (1988), Tabatabi & Bremner (1969), Browman & Tabatabi (1978), and Tabatabi & Bremner (1970), respectively. Soil enzyme activity was measured on a Cecil CE 3021 spectrophotometer (Cecil Instruments, Cambridge, England) at λ = 405 nm (BGl), λ = 690 nm (Ur), λ = 420 nm (AlP and AcP), and λ = 400 nm (Ars). The following substrates were used to determine enzyme activity: p-nitrophenyl β-D-glucopyranoside for BGl, urea for Ur, 4-nitrophenyl phosphate disodium for AlP and AcP, and p-nitrophenyl sulfate for Ars (Sigma - Aldrich).

Formulas proposed by Orwin & Wardle (2004) were used to determine the soil resistance index (RS) on days 1 (D1) and 30 (D30), and the soil resilience index (RL) on day 120 (D120) after Az application to soils:

$$RS(t_0) = 1 - \frac{2|D_0|}{(C_0 + |D_0|)} \quad \text{and}$$

$$RL\,at\,t_x = \frac{2|D_0|}{(|D_0| + |D_x|)} - 1$$

where: $D_0 = C_0 - P_0$, $D_x = C_x - P_x$, C_0 is the enzyme activity under natural conditions over time t_0, P_0 is the enzyme activity of Az disturbed soil over time t_0, C_x is the enzyme activity under natural conditions over time t_x, P_x is the enzyme activity of Az disturbed soil over time t_x.

Data analysis

Each data point in the paper represented the results from the three replicates of each soil mesocosm, and each value was expressed as a mean value for the respective Az treated soil. One-way ANOVA followed by Tukey's test were performed to examine the differences in the means of soil (pH, NO_3-N, NH_4-N, HPO_4, Az) and microbial (RS and RL) parameters among the studied soil mesocosms. Person product moment correlation analysis was performed to examine the linear correlations between the Az soil residues and enzyme resistance/ resilience. Principal component analysis (PCA) was performed using the data matrix of RS and RL to ordinate the enzyme responses to Az. The above statistics were performed with the package PAST (Hammer et al., 2001) at a level of significance $p<0.05$.

Results and Discussion

Soil environment

Different trends of changes in soil parameters were recorded during Az exposition (Table 1).

The Az0's soil variables are shown as actual values, whereas those of Az1, Az2 and Az3 were calculated as percentages of Az0. For each sampling day, values of each soil variable (Az1 – Az3) followed by different letters are significantly different ($p \leq 0.05$) according to Tukey's HSD test. Comparing with Az0, soil content of ammonium nitrogen and phosphates in Az amended soils increased and that of Az residues and nitrate nitrogen decreased over time. Soil acidification also increased along the soils' incubation. The higher soil content of NH_4-N and PO_4 at the end of the experiment might be related to the accumulation and biodegradation of death fungal biomass in Az soils. pH gradual decrease over time might be related to the process of NH_4-N accumulation in soils and/or Az degradation and release of azoxystrobin acid as a main end product of the fungicide transformation in soils (Singh et al., 2010). Az application (D1) increased dramatically soil concentration of nitrate nitrogen (50 % - 78 %) at the beginning of the experiment. Nitrate increase in soils after pesticide application has been reported also in other studies. The authors suggested that this effect might be a result of soil pre-treatment activities (Franzluebbers, 1999) or nitrogen input by fungicide adjuvants (Devare et al., 2007; Mijangos et al., 2009). For example, according to Syngenta, Quadris[R] consists of 22.9% Az and 77.1% of other ingredients. In general, it was found that Az application caused changes in soil environment, and we supposed that it might moderate the fungicide influence on soil enzyme activities (secondary effects of Az).

Resistance and resilience of soil enzymes

Microorganisms are the key players of many soil functions such as biogeochemical cycling and plant productivity, and are essential for the integrity of terrestrial ecosystems. Given the crucial importance of maintaining soil functions, we aimed to investigate the resistance and resilience of soil/ microbial enzymes to Az application. In general, resistance is commonly defined as the ability of a system to withstand a disturbance, while resilience is considered as an ability of a system to recover as soon as possible after the end of the perturbation (Griffiths & Philippot, 2013). In this aspect, the resistance and resilience are the two components of system stability (Loreau et al., 2002). The resistance and resilience of soil enzymes were calculated and they are shown on Fig. 1.

Az application to soils caused immediately (D1) microbial response, manifested by the relatively high resistance (on average, Az1 – Az3) of Ur (0.942), followed by that of BGL (0.840), Ars (0.723), AlP (0.667) and AcP (0.650). High resistance of Ur to Az, especially at concentrations lower than 10 mg kg^{-1}, was recorded also by Baćmaga et al. (2015) and Guo et al. (2015), and both of them mentioned that enzyme inhibition was manifested at longer exposure - 14 days (Guo et al. 2015) and 30 days (Baćmaga et al. 2015) after fungicide amendment of soil. Since the effects of Az was not tested on the other soil enzymes except Ur, we cannot compare our results with earlier findings in this aspect. There are some results concerning the effects of triazoles, acylalanines and mancozeb on Ars (Floch *et al.*, 2011; Saha *et al.*, 2016; Sukul, 2006), but these fungicides have different mechanisms of action on target organisms comparing to Az, making the comparison inaccurate.

Pearson correlation coefficients were calculated on D1 and they evidenced that the RSs of BGl, Ur and Ars did not relate significantly with Az soil residues, in the opposite to that of AcP and AlP (Table 2), indicating again the relatively high resistance of BGl, Ur and Ars to the chemical stress. The next enzyme resistance determination was a month after Az application (D30), when different trends of changes in enzyme resistances were recorded (Fig. 1). Comparing the enzyme RSs to that of D1, the resistance of AlP remained unchangeable, the resistances of BGL, AcP and Ur decreased by 15 % - 25 %, and the resistance of Ars increased by 10 %. On D30, the number of significant relationships between Az soil residues and enzymes increased, indicating increased influence of Az on soil functioning. Considering the negative direction of the above mentioned correlations, we assumed that Az might slowdown the nutrients' transformation in fungicide impacted light textured soils (sandy soils).

Even if enzyme activities are sensitive to Az, the microbial community might still be resilient and able to return quickly to its pre-disturbance functioning. A number of features of microorganisms suggest that the resilience could be their common property, because microorganisms 1) have fast growth rates and they have the potential to recover quickly after disturbance and 2) have a high degree of physiological flexibility (Griffths & Philippot, 2013). In our study, the RL indices calculated on D120 would indicate whether the effect of Az on soil microorganisms has disappeared four months after fungicide application, whether the fungal communities (main contributors to the soil enzyme pool) have been recovered, and whether the soil functions are completely carried out.

The RL values (D120) clearly showed that four months after soil treatment with Az, soil enzyme activities were still not recovered and moreover they were far from their stable natural state (pre-treatment state) (Fig. 1). For example, the values of RLs (on average for Az1 – Az3) were for BGL: - 0.39, Ur: 0.09, AcP: - 0.15, AlP: 0.14, and Ars: - 0.21. Calculating the mean value of overall enzyme resilience per mesocosm, it was recorded negative RL values for Az1 (-0.19) and Az2 (-0.28), and positive RL value for Az3 (0.16). In fact, at Az3 more resilient to Az was Ur, having relatively high (0.86) and positive value of the index. Considering that the metabolic capacity of microbial communities likely reflect the abundance and bioavailability of nutrients (Orwin et al., 2006), we assumed that Az influenced on enzyme RLs by changing the size of soil nutrient pool.

Table 1. Soil parameters at different levels of azoxystrobin (Az) amended soil mesocosms. The Az0's soil variables are shown as actual values, whereas those of Az1, Az2 and Az3 were calculated as percentages of Az0. For each sampling day, values of each soil variable (Az1 – Az3) followed by different letters are significantly different ($p \leq 0.05$) according to Tukey's HSD test. Legend: *Az concentrations on D1 were taken as 100% and they were used for the calculation of the Az soil residues on D30 and D120.

Mesocosm	Day of sampling	Az	HPO_4	NH_4-N	NO_3-N	pH
Az0	D1	-	**2.83 mg kg^{-1}**	**2.24 mg kg^{-1}**	**16.48 mg kg^{-1}**	**5.63**
Az1		100*	96.11^{a}	99.65^{a}	176.30^{a}	93.43^{a}
Az2		100*	86.57^{b}	57.29^{a}	151.37^{b}	91.12^{b}
Az3		100*	113.78^{c}	92.36^{a}	178.14^{a}	86.68^{c}
Az0	D30	-	**7.80 mg kg^{-1}**	**0.67 mg kg^{-1}**	**47.04 mg kg-^{1}**	**6.04**
Az1		48.96^{a}	120.64^{a}	109.30^{a}	123.28^{a}	88.24^{a}
Az2		73.31^{b}	88.59^{a}	145.35^{b}	123.72^{a}	81.46^{b}
Az3		84.51^{b}	96.02^{a}	147.67^{c}	131.26^{b}	83.28^{c}
Az0	D120	-	**23.08 mg kg^{-1}**	**10.47 mg kg^{-1}**	**146.73 mg kg^{-1}**	**5.72**
Az1		10.34^{a}	110.57^{a}	154.98^{a}	111.06^{a}	85.14^{a}
Az2		19.38^{b}	112.56^{a}	76.37^{b}	103.64^{a}	84.79^{a}
Az3		13.14^{ab}	119.84^{a}	113.08^{c}	112.16^{b}	84.09^{a}

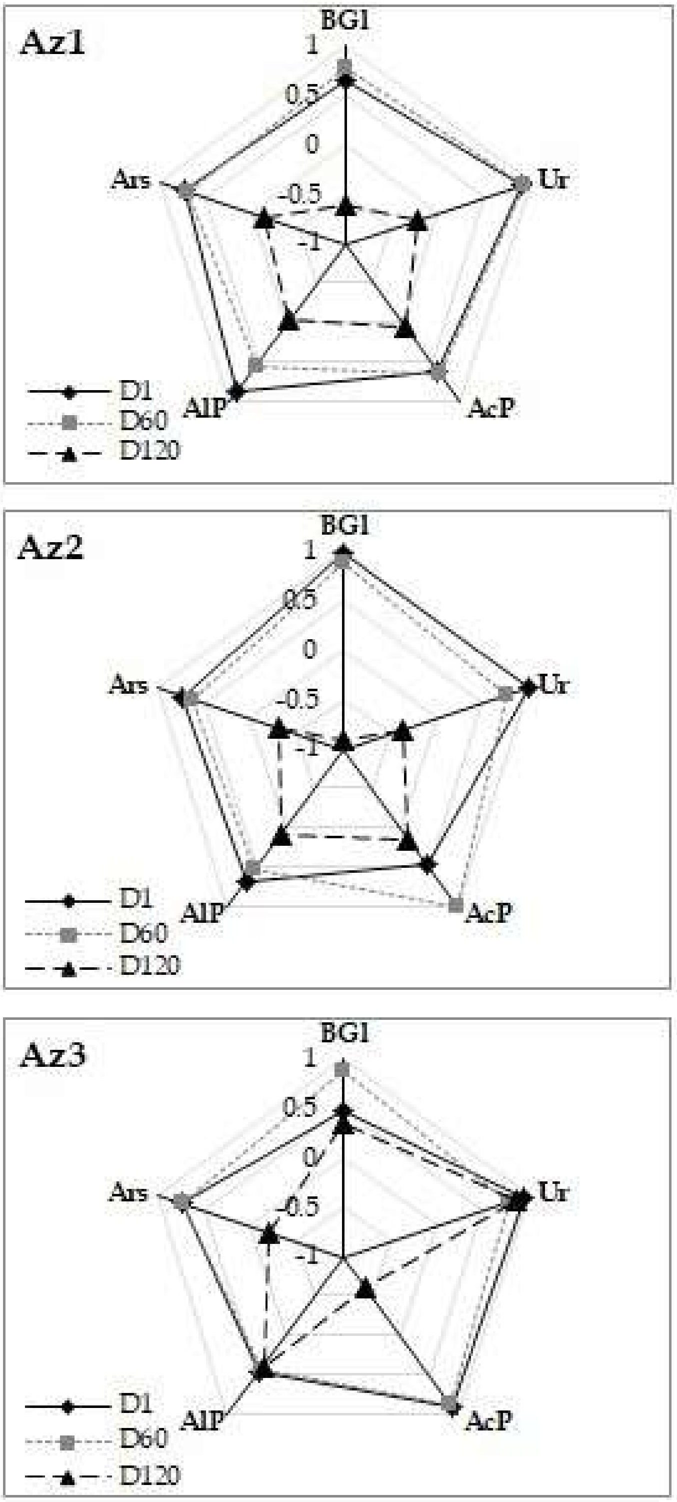

Fig. 1. Resistance (D1 and D30) and resilience (D120) of soil enzymes beta-glucosidase (BGl), urease (Ur), acid (AcP) and alkaline (AlP) phosphatases and arylsulphatase (Ars) after application of Az to soils in increasing concentrations (Az1 – Az3).

We assumed that the highest Az concentration caused 1) the highest death and accumulation of fungal biomass (proteins) in soil, and 2) the highest synthesis of detoxification agents (mainly proteins) - molecules that can later be metabolized by the same microbiota (Degens et al., 2000; Hall, 2002). Accumulation of microbial proteins (easy degradable substrates) in soil could stimulate Ur activity (Tabatabai, 1982) and increase its recovery rate.

On D120, significant relationships were calculated between Az soil residues and the resiliences of Ur, AlP, and AcP (Table 2), indicating that these enzymes were still under the influence of Az. Although, the correlation between Az and BGl was not significant (0.64, p=0.06), it was considered as strong.

Summarizing the RL results, we could conclude that four months after Az application soil enzyme activity is still unrecovered, probably due to the Az toxicity on soil fungi, which are considered to be the main drivers of nutrient turnover in soils (Güsewell & Gessner, 2009).

Principle component analysis (PCA) was used to examine how soil microbial communities from Az amended soils differ with respect to the resistance and resilience of their enzyme activities (Fig. 2).

Table 2. Pearson correlation analysis between Az soil residues and soil enzyme responses in the respective day of sampling. The significant correlation coefficients are in bold.

Soil enzymes	Corr. indices between Az soil residues and enzyme resistance		Corr. indices between Az soil residues and enzyme resilience
	D1	D30	D120
BGl	-0.49 (0.18)	**-0.99** (0.00)	0.64 (0.06)
Ur	0.42 (0.26)	-0.39 (0.29)	**0.74** (0.02)
AcP	**0.76** (0.02)	**0.79** (0.01)	**-0.74** (0.02)
AlP	**-0.99** (0.00)	**-0.98** (0.00)	**0.93** (0.00)
Ars	-0.58 (0.10)	**-0.98** (0.00)	-0.39 (0.29)

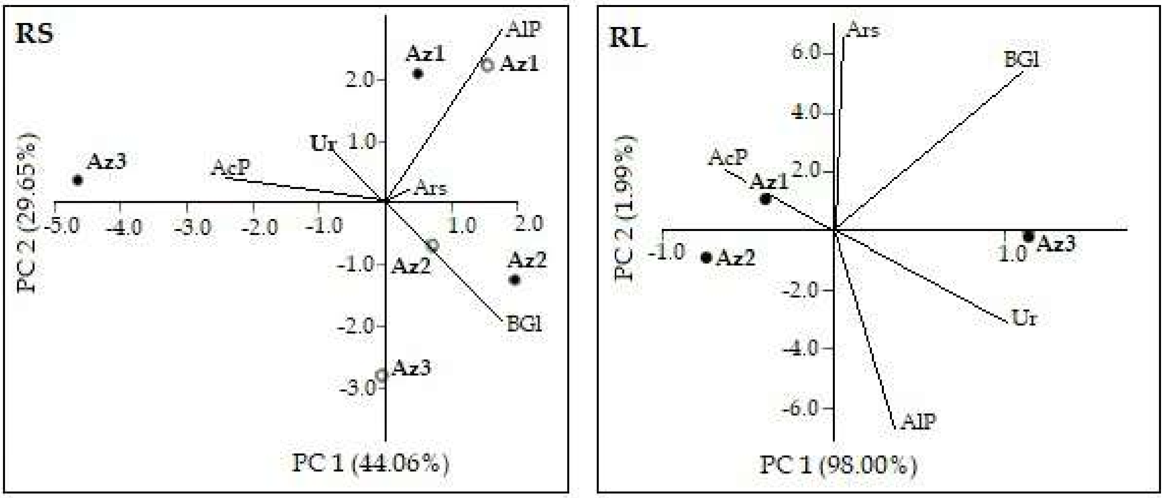

Fig. 2. Principal component analysis (PCA) plots showing similarity in soil enzyme resistance (RS; filled dot – D1 and open dot – D30) and resilience (RL) responses to the soil application of Az in increasing concentrations (Az1 – Az3).

PC 1 and PC 2 together were able to constrain more than 83 % of the variation in the soil enzyme RS and RL. Relationships of RS and RL with the respective primary axes indicated strong correlations of PC 1 with AlP, BGl and AcP (RS), and BGl and Ur (RL). PC 2 correlated strongly with AlP and BGl (RS), and Ars and AlP (RL).

Az1 and Az2 were separated by each other (PC 1), and both of them differed from Az3 (PC 2) due to the resistance of BGl and phosphatases (AcP and AlP). BGL and Ur differentiated the resilience between Az1 and Az2, whereas the resilience of AlP and Ars were the main factors contributed to the differences between Az3, and both Az1 and Az2.

On the PC plots, increasing distance between soil mesocosms equates to higher dissimilarity in enzyme responses (RS and RL) towards Az stress. The distances between the RSs of Az1 on D1 and D30 and between that of Az2 on D1 and D30 were relatively low, indicating minor changes within one month after Az application to soils. It was not the case of Az3, where the distance of RSs between the two sampling events (D1 and D30) was much higher than that between the mesocosms amended with lower Az concentrations. The same pattern of segregation of RLs was manifested for Az amended soil mesocosms on D120.

Conclusions

The effect of increasing concentrations of Az on soil functioning was assessed through the calculation of the resistance and resilience of soil enzymes involved into the turnover of organic carbon (BGl), nitrogen (Ur), phosphorus (AcP and AlP) and sulfur (Ars). The results evidenced that the most sensitive (less resistant) to Az stress was AcP. AcP was also the soil enzyme which recovered (low resilience) most slowly, which assumed that the soil cycling of organophosphates would be slowed down by Az for much more than four months. The other tested enzymes demonstrated high (Ur) to medium (BGL and Ars) resistance to soil chemical perturbation, but their ability to recover was low (Ur and Ars) to extremely low (BGl). Most of the enzyme responses were dependent on Az residues in soils. Summarizing the results, we could conclude that Az application caused stress on soil enzymes, and the recovery of enzyme activities can be moderated in different rates by soil properties, including Az residues and fungicide adjuvants. Considering the fact that fungicides are frequently introduced into soils, their long-term effects on microbially-mediated soil functions should be a subject of a more detailed further research.

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Development of Accurate Chemical Thermodynamic Database for Geochemical Storage of Nuclear Waste. Part I: Models for Predicting Solution Properties and Solid-Liquid Equilibrium in Binary Nitrate Systems of the Type 1-1

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Abstract. In this study we developed very well validated thermodynamic models for solution behavior and solid-liquid equilibrium in 7 nitrate binary systems of the type 1-1 (HNO_3-H_2O, $LiNO_3$-H_2O, $NaNO_3$-H_2O, KNO_3-H_2O, $RbNO_3$-H_2O, $CsNO_3$-H_2O, and NH_4NO_3-H_2O) from low to very high concentration at T = 25°C. The models for nitrate systems described in this study are of high importance, especially in development of strategies and programs for nuclear waste geochemical storage. Models are developed on the basis of Pitzer ion interactions approach. To parameterize models for binary systems we used all available raw experimental osmotic coefficients data (φ) for whole concentration range of solutions, and up to saturation point. Data for supersaturation zone, available for $LiNO_3$-H_2O system, are also included in parameterization. To construct models we used different versions of standard molality-based Pitzer approach. It was established that for three of systems under study (HNO_3-H_2O, $LiNO_3$-H_2O, $RbNO_3$-H_2O) application of extended approach with 4 parameters (β^0, β^1, β^2 and C^φ) and variation of a_2 term in fundamental Pitzer equations leads to the lowest values of standard model-experiment deviation. The recommendations on mean activity coefficients ($g_\pm$) given in literature are model-dependent. Therefore, they are not used in parameterization process. However, these data are used to validate the resulting models. The predictions of new developed here models are in excellent agreement with experimental osmotic coefficients data, and with recommendations on activity in binary solutions from low to very high concentration: up to ≈ 29 $mol.kg^{-1}$ in HNO_3-H_2O, and up to 26.8 $mol.kg^{-1}$ in NH_4NO_3-H_2O. The Deliquescence Relative Humidity (DRH), and thermodynamic solubility product (as ln K^o_{sp}) of six solid phases [$LiNO_3.3H_2O(s)$, $NaNO_3(s)$, $KNO_3(s)$, $RbNO_3(s)$, $CsNO_3(s)$, and $NH_4NO_3(s)$] have been determined on the basis of evaluated binary parameters and using experimental m(sat) solubility data. Model predictions are in good agreement with available reference data.

Key words: Computer chemical and geochemical modeling; Pitzer approach; Binary nitrate systems HNO_3-H_2O, $LiNO_3$-H_2O, $NaNO_3$-H_2O, KNO_3-H_2O, $RbNO_3$-H_2O, $CsNO_3$-H_2O, and NH_4NO_3-H_2O; Solution and solute activity; Solid-liquid phase equilibrium; Geochemical nuclear waste sequestration.

Used symbols:

β^0, β^1, β^2 and C^φ – Pitzer binary parameters
Θ and Y- Pitzer mixing parameters
a_1 and a_2 - terms in fundamental Pitzer equations
φ - osmotic coefficient
$g_\pm$ - mean activity coefficient
a_w - activity of water
m(sat)- molality of saturated solutions ($mol.kg^{-1}$)
m (max)= maximum molality ($mol.kg^{-1}$) of experimental activity data
σ = standard deviation of fit of osmotic coefficients
ln K^o_{sp} - logarithm of thermodynamic solubility product
DRH - Deliquescence Relative Humidity
$D_fG^o_m$-standard molar Gibbs free energy of formation
YMTDB- Yucca Mountain Thermodynamic DataBase
THEREDA –THErmodynamic REference DAtabase
UCSD - University California San Diego

Introduction

Computer models that predict solution behavior and solid-liquid-gas equilibria close to experimental accuracy have wide applicability. They can simulate the complex changes that occur in nature and can replicate conditions that are difficult or expensive to duplicate in the laboratory. Such models can be powerful predictive and interpretive tools to study the geochemistry of natural waters and mineral deposits, solve environmental problems and optimize industrial processes. However, development of comprehensive models for natural systems, with their complexity and sensitivity, is a very difficult, time consuming and challenging task. The specific interaction approach for describing electrolyte solutions to high concentration introduced by Pitzer (1973; 1991) represents a significant advance in physical chemistry that has facilitated the construction of accurate computer thermodynamic models. It was showed that this approach could be expanded to accurately calculate solubilities in complex brines, and to predict the behavior of natural and industrial fluids from very low to very high concentration at standard temperature of 25°C (Harvie et al., 1984; Andre et al., 2019; Lach et al., 2018; Guignot et al., 2019; Park et al. 2009; Kolev et al., 2013; Lassin et al., 2020; Christov, 1996; 1999; 2000; 2001a; b; 2002a; b; 2003a; b; 2004; 2005; Christov et al., 2012), and from 0° to 290°C (Pabalan & Pitzer, 1987; Lassin et al., 2015; Christov & Moller, 2004a; b; Moller et al, 2006; 2007; Christov, 2007; 2009a; b; 2012a; b).

A long term safety assessment of a repository for radioactive waste requires evidence, that all relevant processes are known and understood, which might have a significant positive or negative impact on its safety. It has to be demonstrated, that the initiated chemical reactions don't lead to an un-due release of radionuclides into the environmental geo-, hydro-, and bio-sphere. One key parameter to assess the propagation of a radionuclide is its solubility in solutions interacting with the waste. Solubility estimations can either be based on experimental data determined at conditions close to those in the repository or on thermodynamic calculations. A so called "thermodynamic database" created from experimental data is the basis for thermodynamic model calculations. Since the disposal of radioactive waste is a task encompassing decades, the database is projected to operate on a long-term basis. Chemical models that predict equilibrium involving mineral, gas and aqueous phases over a broad range of solution compositions and temperatures are useful for studying the interactions between used nuclear fuel waste and its surroundings. The reliability of such predictions depends largely on the thermodynamic database. Waters of high salinity are not a typical of many geochemical environments which may be chosen as future nuclear waste repository sites. This suggests that an accurate description of highly saline waters should be required for modeling of chemical interactions in and around nuclear repositories. Currently, the most accurate description of saline waters uses the Pitzer ion interaction model. Extensive thermodynamic databases, which are based on the Pitzer ion interaction model was developed within the Yucca Mountain Project (YMTDB: data0.ypf.r2 (2007)), and Thereda project (THermodynamic REference DAtabase, THEREDA-Final Report) (Altmaier et al. 2011). Unfortunately, many of introduced in YMTDB and in THEREDA databases Pitzer models are concentration restricted and cannot describe correctly the solid-liquid equilibrium in geochemical and industrial systems of interest for nuclear waste programs.

Nitrates are expected to play a significant role in the context of the underground geochemical repository of nuclear waste. More precisely, long-lived, intermediate-level radioactive wastes that are planned to be stored in deep clay formations are composed of dried sludge from effluent treatments that contain significant quantities of nitrate amongst other elements. They are enclosed in specific containers which are placed in underground cavities dug in a very low-permeable argillite host rock. The storage safety analyses show that, despite the protection of the concrete or stainless steel-made external layers of the containers, the formation water of the host rock is likely to migrate and reach the waste during the disposal period. This would result in the potential dissolution of large amounts of nitrate and other elements, resulting in a highly saline, corrosive and oxidative media with a high reactivity towards the containment materials and their surroundings, including the host rock. Therefore, this reactivity must be characterized by development of not concentration restricted thermodynamic models, which accurately describe not only solution behavior at low molality, but also low and high molality solid-liquid phase equilibrium in nitrate systems. The models presented in our previous studies (Lach et al. 2018; Guignot et al. 2019; Lassin et al., 2020) and in the present work is a step towards this objective. It should be noted that Altmaier et al. (2011) do not include models for nitrate solutions and solids. The models introduced in Sandia National Laboratories (2007), including these for NO_3-systems are restricted up to 6 mol.kg^{-1}.

In this study we developed very well validated accurate thermodynamic models for solution behavior and solid-liquid equilibrium in 7 nitrate binary systems of the type 1-1 (HNO_3-H_2O, $LiNO_3$-H_2O, $NaNO_3$-H_2O, KNO_3-H_2O, $RbNO_3$-H_2O, $CsNO_3$-H_2O, and NH_4NO_3-H_2O) from low to very high concentration at 298.15 K. Models are developed on the basis of Pitzer ion interactions approach.

Material and Methods

FThe models for nitrate binary systems have been developed on the basis of Pitzer's semi-empirical equations (Pitzer, 1973; 1991). Since the Pitzer's representation of the aqueous phase is based on the excess free energy, all the activity expressions are consistent, allowing different kinds of data (e.g., osmotic, emf, and solubility measurements) to be used in the parameter evaluations and other thermodynamic functions to be calculated (Christov, 2007; 2009a; 2012b). Pitzer approach has found extensive use in the modeling of the thermodynamic properties of aqueous electrolyte solutions. Several extensive parameter databases have been reported. These include: 25°C database of Pitzer & Mayorga (1973; 1974) (summarized also in Pitzer, 1991); of Kim & Frederick (1988); Sandia National Laboratories (2007) and Altmaier et al. (2011).

The most widely used are databases of Chemical Modelling Group at UCSD (University California San Diego): at 25°C (Harvie et al., 1984), and T-variation (from 0° to 300°C) (Christov & Moller, 2004a; b; Moller et al., 2006; 2007; Christov, 2009a; b). However, some of the models in all of these databases are concentration restricted, and do not include all minerals precipitating from saturated and supersaturated binary and mixed systems. Some of comprehensive minerals solubilitiy databases also contain concentration restricted models for some low-, or high- concentration binary and mixed sub-systems with strong association reactions in unsaturated solutions. The concentration restricted sub-models are developed using experimental activity data in binary solutions, and solubility data in binary and high order systems up to maximum concentration (m(max)), which is much lower than concentration of saturated or supersaturated binary and mixed solutions (m(sat)). Such a restricted models predict minerals solubility, which is in pure agreement with experimental data.

The Pitzer's equations

According to Pitzer theory electrolytes are completely dissociated and in the solution there are only ions interacting with one to another (Pitzer, 1973; Pitzer & Mayorga, 1973). Two kinds of interactions are observed: (i) specific Coulomb interaction between distant ions of different signs, and (ii) nonspecific short-range interaction between two and three ions. The first kind of interaction is described by an equation of the type of the Debye-Hueckel equations. Short-range interactions in a binary system (MX(aq)) are determined by Pitzer using the binary parameters of ionic interactions ($\beta^{(0)}$,$\beta^{(1)}$, C^{φ}, and $\beta^{(2)}$). The Pitzer's equations (1 to 4) are described and widely discussed in the literature (Harvie et al., 1984; Moller et al., 2006; 2007; Christov & Moller, 2004a; 2004b).

Here only the expression for the activity coefficient of the interaction of cation (M) with other solutes, $\gamma_{(M^+)}$ is given:

$$\ln\gamma_M = z_M^2 F + \sum_a m_a(2B_{Ma}(I) + ZC_{Ma}) + \sum_c m_c(2\Phi_{Mc} + \sum_a m_a\psi_{Mca}) + \sum_c\sum_{a<a'} m_a m_{a'}\psi_{Maa'} + |z_M|\sum_c\sum_a m_c m_a C_{ca}$$
$$+ \sum_n m_n(2\lambda_{nM}) + \sum_n\sum_a m_n m_a \varsigma_{naM} \quad (1)$$

Equation (1) is symmetric for anions. The subscripts c and a in eqn 1 refer to cations and anions, and *m* is their molality; z is the charge of the M^+ ion. B and Φ represent measurable combinations of the second virial coefficients; C and ψ represent measurable combinations of third virial coefficients. B and C are parameterized from single electrolyte data, and Φ and ψ are parameterized from mixed solution data. The function F is the sum of the Debye-Hueckel term,

$$-A^{\varphi}\left[\sqrt{I}/(1 + b\sqrt{I}) + (2/b)(\ln(1 + b\sqrt{I})\right], \quad (2)$$

and terms with the derivatives of the second virial coefficients with respect to ionic strength (see Harvie et al., 1984). In Eq. (2), b is a universal empirical constant assigned to be equal to 1.2. A^{φ} (Debye-Hückel limiting law slope for the osmotic coefficient) is a function of temperature, density and the dielectric constant of water (Christov & Moller, 2004b).

For the interaction of any cation M and any anion X in a binary system $MX\text{-}H_2O$, Pitzer assumes that in Eq. (1) B has the ionic strength dependent form:

$$B_{MX} = \beta^{(o)}_{MX} + \beta^{(1)}_{MX}\, g(\alpha_1\sqrt{I}) + \quad (3)$$
$$+\beta^{(2)}_{MX}\, g(\alpha_2\sqrt{I}), \quad (3A)$$

where $g(x) = 2[1 - (1 + x)e^{-x}] / x^2$ with $x = \alpha_1\sqrt{I}$ or $\alpha_2\sqrt{I}$. α terms are function of electrolyte type and does not vary with concentration or temperature.

In Eq. 1, the Φ terms account for interactions between two ions i and j of like charges. In the expression for Φ,

$$\Phi_{ij} = \theta_{ij} + {}^{E}\theta_{ij}(I), \quad (4)$$

θ_{ij} is the only adjustable parameter. The ${}^{E}\theta_{ij}(I)$ term accounts for electrostatic unsymmetric mixing effects that depend only on the charges of ions i and j and the total ionic strength. The ψ_{ijk} parameters are used for each triple ion interaction where the ions are not all of the same sign. Their inclusion is generally important for describing solubilities in concentrated multicomponent systems. Therefore, according to the basic Pitzer equations, at constant temperature and pressure, the solution model parameters to be evaluated are: 1) pure electrolyte $\beta^{(0)}$, $\beta^{(1)}$, and C^{φ} for each cation-anion pair; 2) mixing θ for each unlike cation-cation or anion-anion pair; 3) mixing ψ for each triple ion interaction where the ions are all not of the same sign.

Fluids commonly encountered in natural systems include dissolved neutral species (such as carbon dioxide ($CO_{2(aq)}$, $SiO_{2(aq)}$, and $Al(OH)_3^o(aq)$). To account neutral specie interactions in aqueous solutions the UCSD Chemical Modelling Group included in their models additional terms to Pitzer equations, denoted as $\lambda_{N,X}$ or $\lambda_{N,A}$, and $\zeta_{N, A,X.}$ (Eq. (1)) (Harvie et al., 1984; Moller et al., 2006, 2007).

The $\beta^{(2)}$ parameter (Eqn. 3A) for 2-2 type of electrolytes

Pitzer & Mayorga (1973) did not present analysis for any 2-2 (e.g. $MgSO_4$-H_2O) or higher {e.g. 3-2: $Al_2(SO_4)_3$-H_2O} electrolytes. Indeed, they found that three β^0, β^1, and C^{φ} parameters approach (see Eqn. 1) could not accurately fit the activity data for these types of solutions. For these electrolytes mean activity ($\gamma_{\pm}$) and osmotic (φ) coefficients drop very sharply in dilute solutions, while showing a very gradual increase, with a very wide minimum at intermediate concentration. Pitzer concluded that this behaviour is due to ion association reactions and that the standard approach with three evaluated solution parameters cannot reproduce this behaviour. This lead to a further (Pitzer & Mayorga, 1974) modification to the original equations for the description of binary solutions: parameter $\beta^2(M,X)$, and an associated $\alpha_2\sqrt{I}$ term are added to the B_{MX} expression (see Eqn. (3A). Pitzer presented these parameterizations assuming that the form of the functions (i.e. 3 or 4 β and C^{φ} values, as well as the values of the α terms) vary with electrolyte type. For binary electrolyte solutions in which either the cationic or anionic species are univalent (e.g. NaCl, Na_2SO_4, or $MgCl_2$), the standard Pitzer approach use 3 parameters (i.e. omit the β^2 term) and α_1 is equal to 2.0. For 2-2 type of electrolytes the model includes the β^2 parameter and α_1 equals to 1.4 and α_2 equals to 12. This approach provides accurate models for many 2-2 binary sulfate (Pitzer & Mayorga, 1974; Christov, 1999; 2000; 2003a) and selenate (Ojkova et al., 1998; Christov, 1997; 2003a; Barkov et al., 2001), electrolytes, giving excellent representation of activity data covering the entire concentration range from low molality up to saturation and beyond.

Inclusion of "standard Pitzer approach" $\beta^{(2)}$ parameter into a models for 1-1, 2-1, 3-1, 4-1, 1-2, 1-3, and 3-2 type of electrolytes

Some authors found that there are some restrictions limited the potential of the model to describe correctly activity and solubility properties in some binary electrolyte systems with minimum one univalent ion (see Gruszkiewicz & Simonson, 2005; Petrenko & Pitzer, 1997; Sterner et al., 1998; Christov, 1996; 1998; 1999; 2000; 2001a; 2004; 2005; Christov et al., 2012; Andre et al., 2019; Lach et al., 2018; Guignot et al., 2019; Lassin et al., 2020), and of 3-2 type (see Reardon, 1988; Christov, 2001; 2002a; b; 2003b) at very high molality using classical 3 parameters (β^0, β^1, and C^{φ}) approach. According to discussion in Christov (2012a), there is one major factor which determined these restrictions: type of φ (osmotic coefficient) vs. m, or $\gamma_{\pm}$ (activity coefficient) vs. m dependences at high concentration. For all these systems φ vs. m, or $\gamma_{\pm}$ vs. m curves have a wide maximum at molality approaching molality of saturation: "LiCl(aq) type": see Lassin et al. (2015), "$CaCl_2$(aq) type": see Sterner et al. (1998); "$FeCl_2$(aq) and $FeCl_3$(aq) type": see Andre et al. (2019), Christov (2004); "$Al_2(SO_4)_3$(aq) and $Cr_2(SO_4)_3$(aq) type": see Christov (2001; 2002a; b; 2003b). On next figures (Fig. 1ab) we give a comparison of reference model (Felmy et al., 1994 and Kim & Frederick, 1988a) calculated (lines) osmotic coefficients (φ) of HNO_3 and $LiNO_3$ in binary solutions HNO_3-H_2O (Fig. 1a) and $LiNO_3$-H_2O (Fig. 1b) against molality at T = 298.15 K with recommendations in literature (symbols) (Mikulin, 1968; Hammer &

Wu, 1972). The comparison given on Fig. 1 shows that the 3 parameters (β^0, β^1, and C^φ) model of Felmy et al. (1994) for HNO_3-H_2O (Fig. 1a), included in YMTDB, is in disagreement with the data at high molality. The model of Kim and Frederick (1988a) based on 3 parameters approach (Fig. 1b) is in a very pure agreement with the data at low molality for $LiNO_3$-H_2O solutions. According to the data (see symbols on Fig. 1a and on 3a) the φ vs. molality dependences for HNO_3-H_2O and $LiNO_3$-H_2O solutions have a wide maximum at very high molality.

To describe the high concentration solution behaviour of systems showing a "smooth" maximum on $\gamma_\pm$ vs. m dependence, and to account strong association reactions at high molality, Christov (1996; 1999; 2000; 2005) used a very simple modelling technology: introducing into a model a fourth ion interaction parameter from basic Pitzer theory {$\beta^{(2)}$}, and varying the values of α_1 and α_2 terms. The author also found that by variation of the values of α_1 and α_2 terms it is possible to vary the concentration range of binary solutions at which association reactions become more important and should be account by introducing $\beta^{(2)}$ parameter. According to Christov (2005), model which uses α_1 = 1.4 and α_2 = 12 accounts association only at low molality solutions (see Christov & Moller (2004b) for $Ca(OH)_2$-H_2O model). According to previous studies of one of the authors (Christov) an approach with 4 ion interaction parameters ($\beta^{(0)}$,$\beta^{(1)}$, $\beta^{(2)}$,and C^φ), and accepting α_1 = 2, and varying in α_2 values can be used for solutions for which ion association occurs in high molality region. This approach was used for binary electrolyte systems of different type: 1-1 type {such as HNO_3-H_2O (Christov, 2005), and LiCl-H_2O (Lassin et al., 2015)}, 2-1 {such as $NiCl_2$-H_2O, $CuCl_2$-H_2O, $MnCl_2$-H_2O, $CoCl_2$-H_2O: (Christov, 1996; 1999; 2000); $FeCl_2$-H_2O: (Christov, 2004); $Ca(NO_3)_2$-H_2O: (Lach et al., 2018); $UO_2(NO_3)_2$-H2O (Lassin et al., 2020)}, 1-2 {such as $Na_2Cr_2O_7$-H_2O: (Christov, 2001a); $K_2Cr_2O_7$-H_2O: (Christov, 1998)}, 3-1 {such as $FeCl_3$-H_2O: (Christov, 2004); $Ln(NO_3)_3$(aq): (Guignot et al., 2019)}, and 3-2 {such as $Al_2(SO_4)_3$-H_2O, $Cr_2(SO_4)_3$-H_2O, and $Fe_2(SO_4)_3$-H_2O: (Christov, 2001b; 2002a; b; 2003b; 2004; 2005)}. The resulting models reduce the sigma values of fit of experimental activity data, and extend the application range of models for binary systems to the highest molality, close or equal to molality of saturation {m(sat)}, and in case of data availability: up to supersaturation. For example, aqueous complexes free 4 parameters model for LiCl-H_2O system predicts LiCl.nH_2O(s) solubilities from 0°C to 200°C and up to 40 mol.kg^{-1} (Lassin et al., 2015). The resulting accurate 4 - parameters solution models are used directly to determine $\ln K^o_{sp}$ values of precipitated solid phases using solubility approach (Harvie et al., 1984; Christov, 1996; 2012a; 2012b; Christov & Moller, 2004a; b). Therefore, the developed not high molality restricted parameterization, were used without any changes for development of solid-liquid equilibrium models for high order systems. Thus, models for $Al_2(SO_4)_3$(aq) and $Cr_2(SO_4)_3$(aq) are used without additional adjustments to construct a model for multi-component (Na+K+NH_4+Mg+Al+Cr+SO_4+H_2O) system (Christov, 2001b, 2002a, 2002b, 2003b). Four parameters (β^0, β^1, β^2, and C^φ) models for $NiCl_2$(aq), $CuCl_2$(aq), $MnCl_2$(aq), and $CoCl_2$(aq) are used for construction of Na-K-Rb-Cs-Ni-Co-Cu-Mn-Cl-H_2O model (Christov, 1996, 1999, 2000). Four parameters models for $FeCl_2$(aq)and $FeCl_3$(aq) are directly used in development of high accuracy minerals solubility model for (Na+K+Mg+Fe(II) +Fe(III)+Cl+SO_4+H_2O) system (Christov, 2004; Andre et al., 2019). A model for binary systems $Na_2Cr_2O_7$(aq), $K_2Cr_2O_7$(aq) was used without any changes to develop a comprehensive model for: (Na+K+Cl+SO_4+Cr_2O_7+H_2O) system (Christov, 1998, 2001a), and $Ca(OH)_2$(aq) model is used as s strong base for H+Na+K+Ca+OH+Cl+SO_4+H_2O model from from 0°C to 300°C (Christov and Moller, 2004b).

Determination of thermodynamic solubility product (K^o_{sp}) of precipitates

In this study we determine the thermodynamic solubility products (as K^o_{sp}) of solid phases, precipitating from saturated nitrate binary solutions (s.a. anhydrous $NaNO_3(s)$, and hydrate $LiNO_3.3H_2O(s)$ precipitating in $NaNO_3$-H_2O and $LiNO_3$-H_2O). The K^o_{sp} have been determined on the basis of evaluated binary parameters and using experimental m(sat) solubility data, using the following relationships (Christov, 2005, 2009a, 2009,b, 2012a, 2012b):

$$K^o sp (NaNO_3) = \gamma_{(\pm)}(sat)^2 .m(sat)^2$$
$$K^o sp(LiNO_3.3H_2O) = \gamma_{(\pm)}(sat)^2 .m(sat)^2 .a_w(sat)^3 \quad (5)$$

The ($\beta^{(0)}$, $\beta^{(1)}$, $\beta^{(2)}$,and C^{φ}) evaluations are directly used to determine water activity (a_w(sat)) and mean activity coefficient ($\gamma_{(\pm)}$(sat)) values at saturation. Next, experimentally determined molalities (m(sat) of the saturated binary solutions are used to calculate the thermodynamic solubility product K^o_{sp} . Than, the calculated $K^o sp$ values can be directly used to calculate standard chemical potential ($D_fG^o{}_m$) of nitrate solids (Barkov et al., 2001; Christov, 2000; 2005):

K^o_{sp} vs. standard chemical potential:

$$RTln\ K^o sp\ (LiNO_3.3H_2O) = D_fG^o{}_m(LiNO_3.3H_2O) - D_fG^o{}_m(Li^+) - D_fG^o{}_m(NO_3^-) - 3D_fG^o m\ (H_2O) \quad (6)$$

Results and Discussion

Model parameterization and validation of models for binary 1-1 nitrate systems

Validation of reference models for HNO_3-H_2O, $NaNO_3$-H_2O, and KNO_3-H_2O systems

The main goal of this study is to develop Pitzer approach based thermodynamic models for solution behavior and solid-liquid equilibrium in nitrate binary systems HNO_3-H_2O, $LiNO_3$-H_2O, $NaNO_3$-H_2O, KNO_3-H_2O, $RbNO_3$-H_2O, $CsNO_3$-H_2O, and NH_4NO_3-H_2O at 25°C. Pitzer parameters for all 7 nitrate systems under study are evaluated by many authors. The models of El Guendouzi and Marouani (2003) for $NaNO_3$-H_2O, KNO_3-$H_2O$and NH_4NO_3-H_2O are constructed only using their own low molality experimental data. Pitzer & Mayorga (1973) evaluate binary parameters for all 7 nitrate systems under study. Their standard approach 3 parameters models are valid up to m(max) molality equals to 6 m, and using osmotic coefficients (φ) data from Robinson and Stokes (1965). The m(max) parametrization of Pitzer & Mayorga (1973) is higher than the m(sat) molality of saturation (Silcock, 1979; Mikulin, 1968) for binary nitrate systems KNO_3-H_2O, $RbNO_3$-H_2O, and $CsNO_3$-H_2O (see also Table 1). The sigma value of Pitzer & Mayorga (1973) parameterization for $RbNO_3$-H_2O, and $CsNO_3$-H_2O is 0.001 and 0.002, respectively.

The models of Pitzer & Mayorga (1973) for $LiNO_3$-H_2O, $NaNO_3$-H_2O, and NH_4NO_3-H_2O are valid up to molality, which is much lower than the maximum molality of available experimental osmotic coefficients (φ) data (Hammer & Wu, 1972; Mikulin, 1968) and m(sat) molality of saturation (see Table 1). Their model for HNO_3-H_2O is valid only up to 3 m, while the data are available up to 29.3 m (Hammer & Wu, 1972; Mikulin, 1968). In our previous study (Lach et al., 2018) we present a Pitzer parameterization to predict solution properties and salt solubility in the H-Na-K-Ca-Mg-NO_3-H_2O system. All models for binary sub-systems were parameterized using all available experimental data up to saturation. The models for HNO_3-H_2O and KNO_3-H_2O includes formation of neutral aqueous complexes $HNO_3^o(aq)$ and $KNO_3^o(aq)$. In a previous study of Christov (2005) an extended 4-parameters model for HNO_3-H_2O system is presented. The extended approach with four Pitzer ion interaction binary parameters ($\beta^{(0)}$, $\beta^{(1)}$, $\beta^{(2)}$,and C^{φ}), accepting α_1 =2, and varying in the values of α_2 term gives an excellent agreement with osmotic coefficients data (σ=0.0043). The model is valid up to m(max) of data, equals to 29.3 m.

On next Figures 2abc we compare reference model calculated (lines) osmotic coefficients (φ) of HNO_3, $NaNO_3$ and KNO_3 in binary solutions HNO_3-H_2O, $NaNO_3$-H_2O and KNO_3- H_2O against molality at T = 25°C with recommendations in literature (symbols). Heavy solid lines represent the predictions of the accepted models of Christov (2005) for HNO_3-H_2O, of Lach et al. (2018) for $NaNO_3$-H_2O, and of Pitzer & Mayorga (1973) for KNO_3- H_2O. Dashed-dotted line represents the predictions of the model of Felmy et al. (1984) for HNO_3-H_2O, of Pitzer & Mayorga (1973) for $NaNO_3$-H_2O, and of Lach et al. (2018) for KNO_3- H_2O. The experimental data on Figs. 2abc are taken from Mikulin (1968) up to saturated solutions (open squares on 2a, and open triangles on 2b and 2c), from Hammer & Wu (1972) (stars on 2a) and El Guendouzi & Marouani (2003) (open squares on 2b and 2c). It should be noted that all three sets of experimental data are in good agreement (see Fig. 2). The vertical lines on 2b and 2c denote the molality of stable crystallization of solid phases $NaNO_3(cr)$ and $KNO_3(cr)$, taken from Mikulin (1968). As it is shown the four parameters model of Christov (2005) for HNO_3-H_2O, and the 3 parameters models of Lach et al. (2018) for $NaNO_3$-H_2O, and of Pitzer and Mayorga (1973) for KNO_3- H_2O are in very good agreement with raw experimental data from low to very high concentration. Therefore, these 3 models are accepted in our further calculations. The results given on Fig. 2a and 2b also show that inclusion of neutral species $HNO_3^o(aq)$ and $KNO_3^o(aq)$ into a models for HNO_3-H_2O and KNO_3- H_2O systems is not necessary. The inclusion of complex reactions only complicates, and do not improve the models.

Parametrization of models for $LiNO_3$-H_2O, $RbNO_3$-H_2O, $CsNO_3$-H_2O, and NH_4NO_3-H_2O.

The remaining models developed in this study are those for binary systems $LiNO_3$-H_2O, $RbNO_3$-H_2O, $CsNO_3$-H_2O, and NH_4NO_3-H_2O. New sets of Pitzer ion interaction binary parameters are evaluated using available raw experimental osmotic coefficients (φ) data for whole molality range of solutions. The φ vs. m data for all 4 solutions are given in Hammer and Wu (1972), Mikulin (1968), and Robinson and Stokes (1965). El Guendouzi & Marouani (2003) present the φ data for $LiNO_3$-H_2O and NH_4NO_3-H_2O solutions. All reference φ vs. m data sets are in a good agreement (see also symbols on Fig. 3a,b,e). However, the data of Mikulin (1968) cover the whole molality range of unsaturated and saturated solutions. In case of $LiNO_3$-H_2O Mikulin also reported data for supersaturated solutions (from 12.45 m to 20 m). In this study we parameterize the models using 1) very low molalty (to 0.1 m) data of Hammer and Wu (1972), 2) all data of Mikulin (1968) for whole molalty range of unsaturated solutions from 0.1 m to m(max), 3) the data points at saturation (φ(sat)) (from Mikulin, 1968), and 4) data for supersaturated $LiNO_3$-H_2O solutions (from Mikulin, 1968).

In parameterization process we used the value of Debye-Hückel term ($A^φ$) equals to 0.39147, as given in Christov (2009a; 2009b; 2012a). Following the parameterization scheme described in paragraphs 2.2 and 2.3 the model for all 4 binary nitrate solutions is parameterized using two different approaches: (I) standard for 1-1 electrolytes approach with 3 ion interaction binary parameters ($\beta^{(0)}$, $\beta^{(1)}$, and $C^φ$) and setting α_1 term equals to 2, and α_2 = 0.0, and (II) an extended approach with four Pitzer ion interaction binary parameters ($\beta^{(0)}$, $\beta^{(1)}$, $\beta^{(2)}$,and $C^φ$) and varying in the values of α_1 and α_2 terms. As a first step in parameterization we used classical 3 parameters approach (I) and evaluate binary parameters using all available raw φ data for whole molality range of solutions. As a next step, using the same φ data we re-parameterize the models on the basis of extended approach (II), and using two α – combinations: (IIa) α_1 =2 and α_2 = 1, and (IIb) α_1 =2 and α_2 = -1 (Christov, 1996; 1999; 2000; 2004; 2005). It was found that more combinations in "alfa" values do not improve the fit of data used in parameterization. The main criterion in the choice of established parameterization was the value of standard deviation (σ) of fit of used φ data, i.e. parameterization with the lowest sigma value is accepted. For definition of sigma (σ) see Christov & Moller (2004b). It was found that similarly to accepted reference binary models for $NaNO_3$-H_2O and KNO_3-H_2O), for 2 of studied systems $CsNO_3$-H_2O and NH_4NO_3-H_2O, the approach with 3 parameters ($\beta^{(0)}$, $\beta^{(1)}$, $C^φ$) give an acceptable agreement with the data, up to much higher molality, than the available parameteri-zations (Pitzer & Mayorga (1973), and El Guendouzi & Marouani (2003). The new model for $CsNO_3$-H_2O fits the osmotic coefficients data with a sigma equals to 0.00052, which is almost four times less than sigma value of parameterization of Pitzer & Mayorga (1973) (σ = 0.002). For these systems introducing into a model of fourth ($\beta^{(2)}$) parameter do not improve considerably the fit of data. The new parameterization for $CsNO_3$-H_2O and NH_4NO_3-H_2O systems is not only concentration-extended up to molality of saturation (m(sat)), but also provide a better fit of data (lowest sigma), than the concentration restricted models presented in literature (Pitzer & Mayorga, 1973; Kim & Frederick (1988) and El Guendouzi & Marouani, 2003). The new NH_4NO_3-H_2O parameterization fits the data up to m(sat) =26.8 m with a sigma equals to 0.0018, which is three times less than sigma value of parameterization of Kim & Frederick (1988) (σ = 0.0054).

For $LiNO_3$-H_2O system we construct a model on the basis of extended approach (2), and using α_1 =2 and α_2 = -1. The resulting model fits the data up to supersaturation zone (m(max) = 20m) with sigma value equals to 0.00137, which is much less than the value of Kim & Frederick (1988) parameterization (σ = 0.0064). The new model for $RbNO_3$-H_2O also is based on 4 parameters approach and uses α_1 = 2 and α_2 = 1. The model fits the data with σ = 0.000373, which is much less than the values of models of Pitzer & Mayorga (1973) (σ = 0.001), and of Kim & Frederick (1988) (σ = 0.0023).

On next Figures 3(abcde) we present a comparison of osmotic coefficients in nitrate binary solutions $LiNO_3$-H_2O, $RbNO_3$-H_2O, $CsNO_3$-H_2O, and NH_4NO_3-H_2O calculated by the accepted models developed here (heavy solid lines), models developed by other authors (dashed lines, light solid lines; Pitzer and Mayorga (1973), Kim and Frederick (1988)), and with the recommended values given in literature (symbols) at 25°C. The vertical lines on the figures denote the molality of solutions saturated with corresponding nitrate solid phase (m(sat)), taken from Mikulin (1968). Excellent model – experiment agreement has been obtained for all four systems and from low up to very high molality (see Fig. 3a and 3e). As is shown on Fig. 3b, the new model for $LiNO_3$-H_2O is in excellent agreement not only with the data at high molality, but contrary to the model of Kim & Frederick (1988) (see Fig. 1b) also in low molality range.

The models for all 7 nitrate binary systems under study are also validated by comparison with recommendations given in literature (Hammer & Wu, 1972; Mikulin, 1968) on the mean activity coefficients ($g_{\pm}$). These recommendations on $g_{\pm}$ are model-dependent. Therefore, they are not used in parameterization process, and only to validate the resulting models. The comparisons between model predictions and reference recommendations, which are not given here, show an excellent agreement from low to very high concentrations.

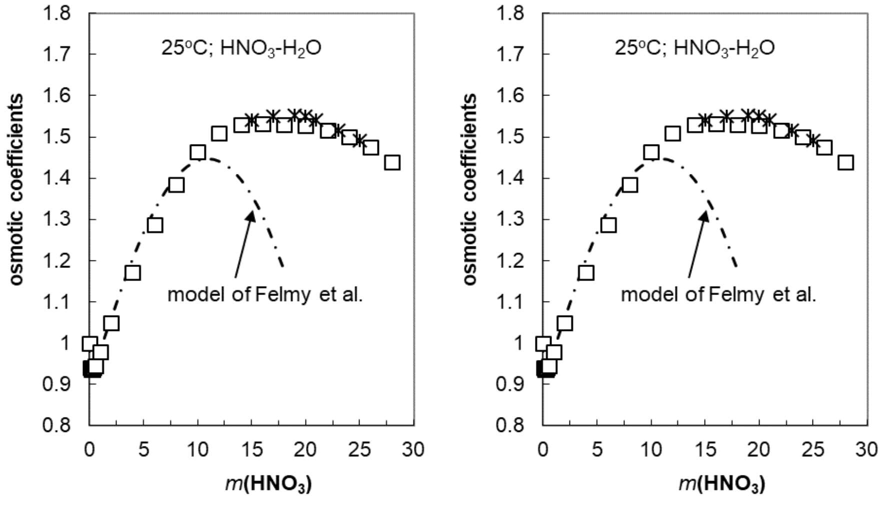
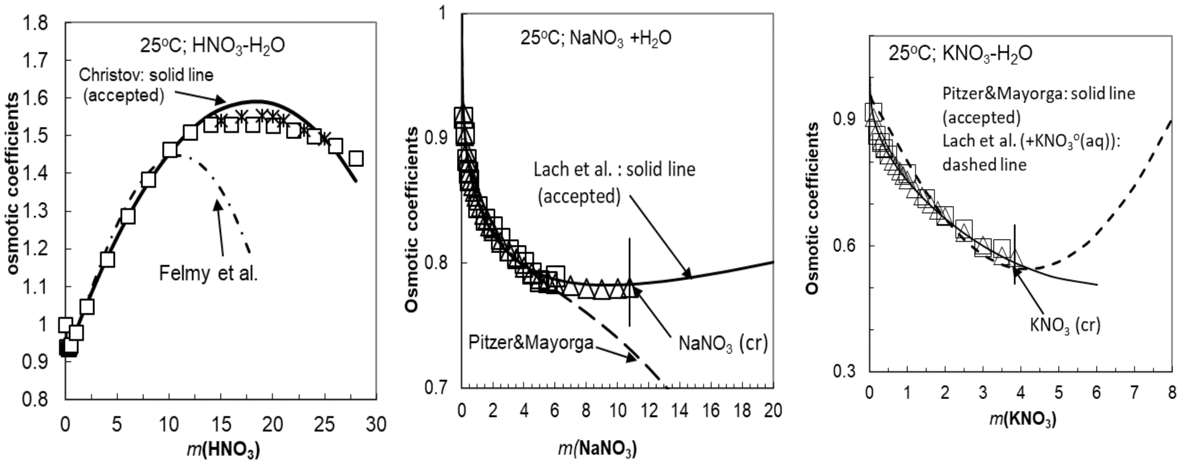
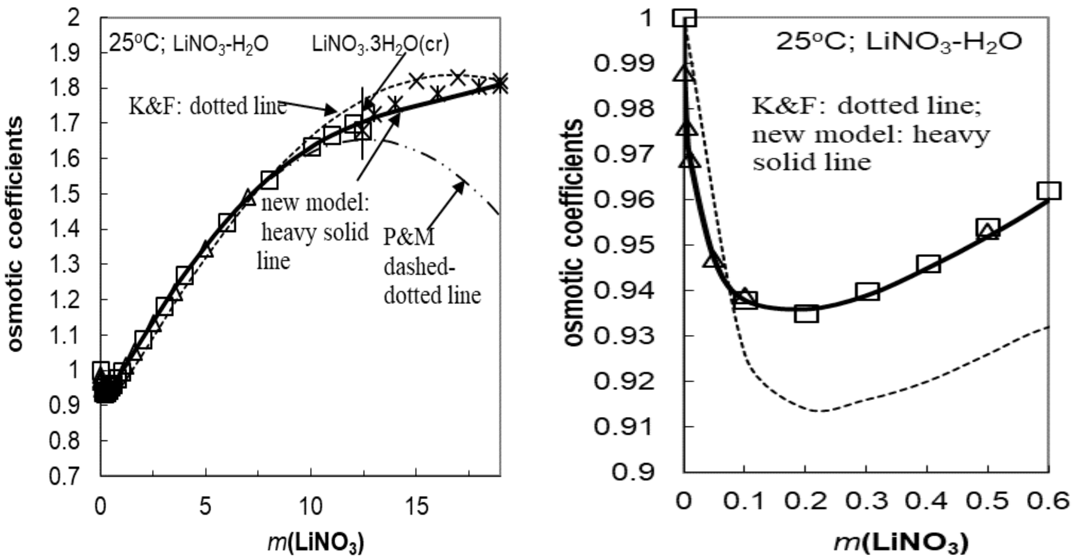


Fig. 1a,b. Comparison of reference model calculated (lines) osmotic coefficients (φ) of HNO_3 (and $LiNO_3$ in binary solutions HNO_3-H_2O (Fig. a) and $LiNO_3$-H_2O (Fig. b) against molality at T = 25°C with recommendations in literature (symbols) (Mikulin, 1968; Hammer & Wu, 1972). The (b) figure is an enlargement of the low molality corner. Dashed-dotted line on (a) represent the predictions of the model of Felmy et al. (1994), and dotted line on (b) are predictions of the model of Kim & Frederick (1988a).

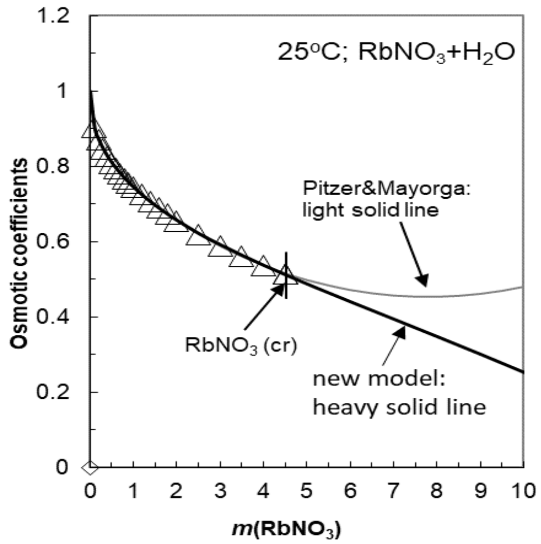


a) b) c)

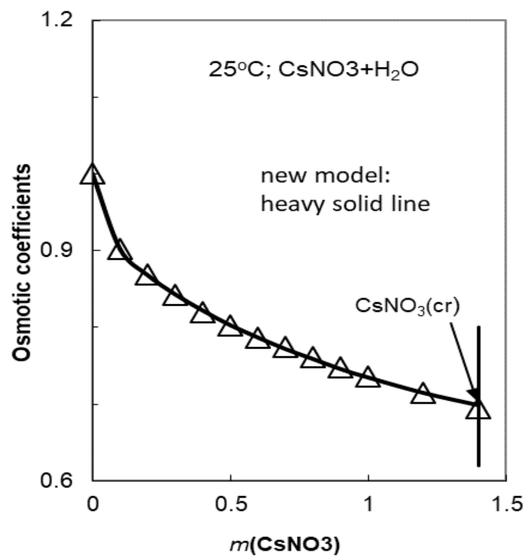
Fig. 2a,b,c. Comparison of reference model calculated (lines) osmotic coefficients (φ) of HNO_3, $NaNO_3$ and KNO_3 in binary solutions HNO_3-H_2O, $NaNO_3$-H_2O and KNO_3- H_2O against molality at T = 298.15 K with recommendations in literature (symbols). Heavy solid lines represent the predictions of the accepted models of Christov (2005) for HNO_3-H_2O, of Lach et al. (2018) for $NaNO_3$-H_2O, and of Pitzer & Mayorga (1973) for KNO_3- H_2O. Dashed-dotted lines represent the predictions of the model of Felmy et al. (1984) for HNO_3-H_2O, of Pitzer & Mayorga (1973) for $NaNO_3$-H_2O, and of Lach et al. (2018) for KNO_3- H_2O. The model of Lach et al. (2018) includes neutral specie KNO_3^o(aq). The experimental data are taken from Mikulin (1968) up to saturated solutions (open squares on (a), open triangles on (b) and (c)), Hammer & Wu (1972) (stars on (a)), and El Guendouzi & Marouani (2003) (open squares on (b) and (c)). The vertical lines on b and c denote the molality of stable crystallization of solid phases $NaNO_3$(cr) and KNO_3(cr), taken from Mikulin (1968).



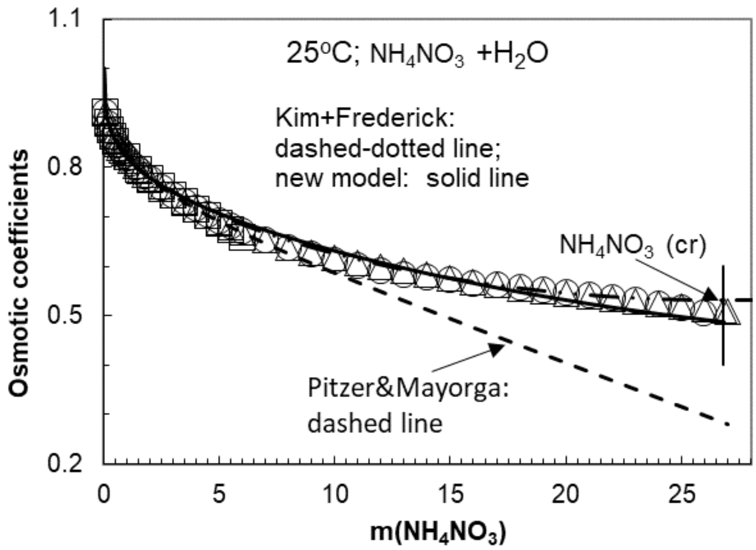
a) b)



c)



d)



e)

Fig. 3a,b,c,d,e. Comparison of model calculated (lines) osmotic coefficients (φ) of $LiNO_3$(Fig. a and b), $RbNO_3$ (Fig. c), $CsNO_3$ (Fig. d) and NH_4NO_3 (Fig. e) in binary solutions $LiNO_3$-H_2O, $RbNO_3$-H_2O, $CsNO_3$-H_2O and NH_4NO_3-H_2O against molality at T = 298.15 K with recommendations in literature (symbols). The (b) figure is an enlargement of the low molality corner. Heavy solid lines represent the predictions of the developed in this study and accepted models. Dashed-dotted, dashed and light solid lines represent the predictions of the reference models of Kim & Frederick (1988) (Fig. a and b), and of Pitzer & Mayorga (1973) (Fig. c and e). The experimental data are taken from Mikulin (1968) (open squares on 3ab and open triangles on 3cde), Hammer & Wu (1972) (open triangles and stars on 3ab and open circles on 3e), and El Guendouzi & Marouani (2003) (open squares on 3e). The vertical lines denote the molality of stable crystallization of solid nitrate phases.

Conclusions

Deliquescence of single inorganic salt or their mixture is a process of spontaneous solid-liquid phase change. It is a process in which a soluble solid substance sorbs water vapor from the air to form a thermodynamically stable saturated aqueous solution on the surface of the particle. It is occurs when relative humidity (RH) in the gas-phase environment is at, or above deliquescence relative humidity (DRH) of the salt, or mutual deliquescence relative humidity (MDRH) of a salt mixture. Within the solid-liquid equilibrium model, relative humidity is related to water activity, a_w (Clegg & Brimblecombe, 1995; Christov, 2009a; b; 2012a; b):

$$a_w = P_w / P^o_w = RH/100, \qquad (7)$$

where P_w and P^o_w are the vapor pressure of the saturation solution and pure water, respectively, at given temperature. As a result, both DRH and MDRH of saturated surface solutions depend of temperature, the salt stoichiometry, and the solution composition. This process is of interest in many areas, such as heterogeneous chemistry of inorganic salts, corrosion of metals in wet atmosphere, in studies of chemistry of sea-type aerosol atmospheric system, and especially in development of strategies and programs for nuclear waste geochemical storage. Because of very high complicity of experiments, the relative humidity DRH experimental data are sparse. Therefore, different sophisticated thermodynamic models have been proposed and developed to describe the deliquescence behavior of inorganic salts at wet conditions. In our previous studies it was showed that calculations based on not high concentration restricted Pitzer models can be used for accurate determinations of both DRH and MDRH of saturated solutions in a wide range of temperatures, and compositions (Christov, 2009a; b; 2012a, 2012b).

On the basis of evaluated binary parameters ($\beta^{(0)}$, $\beta^{(1)}$, $\beta^{(2)}$,and C^{φ}) in this study we also determine water activity (a_w) and Deliquescence Relative Humidity (DRH (%)) (eqn. 7) of solid phases crystallizing from saturated binary nitrate solutions [$LiNO_3.3H_2O$(s), $NaNO_3$(s), KNO_3(s), $RbNO_3$(s), $CsNO_3$(s), and NH_4NO_3(s)]. The results of calculations are given in Table 1. The model DRH predictions are in excellent agreement with the experimental data determined using isopiestic method, and given in Mikulin (1968). According to model calculations the solid-liquid phase change of $LiNO_3.3H_2O$(s) occurs at lowest relative humidity of environment, although the m(sat)-solubility of NH_4NO_3(s) is more than two times higher.

Table 1. Comparison between model calculated and recommended values of the Deliquescence Relative Humidity [DRH (%) = a_w^s x 100; where a_w^s is activity of water at saturation] and of the logarithm of the thermodynamic solubility product (K^o_{sp}) of nitrate solid phases crystallizing from saturated binary solutions at T = 25°C. *Legend:* [a]Experimental data of Mikulin (1968); [b]Calculated values of Lach et al. (2018).

Salt composition	$m^s/(mol.kg^{-1})^a$	DRH, %		lnK^o_{sp}	
		This study	Reference data	This study	Reference data
$LiNO_3$ $3H_2O$(cr)	12.45	46.9	47.06 [a]	4.9765	-
$NaNO_3$(cr)	10.878	73.72	73.8 [a] 73.76[b]	2.5043	2.502 [b]
KNO_3(cr)	3.84	92.73	92.4 [a] 92.6 [b]	-0.2576	-0.1557 [b]
$RbNO_3$(cr)	4.52	92.02	91.9 [a]	-0.2279	-
$CsNO_3$(cr)	1.4	96.54	96.5 [a]	-1.3275	-
NH_4NO_3(cr)	26.8	62.4	61.1 [a]	2.4731	-

As a next step, using the accepted and new developed parameterizations, and experimentally determined molalities (m(sat) of the saturated binary solutions (Mikulin, 1968) we calculate the logarithm of the thermodynamic solubility product (ln K^{o}_{sp}) of six nitrate solid phases crystallizing from saturated binary nitrate solutions at T = 298.15 K (eqn. (5)). The model calculations are given in Table 1. A good agreement has been obtained for $NaNO_3$(s), and KNO_3(s) with calculations of Lach et al. (2018). The small ln K^{o}_{sp} difference (of 0.1 logarithm units) for KNO_3(s) is mainly due on the different m(sat) values used in calculations (see eqn. (5)). Note that the widely used databases of Pitzer (1991), Pitzer & Mayorga (1973) and Kim & Frederick (1988) do not consider solid phases.

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Macropterous Ground Beetles (Coleoptera: Carabidae) Prevail in European Oilseed Rape Fields

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Abstract. During a research conducted in oilseed rape (*Brassica napus* L.) fields in four European countries (Bulgaria, Germany, Romania and Switzerland), species composition and ecological structure of the ground beetle (Coleoptera: Carabidae) fauna associated with the rape were studied. Field work was carried out in 2017 (2018 in Bulgaria). Pitfall traps (5 in each site) were set in each sampling site in each country. Captured beetles belonged to 179 species and 51 genera. The most diverse were genera *Harpalus* Latreille, 1802 and *Amara* Bonelli, 1810 (21 species each), followed by the genera *Carabus* Linnaeus, 1758 (15 species), *Pterostichus* Bonelli, 1810 (10 species), *Microlestes* Schmidt-Goebel, 1846 and *Poecilus* Bonelli, 1810 (9 species each), and *Brachinus* Weber, 1801 and *Ophonus* Dejean, 1821 (8 species each). In Bulgaria were found 107 species, in Germany – 68 species, in Romania – 71 species, in Switzerland – 45 species. Fourteen species were common in all countries. Macropterous species represented 65% (116 species) of all collected carabid species (in all countries). Pteridimorphic species were 20% of all (36 species), and brachypterous were only 12% (21 species). For 6 species (3%) there were no data about their wing morphology. The results were similar in each country. Macropterous species were 73% (78 species) in Bulgaria, 60% (41 species) in Germany, 68% (48 species) in Romania, and 69% (31 species) in Switzerland. Macropterous beetles prevailed in number of specimens too (79% of the specimens in all countries). The prevalence of the macropterous carabids reflects their higher mobility and adaptiveness.

Key words: carabids, agrocoenoses, ecology, Europe, wing morphology, dispersal power, flight ability.

Introduction

Wing polymorphism in carabid beetles (Coleoptera: Carabidae) is well known and relatively well studied, as constantly macropterous, constantly brachypterous or apterous as well as di- and polymorphic species are reported (Lindroth, 1949; Haeck, 1971; Den Boer, 1977; Den Boer et al., 1980; Brandmayer, 1983; Kavanaugh, 1985; Desender et al., 1986; Kromp, 1999; Kotze & O'Hara, 2003; Venn, 2016, etc.). In fact, ground beetles are probably the best studied group of animals in this respect (Kotze et al., 2011). Recently, Venn (2016) presented a review of studies on the topic.

The degree of hind wing development allows three groups to be distinguished: macropterous (winged) species have fully developed hind wings in all individuals, whereas brachypterous (wingless) species have reduced vestigial wings. In wing dimorphic species, some individuals have fully developed wings, others only vestigial ones (Den Boer et al., 1980; Kromp, 1999). Furthermore, wing morphology of ground beetles can vary considerably, even within the same species, and this variation suggests that the term wing-polymorphic is more appropriate than dimorphic (Desender, 1989; Venn, 2007, 2016).

The dispersal power of beetles could be estimated by measuring their wing morphology (Den Boer et al., 1980; Gutierrez & Menendez, 1997; Matalin 1994, 2003; Kotze & O'Hara, 2003; Venn 2016). The migratory component comprises mainly macropterous species, whereas the stable component comprises mainly brachypterous species and predominantly brachypterous morphs of dimorphic species (Chernov & Makarova, 2008). Good flyers, as a rule, have larger areals, and flightless beetles have smaller ranges (Kryzhanovskij, 1965). The dispersal and migration ability depends on the proportion of macropterous specimens in a given population (Lindroth, 1949) and functionality of the wing muscles (Desender, 1989), and macropterous, dimorphic and brachypterous species differ in patterns of spatial distribution and co-occurrences (Zalewski & Ulrich, 2006).

It is known that habitat type and disturbance influence wing morphology of carabids (Venn, 2016). Darlington (1943) found that full-winged species predominate among arboreal carabids due to the necessity of frequent dispersal in patchy and unstable habitats, and epigeic carabid species from stable habitats have no reason to fly, and therefore evolve brachypterous forms. A number of studies have suggested that in areas with increased disturbance the numbers of specialist, large bodied and poorly dispersing species decrease in abundance, whilst generalist, small bodied effective dispersers increase (Den Boer et al., 1980; Rushton et al., 1989; Blake et al., 1994, Niemelä et al., 2000; Grandchamp et al., 2002; Mazzei et al., 2015; Barber et al., 2017). The more stable the occupied habitats are, the more natural selection will reduce relative wing size, and the numbers of flightless species will rise (Den Boer et al., 1980; Gnetti et al., 2015). Wing morphology is also studied in relation to the trophic level of carabids, and showed that wing dimorphic species occupied higher trophic levels than winged species (Zalewki et al., 2015).

According to Holliday (1991) there may be a general pattern of ground beetle community succession, with early stages typified by small, phytophagous species with strong dispersal capability, and mature stages containing more large, flightless carnivores.

In this study the carabid diversity in oilseed rape (*Brassica napus* L.) fields in four European countries (Bulgaria, Germany, Romania and Switzerland) was researched. It aimed at establishing the composition of the carabid fauna in relation to their wing morphology.

Material and Methods

Field work was carried out in 2017 in Germany, Romania and Switzerland, and in 2018 in Bulgaria. Pitfall traps (5 in each site) with salt and 6% acetic acid saturated solution (with small amount of dishwasher detergent) as a preserving fluid were set in each sampling site in each country. The sampling periods were three in all countries and they were during the flowering, during the ripening and after the harvest of the oilseed rape. Thus, due to the specific conditions in the different countries, the periods of research were different, as well as the number of the sampling sites (Table 1). All carabids were determined to species level using the keys of Hůrka (1996), Turin et al. (2003), Luff (2007), Arndt et al. (2011). Species were classified into three groups: winged or macropterous (always possessing wings), wing dimorphic/polymorphic (only part of the population being fully winged), and brachypterous (wingless), according to the commonly accepted classification of Den Boer et al. (1980).

For the assessment of the taxonomic similarity, the classification of Zlotin (1975) was used.

Frequency of occurrence was calculated using the formula: F = (p/P).100%, where p is number of the countries where the species occur (no matter of its abundance), and P is the number of the studied countries, i.e. P = 4.

The data were processed with MS Excel and PRIMER 6 (Clarke & Gorley, 2005).

Results and Discussion

During the study altogether 37912 carabid beetles were collected. They belonged to 179 species and 51 genera (Appendix 1). The most diverse were genera *Harpalus* and *Amara* (21 species each), followed by the genera *Carabus* (15 species), *Pterostichus* (10 species), *Microlestes* and *Poecilus* (9 species each), and *Brachinus* and *Ophonus* (8 species each).

In Bulgaria were collected 5018 specimens from 107 species, in Germany – 14285 specimens from 68 species, in Romania – 7576 specimens from 71 species, in Switzerland – 11033 specimens from 45 species (Appendix 1). Fourteen species were common in all countries. It is noticeable that in countries with less species diversity there is greater abundance of established beetles, which proves the ecological effect of concentration of dominance and speaks of the presence of some catastrophic effect in the biocoenoses. This could be, for example, the intensification of the agriculture.

Macropterous species represented 65% (116 species) of all collected carabid species (in all countries). Pteridimorphic species were 20% of all (36 species), and brachypterous were only 12% (21 species). For 6 species (3%) there were no data about their wing morphology (Appendix 1, Fig. 1A). Macropterous beetles prevailed in number of specimens too (79% of the specimens in all countries) (Appendix 1, Fig. 1B).

The results were similar in each country. Macropterous species were 73% (78 species) in Bulgaria, 60% (41 species) in Germany, 68% (48 species) in Romania, and 69% (31 species) in Switzerland. Pteridimorphic species were 17% (18 species) in Bulgaria, 32% (22 species) in Germany, 15% (11 species) in Romania, and 29% (13 species) in Switzerland. Brachypterous species were less abundant in all four countries (Fig. 2).

Table 1. Number of sampling sites (Ss) and sampling periods in each country (2017 in Germany, Romania and Switzerland, and 2018 in Bulgaria).

Country	Ss	Flowering	Ripening	After the harvest
Bulgaria	10	19-22.IV – 14-16.V	14-16.V – 11-13.VI	25-27.VII – 24-26.VIII
Germany	9	4-9.V– 23-29.V	21-30.VI – 9-19.VII	16.VIII-15.IX – 4.IX-11.X
Romania	10	3-5.V – 23-24.V	13-15.VI – 5-7.VII	20-22.VIII – 9-10.IX
Switzerland	8	11-12.IV – 3-5.V	1-20.VI – 20.VI-12.VII	2-3.VIII – 22-23.VIII

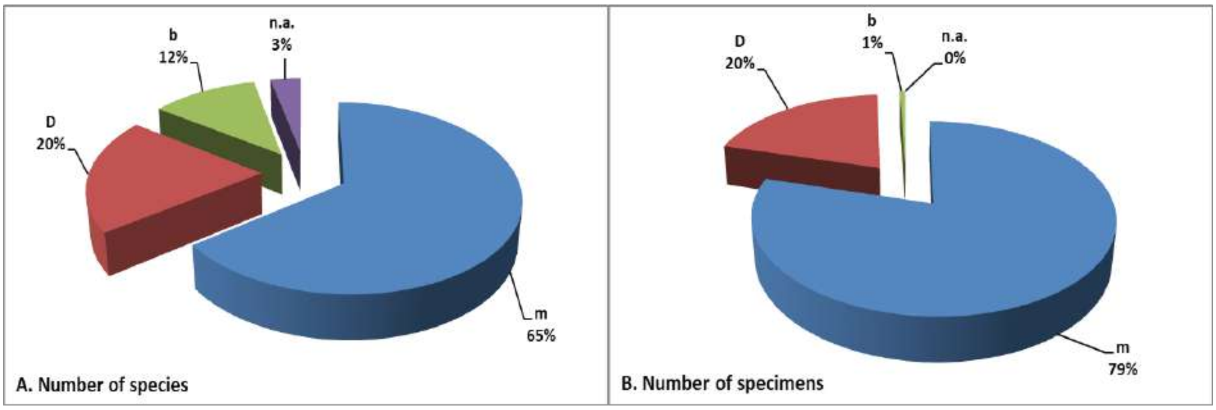


Fig. 1. Wing morphology of carabids in all countries. A. Number of species. B. Number of specimens. m – macropterous, D – wing di(poly)morphic, b – brachypterous, n.a. – no data.

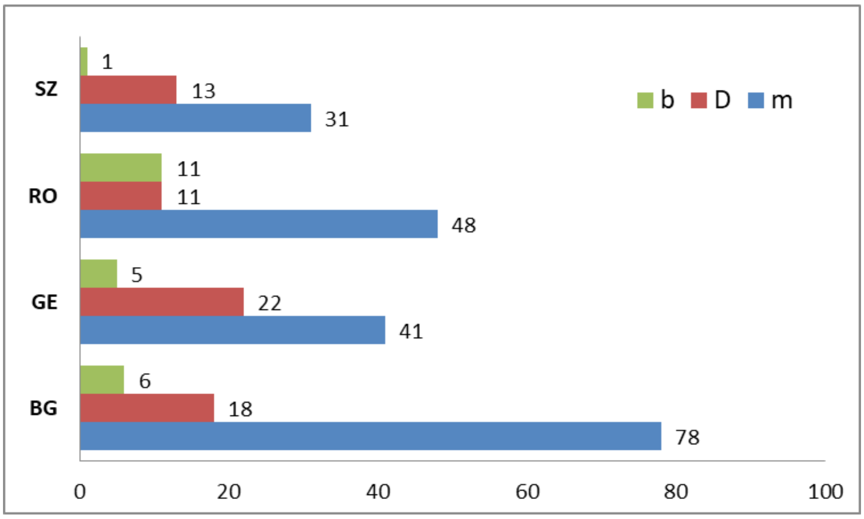


Fig. 2. Numbers of macropterous (m), di(poly)morphic (D) and brachypterous (b) species in four countries.

The prevalence of the macropterous carabids reflects their higher mobility and adaptiveness. Since macropterous wings are mainly used for dispersal flights, winged species seem normally especially abundant in scattered or disturbed habitats, e.g. cultural land. On the other hand, brachypterous species often are stenotopic (e.g. forest) inhabitants with a low dispersal ability (Kryzhanovskij, 1965; Kromp, 1999; Chernov & Makarova, 2008). Carabid communities in earliest stages of restoration of grasslands were also numerically dominated by small, winged species (Barber et al., 2017). In contrast, all species collected in high numbers in spruce forests were brachypterous (Gnetti et al., 2015).

Our results are in accordance with Gray's hypothesis, that the proportion of flight capable pioneer species should increase with increasing disturbance, and the proportion of flightless species should decrease (Gray, 1989), as it was also suggested by Magura et al. (2010). Gobbi & Fontaneto (2008) also found that short winged, large and predatory species were negatively related to human impact. Habitats with a high degree of disturbance have a lower proportion of brachypterous carabids, as those species are sensitive to unstable and variable conditions, such as in agroecosystems. Similarly, measuring the potential flight ability of carabids, Venn (2007) found that the proportion of macropterous individuals was greater, and the wing-length of brachypterous individuals was longer in the populations of disturbed sites. Ground beetle species able to fly were better represented (72%) in the younger, disturbed and less stable riparian alder stand in the study of Mazzei et al. (2015). Similar results were obtained in urban park grasslands under different mowing regimes by Venn & Rokala (2005) and in urban golf courses by Saarikivi et al. (2010).

It is considered (Lindroth, 1992; den Boer, 1971; Venn, 2007) that the proportion of macropterous individuals is indicative of the age and stability of the population. A stable and long established population should contain almost exclusively brachypterous individuals, as dispersal ability is not advantageous in these circumstances, which is not the case in our study. Such results were obtained by Kavanaugh (1985), who found 73% brachypterous carabid taxa in an Alpine habitat. During the last decades, many typical natural habitats were destroyed or declined in whole Europe, particularly in lowlands, where extensive lands were transformed into agrolandscapes. That is why brachypterous, large and specialist ground beetles are declining too (Kotze & O'Hara, 2003).

Comparing two riparian alder forests subject to different disturbance factors, Mazzei et al. (2015) also found that the younger stand is a less stable environment with fewer brachypterous species. Young sites were typified by small, macropterous, phytophagous species, while older sites contained larger species more likely to be flightless and carnivorous, in a study in restored grasslands (Barber et al., 2017). Across a coastal heathland successional gradient winged and phytophagous species predominated in the earliest successional stages too (Schirmel et al., 2012). Woodcock et al. (2012) showed that flightless beetle species and those relying on a more limited food breadth took longer to colonize early successional habitats, which explains their smaller presence in the studied rape fields.

The similarity between four countries (Fig. 3), calculated on the basis of the abundance of all macropterous, dimorphic, brachypterous and not determined species, showed that Bulgarian sample significantly distinguishes from the other countries and separates from them on a very low level of similarity, according to Zlotin (1975). Romania also distinguishes from Germany and Switzerland on an average level of similarity. The last two countries seem grouped, although their similarity is not very high. This is in accordance with the established similar ratios between the species diversity and abundance in these countries.

In relation of their frequency of occurrence, carabid species were separated in four classes (see Appendix 1): with occurrence of 25% (occurring in only one country), 50% (occurring in two countries), 75% (occurring in three countries) and 100% (constant species, occurring in all countries). Most of the species were with occurrence of 25% (Fig. 4), which is normal given the fact that every country has its own set of species. It is, however, notable that the brachypterous species where mainly in the class of the "local" species, and only two of them had occurrence of 50%. This showed the lower dispersal power of those species, in contrast of the findings of Zalewski & Ulrich (2006), where the macropterous species occupied fewer sites than dimorphic and brachypterous species. Common species with occurrence of 100% in our study where mostly winged, as only one species was dimorphic.

According to the abundance of the macropterous, dimorphic and brachypterous species, our study showed that the most abundant were common (F = 100%) macropterous species (Fig. 5). They totally predominated over all other species, which once again confirmed the already established trend for concentration of domination. This concentration is resulting from the extremely high abundances of *Poecilus cupreus* in Switzerland, Germany and Romania, *Anchomenus dorsalis* in Switzerland and Romania, *Nebria brevicollis* in Germany, and *Brachinus explodens* in Romania. These results are totally in contrast of the findings of Zalewski & Ulrich (2006), where the macropterous species had lower site abundances and occupied fewer sites than dimorphic and brachypterous species. Probably the reason is in the type of the habitat, since they performed their research in natural sites, whilst ours were conducted in agrocoenoses. Also in contrast to our results, Work et al. (2008) did not observe a clear association between frequency–abundance relationships and dispersal ability, probably due to the lack of quantitative evidence of dispersal ability of some species.

Macropterous, flight capable species are supposed to have higher dispersal abilities than dimorphic or brachypterous ones, they are better adapted to ecosystems with frequent disturbance and their higher abundance might be attesting to their faster dispersion and colonization of new habitats (Kryzhanovskij, 1965; Chernov & Makarova, 2008; Hendrickx et al., 2009; Venn, 2016).

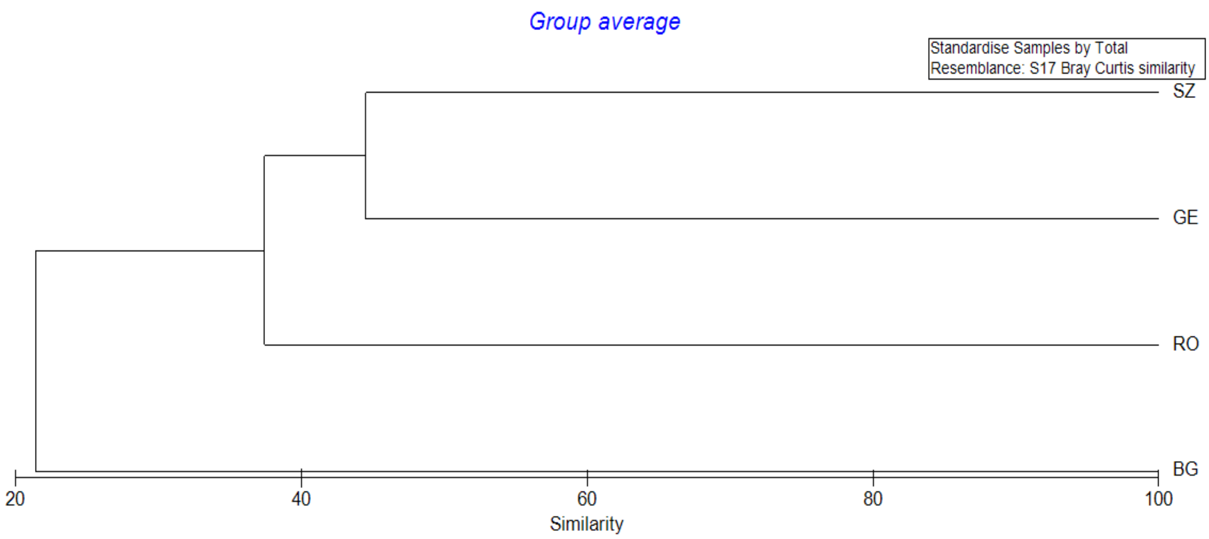
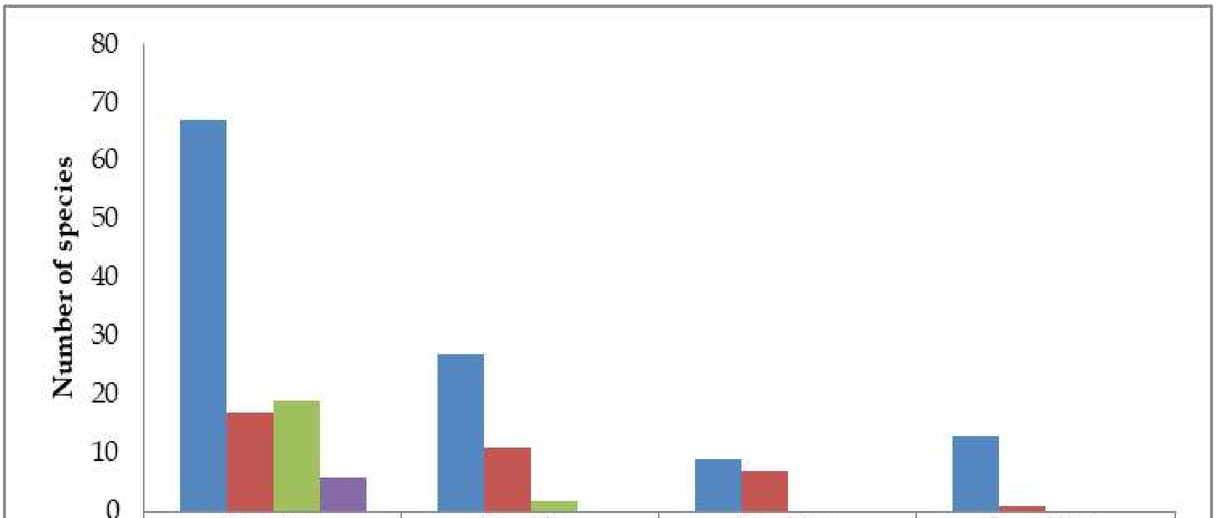
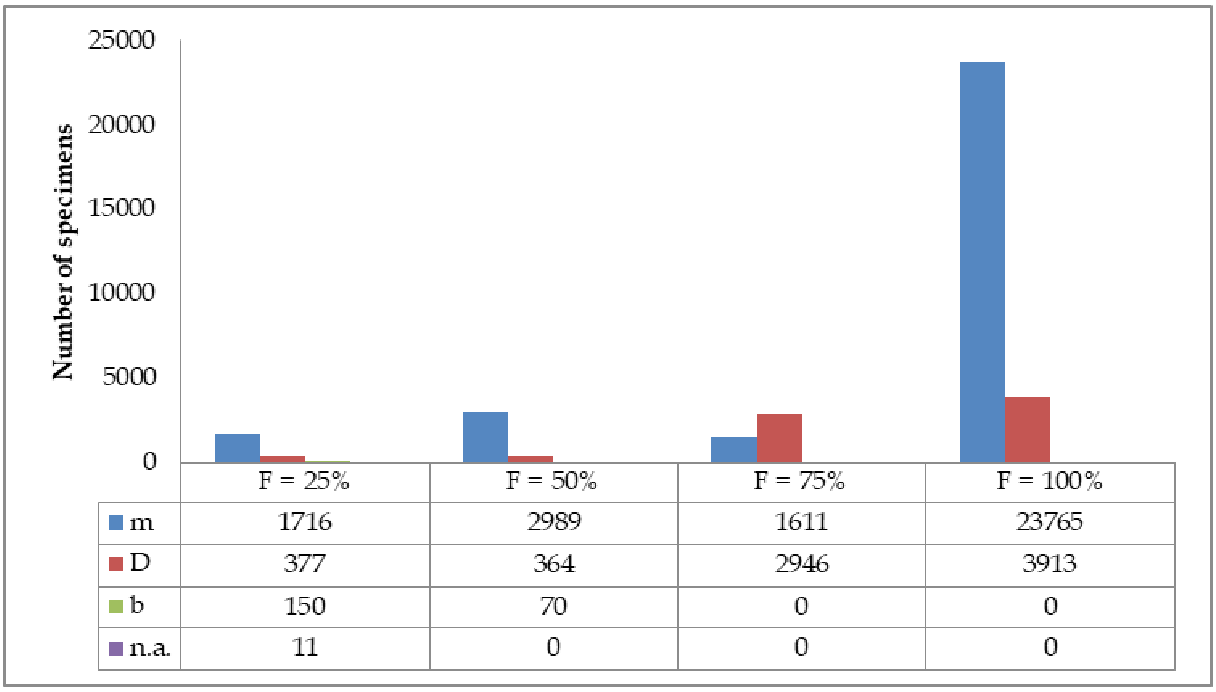


Fig. 3. Group average dendrogram of the similarity between four countries, calculated on the basis of the abundance of macropterous, dimorphic, brachypterous and not determined species. BG – Bulgaria. GE – Germany, RO – Romania, SZ – Switzerland.



	F = 25%	F = 50%	F = 75%	F = 100%
m	67	27	9	13
D	17	11	7	1
b	19	2	0	0
n.a.	6	0	0	0

Fig. 4. Number of species in the four occurrence classes (with frequency of occurrence, respectively 25%, 50%, 75% and 100%): m – macropterous, D – wing di(poly)morphic, b – brachypterous, n.a. – no data.



	F = 25%	F = 50%	F = 75%	F = 100%
m	1716	2989	1611	23765
D	377	364	2946	3913
b	150	70	0	0
n.a.	11	0	0	0

Fig. 5. Number of specimens in the four occurrence classes (with frequency of occurrence, respectively 25%, 50%, 75% and 100%): m – macropterous, D – wing di(poly)morphic, b – brachypterous, n.a. – no data.

In a study of the influence of dispersal ability of ground beetles from 15 lake islands and 2 mainland sites in northern Poland, Zalewski & Ulrich (2006) found similar share of the macropterous, dimorphic and brachypterous species as we did, respectively 66%, 22% and 11%. The presence of more beetles with fully or differently developed wings is also probably connected with their possible chance to avoid hazards in the form of agricultural treatments (Kromp, 1999). Macropterous carabids dominated and brachypterous carabid beetles were very few in assemblages in both conventional and non-inversion tillage systems in oilseed rape fields (Kosewska, 2016). Comparing forest and open areas without any land management practice, Shibuya et al. (2014) also found that macropterous carabid beetles are more common in disturbed habitats. Lower proportion of macropterous individuals was found in vineyards with lower agricultural intensification during a study of the effect of local vegetation management on carabid wing-morphology composition (Rusch et al., 2016).

Conclusions

Oilseed rape fields, being young and less stable habitats, harbor more macropterous ground beetles, while brachypterous species with lower dispersion abilities seem to be more vulnerable to anthropogenic interference in the crops.

The prevalence of the macropterous carabids reflects their higher mobility and adaptiveness, and evidences the initial stage of formation of cenoses, as well as the unstable state of carabid populations in the oilseed rape fields in all studied countries.

The combination of less species diversity and greater abundance of the established beetles in Germany and Switzerland might be a sign of some catastrophic effect in the biocoenoses there, e.g. stronger intensification of the agriculture.

Intensification of the agriculture leads to the decline of natural habitats and associated biota worldwide, and in this study the ground beetles were used as a model, as they are well studied bioindicators and have a proved role in the ecosystems as valuable pest control factor.

Since the ecosystem functions, such as pest control (and pollination), are directly dependent on the invertebrate predators (and pollinators) diversity, it is relevant to keep their habitats stable and keep them from disturbance and destruction. Environmental sustainability should be included in the agriculture standards and practices.

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Appendix 1. Species list and numbers of specimens of the ground beetles established in the oilseed rape fields: BG – Bulgaria, GE – Germany, RO – Romania, SZ – Switzerland; Wing development: m – macropterous, b – brachypterous, D – dimorphic, n.a. – no data; F – occurrence, referring to the number of countries where the species was found (in %).

Species	BG	GE	RO	SZ	Wings	F
Acinopus (s.str.) *picipes* (Olivier, 1795)	49				m	*25*
A. (Oedematicus) megacephalus (P. Rossi, 1794)	54				m	*25*
Acupalpus (s.str.) *exiguus* Dejean, 1829		2			m	*25*
Acupalpus (s.str.) *meridianus* (Linnaeus, 1760)	3	2	2	4	m	*100*
Acupalpus (Ancylostria) interstitialis Reitter, 1884	5		12		m	*50*
Agonum (s.str.) *muelleri* (Herbst, 1784)				308	m	*25*
Agonum (Europhilus) piceum (Linnaeus, 1758)		1			m	*25*
A. (Olisares) viridicupreum (J.A.E. Goeze, 1777)	1		3		m	*50*
Amara (s.str.) *aenea* (De Geer, 1774)	292	58	19	9	m	*100*
Amara (s.str.) *anthobia* Villa et Villa, 1833	2				m	*25*
Amara (s.str.) *communis* (Panzer, 1797)	3	2	19		m	*75*
Amara (s.str.) *convexior* Stephens, 1828		3			m	*25*
Amara (s.str.) *eurynota* (Panzer, 1796)	1		4	9	m	*75*
Amara (s.str.) *familiaris* (Duftschmid, 1812)	2	136	5	2	m	*100*
Amara (s.str.) *lucida* (Duftschmid, 1812)			2		m	*25*
Amara ((s.str.) *lunicollis* Schiødte, 1837		13			m	*25*
Amara (s.str.) *ovata* (Fabricius, 1792)	9	239	126	583	m	*100*
Amara (s.str.) *proxima* Putzeys, 1866				1	m	*25*
Amara (s.str.) *saphyrea* Dejean, 1828	1		1		m	*50*
Amara (s.str.) *similata* (Gyllenhal, 1810)	27	224	310	296	m	*100*
Amara (s.str.) *tibialis* (Paykull, 1798)		2			m	*25*
Amara (Bradytus) apricaria (Paykull, 1790)	1				m	*25*
Amara (Bradytus) consularis (Duftschmid, 1812)	1				m	*25*
Amara (Bradytus) fulva (O. F. Müller, 1776)	1	5			m	*50*
Amara (Curtonotus) aulica (Panzer, 1796)		1	4		m	*50*
Amara (Percosia) equestris (Duftschmid, 1812)			2		m	*25*
Amara (Zezea) chaudoiri Schaum,1858	3		1		m	*50*
Amara (Zezea) fulvipes (Audinet-Serville, 1821)	1				m	*25*
Amara (Zezea) plebeja (Gyllenhal, 1810)		4			m	*25*
Amblystomus metallescens (Dejean, 1829)	1				m	*25*
Amblystomus rectangulus Reitter, 1883	1				n.a.	*25*
Anchomenus dorsalis (Pontoppidan, 1763)	297	246	875	842	m	*100*
Anisodactylus (s.str.) *binotatus* (Fabricius, 1787)		5		34	m	*50*
A. (Pseudanisodactylus) signatus (Panzer, 1796)			17		m	*25*
Apotomus clypeonitens G. Müller, 1943	1				m	*25*
Asaphidion flavipes (Linnaeus, 1760)	6	88		4	m	*75*
Badister (s.str.) *bullatus* (Schrank, 1798)			1		m	*25*
Badister (s.str.) *unipustulatus* Bonelli, 1813		1			m	*25*
Badister (Trimorphus) sodalis (Duftschmid, 1812)			1	2	D	*50*
Bembidion (Metallina) lampros (Herbst, 1784)		111	4	52	D	*75*
Bembidion (Metallina) properans (Stephens, 1828)	20	38		42	D	*75*
B. (s.str.) *quadrimaculatum* (Linnaeus, 1760)		27		61	m	*50*
B. (Peryphanes) deletum Audinet-Serville, 1821				1	m	*25*
Bembidion (Peryphus) tetracollum Say, 1823		6			D	*25*
Bembidion (Phyla) obtusum Audinet-Serville, 1821		18		3	D	*50*
Brachinus (Brachinus) alexandri F. Battoni, 1984	2				m	*25*
Brachinus (s.str.) *crepitans* (Linnaeus, 1758)	3		398	13	m	*75*
Brachinus (s.str.) *ejaculans* Fischer-Waldheim, 1828	63				m	*25*
Brachinus (s.str.) *elegans* Chaudoir, 1842	11		1206		m	*50*

Brachinus (s.str.) *psophia* Audinet-Serville, 1821	148				D	*25*
Br. (Brachynidius) bodemeyeri Apfelbeck, 1904			1		n.a.	*25*
Br. (Brachynidius) explodens Duftschmid, 1812	189	35	1500	159	m	*100*
Br. [sp. incertae sedis] *nigricornis* Gebler, 1830	1				n.a.	*25*
Calathus (s.str.) *fuscipes* Goeze, 1777	23	3666	219	5	D	*100*
Calathus (Neocalathus) ambiguus (Paykull, 1790)	4	169	1		m	*75*
Calathus (Neocalathus) cinctus Motschulsky, 1850	6	70			D	*50*
C. (Neocalathus) melanocephalus (Linnaeus, 1758)	1	2	2		D	*75*
Calathus (Neocalathus) mollis (Marsham, 1802)		1			D	*25*
Calosoma (s.str.) *sycophanta* (Linnaeus, 1758)	2				m	*25*
Calosoma (Campatita) auropunctatum (Herbst, 1784)	692		10		m	*50*
Carabus (Archicarabus) montivagus Palliardi, 1825	2				b	*25*
Carabus (Archicarabus) nemoralis O. F. Müller, 1836		4			b	*25*
Carabus (Archicarabus) wiedemanni Ménétriés, 1836	1				b	*25*
Carabus (s.str.) *granulatus* Linnaeus, 1758	2	68		1	D	*75*
Carabus (Chrysocarabus) auronitens Fabricius, 1792				1	b	*25*
Carabus (Eucarabus) ulrichii Germar, 1824			2		b	*25*
Carabus (Megodontus) violaceus Linnaeus, 1758			36		b	*25*
Carabus (Morphocarabus) hampei Kuster, 1846			1		b	*25*
Carabus (Pachystus) glabratus Paykull, 1790			1		b	*25*
Carabus (Pachystus) hortensis Linnaeus, 1758		1			b	*25*
Carabus (Procrustes) coriaceus Linnaeus, 1758	45		20		b	*50*
Carabus (Tachypus) auratus Linnaeus, 1761		10			b	*25*
Carabus (Tachypus) cancellatus Illiger, 1798			11		b	*25*
Carabus (Tomocarabus) convexus Fabricius, 1775	1		4		b	*50*
C. (Trachycarabus) scabriusculus G.-A. Olivier, 1795			1		b	*25*
Carterus (Carterus) dama (P. Rossi, 1792)	2				n.a.	*25*
Cicindela (Cicindela) campestris Linnaeus, 1758	4		1		m	*50*
Chlaenius (Chlaeniellus) vestitus (Paykull, 1790)	1				m	*25*
*Chlaenius (Dinodes) decipiens (*L. Dufour, 1820)	70		6		m	*50*
Chl. (Trichochlaenius) aeneocephalus Dejean, 1826	498				m	*25*
Clivina (Clivina) fossor (Linnaeus, 1758)		29	6	21	D	*75*
Cychrus caraboides (Linnaeus, 1758)		1			b	*25*
Cylindera (s.str.) *germanica* (Linnaeus, 1758)			87		m	*25*
Demetrias (s.str.) *atricapillus* (Linnaeus, 1758)		5			m	*25*
Diachromus germanus (Linnaeus, 1758)	1	1		9	m	*75*
Dixus obscurus (Dejean, 1825)	5				n.a.	*25*
Dolichus halensis (Schaller, 1783)			15		m	*25*
Drypta (s.str.) *dentata* (P. Rossi, 1790)	1				m	*25*
Gynandromorphus etruscus (Quensel, 1806)	19				m	*25*
Harpalus (s.str.) *affinis* (Schrank, 1781)	4	135	18	294	m	*100*
Harpalus (s.str.) *caspius* (Steven, 1806)	1		17		m	*50*
Harpalus (s.str.) *cupreus* Dejean, 1829	25				m	*25*
Harpalus (s.str.) *dimidiatus* (P. Rossi, 1790)				16	m	*25*
Harpalus (s.str.) *distinguendus* (Duftschmid, 1812)	714	59	64		m	*75*
Harpalus (s.str.) *flavicornis* Dejean, 1829	52		1		D	*50*
Harpalus (s.str.) *fuscicornis* Ménétriés, 1832	2				m	*25*
Harpalus (s.str.) *hospes* Sturm, 1818	5		6		m	*50*
Harpalus (s.str.) *latus* (Linnaeus, 1758)		3			m	*25*
Harpalus (s.str.) *luteicornis* (Duftschmid, 1812)				1	m	*25*
Harpalus (s.str.) *pygmaeus* Dejean, 1829	14				m	*25*
Harpalus (s.str.) *rubripes* (Duftschmid, 1812)	8	24	1		m	*75*
Harpalus (s.str.) *serripes* (Quensel, 1806)	82				m	*25*
Harpalus (s.str.) *smaragdinus* (Duftschmid, 1812)	11				m	*25*
Harpalus (s.str.) *subcylindricus* Dejean, 1829	13		3		m	*50*
Harpalus (s.str.) *tardus* (Panzer, 1796)	10	4			m	*50*

H. (s.str.) *xanthopus* Gemminger et Harold, 1868	1	1			m	*50*
H. (Pseudoophonus) calceatus (Duftschmid, 1812)	1		4	1	m	*75*
Harpalus (Pseudoophonus) griseus (Panzer, 1796)	1		21		m	*50*
Harpalus (Pseudophonus) rufipes (De Geer, 1774)	39	267	640	268	m	*100*
H. (Semiophonus) signaticornis (Duftschmid, 1812)	10	187	4	1	m	*100*
Laemostenus (Pristonychus) cimmerius (Fischer-Waldheim, 1823)	1				b	*25*
Laemostenus (Pristonychus) terricola (Herbst, 1784)			1		D	*25*
Licinus (s.str.) *depressus* (Paykull, 1790)	2				D	*25*
Limodromus assimilis (Paykull, 1790)		84		1	m	*50*
Loricera (s.str.) *pilicornis* (Fabricius, 1775)		444		34	m	*50*
Microlestes apterus Holdhaus, 1904			1		b	*25*
Microlestes corticalis (L. Dufour, 1820)	26				m	*25*
Microlestes fissuralis (Reitter, 1901)	114				D	*25*
Microlestes fulvibasis (Reitter, 1901)	33				b	*25*
Microlestes maurus (Sturm, 1827)	13		2		D	*50*
Microlestes minutulus (Goeze, 1777)	215	1		1	D	*75*
Microlestes negrita (Wollaston, 1854)	9				D	*25*
Microlestes plagiatus (Duftschmid, 1812)	1				m	*25*
Microlestes schroederi Holdhaus, 1912	6				m	*25*
Nebria (s.str.) *brevicollis* (Fabricius, 1792)	8	1474	4	2	m	*100*
Notiophilus aestuans Dejean, 1826	1	38			D	*50*
Notiophilus biguttatus (Fabricius, 1779)	1	41			D	*50*
Notiophilus germinyi Fauvel, 1863			1		D	*25*
Notiophilus palustris (Duftschmid, 1812)		4			D	*25*
Ophonus (Hesperophonus) azureus (Fabricius, 1775)	14		3		D	*50*
Ophonus (Hesperophonus) cribricollis (Dejean, 1829)	38				m	*25*
O. (Metophonus) brevicollis (Audinet-Serville, 1821)			1		m	*25*
Ophonus (Metophonus) puncticollis (Paykull, 1798)			4		m	*25*
Ophonus (Metophonus) rufibarbis (Fabricius, 1792)			1		m	*25*
Ophonus (s.str.) *ardosiacus* (Lutshnik, 1922)			1	1	m	*50*
Ophonus (s.str.) *diffinis* (Dejean, 1829)	1				m	*25*
Ophonus (s.str.) *sabulicola* (Panzer, 1796)	3		8		m	*50*
Parophonus (s.str.) *laeviceps* (Ménétriés, 1832)	36				m	*25*
Parophonus (s.str.) *mendax* (P. Rossi, 1790)	26				m	*25*
Parophonus (s.str.) *maculicornis* (Duftschmid, 1812)	1			1	m	*50*
Parophonus (s.str.) *planicollis* (Dejean, 1829)	6				m	*25*
P. (Ophonomimus) hirsutulus (Dejean, 1829)	3				m	*25*
Pedius inquinatus (Sturm, 1824)	6				D	*25*
Poecilus (Ancholeus) puncticollis (Dejean, 1828)	8				m	*25*
Poecilus (s.str.) *anatolicus* (Chaudoir, 1850)	9				m	*25*
Poecilus (s.str.) *cupreus* (Linnaeus, 1758)	543	4014	1760	7126	m	*100*
Poecilus (s.str.) *cursorius* (Dejean, 1828)	193				m	*25*
Poecilus (s.str.) *koyi* Germar, 1823			3		m	*25*
Poecilus (s.str.) *kugelanni* (Panzer, 1797)		1			m	*25*
Poecilus (s.str.) *lepidus* (Leske, 1785)		33			D	*25*
Poecilus (s.str.) *punctulatus* (Shaller, 1783)		2			m	*25*
Poecilus (s.str.) *versicolor* (Sturm, 1824)		92	2		m	*50*
Polystichus connexus (Geoffroy in Fourcroy, 1785)	2				m	*25*
Pterostichus (Adelosia) macer (Marsham, 1802)	12		3		m	*50*
Pterostichus (Argutor) vernalis (Panzer, 1796)		12		7	D	*50*
Pterostichus (Bothriopterus) oblongopunctatus (Fabricius, 1787)		2			D	*25*
Pt. (Feronidius) hungaricus (Dejean, 1828)			33		b	*25*
Pterostichus (Feronidius) melas (Creutzer, 1799)			9		b	*25*
Pt. (Petrophilus) melanarius (Illiger, 1798)		1654	13	663	D	*75*
Pterostichus (Phonias) strenuus (Panzer, 1796)		9			D	*25*
Pterostichus (Platysma) niger (Schaller, 1783)		4		1	D	*50*

Pt. (Pseudomaseus) anthracinus (Illiger, 1798)	1			73	D	*50*
Pterostichus (Steropus) madidus Fabricius, 1775		35			D	*25*
Scybalicus oblongiusculus (Dejean, 1829)	1				m	*25*
Stenolophus (s.str.) *abdomialis* Gene, 1836	1				m	*25*
Stenolophus (s.str.) *teutonus* (Schrank, 1781)				1	m	*25*
Stomis (s.str.) *pumicatus* (Panzer, 1796)				2	D	*25*
Syntomus obscuroguttatus (Duftschmid, 1812)	43	1			m	*50*
Syntomus pallipes (Dejean, 1825)	2				D	*25*
Synuchus (s.str.) *vivalis* (Illiger, 1798)		2			D	*25*
Tachys (Paratachys) bistriatus (Duftschmid, 1812)	3				m	*25*
Tachys (s.str.) *scutellaris* (Stephens, 1828)				11	m	*25*
Tachyura (s.str.) *parvula* (Dejean, 1831)	1				m	*25*
Thalassophilus longicornis (Sturm, 1825)		3			m	*25*
Trechus (Epaphius) secalis Paykull G., 1790		1			b	*25*
Trechus (s.str.) *irenis* Csiki, 1912	1				n.a.	*25*
Trechus (s.str.) *quadristriatus* (Schrank, 1781)	42	360	8	66	m	*100*
Zabrus (s.str.) *tenebrioides* (Goeze, 1777)	7		3		m	*50*
Zuphium olens (Rossi, 1790)	13				m	*25*
Number of specimens = 37912	**5018**	**14285**	**7576**	**11033**		
Number of species = 179	107	68	71	45		
Number of **m** species = 116	78	41	48	31		
Number of **D** species = 36	18	22	11	13		
Number of **b** species = 21	6	5	11	1		
Number of **n.a.** species = 6	5		1			

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Set of Tests for Chlorpyrifos Toxicity Screening

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Abstract. A set of test systems/endpoints for chlorpyrifos screening has been proposed in the present research. The set includes *Myzus persicae*, rats, *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae*, and several endpoints: aphids' mortality; mitotic index; chromosomal aberrations and micronuclei on rats; spot-test, clonal assay, induction of superoxide anions, Zimmermann's test, test of "visible" mutant colonies, CFGE on microalgae and yeast. The aim of the present study was to evaluate the reliability of the proposed by us set of test systems/endpoints for chlorpyrifos toxicity screening. Chlorpyrifos (CPF) treatment was for 5 sec with concentrations in the range: 5 – 10000ppm on aphids; 25.6ppm on rats; for 30 min with 6.5 to 100ppm on *Chlamydomonas reinhardtii* and 100 – 10000ppm on *Saccharomyces cerevisiae*. Dose-dependent mortality of aphids was found. Further, a 2-fold reduction in the mitotic index, about 7-fold increase in chromosomal aberrations, and about 4-fold increase in the total number of micronucleated polychromatic erythrocytes were measured in rats. The LD_{50} values for aphids, *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae* were calculated - 31.5, 36.56 and 66.05 ppm, respectively. The mutagenic potential was expressed mainly of low size and pigment mutations in *Chlamydomonas reinhardtii*, and reverse point mutations in *Saccharomyces cerevisiae*. A correlation between the recombinogenic and pro-oxidant activity of CPF in yeast was found. The cytotoxic, DNA damaging and mutagenic activity did not follow the dose response model in yeast. Based on our data, CPF possesses clastogenic effect on rats, and pro-oxidative, cytotoxic and recombinogenic effect on *Saccharomyces cerevisiae*. Aphids and *Chlamydomonas reinhardtii* are found to be the most susceptible to CPF. Experimental evidence supporting the suggestion that CPF damages photosynthetic pigments and chloroplasts DNA in algae. It could be concluded that the application of the proposed set of test systems/endpoints could provide concise information concerning the genotoxicity of chlorpyrifos.

Key words: Chlorpyrifos; *Myzus persicae*; Wistar rats; *Chlamydomonas reinhardtii*; *Saccharomyces cerevisiae*; toxicity.

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Introduction

Chlorpyrifos (CPF; O, O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate)) is widely applied broad-spectrum organophosphorus insecticide, mainly known to inhibit the acetylcholinesterase activity, to induce oxidative stress and to damage DNA (Hatami et al., 2019). Contradictory data exist concerning its effectiveness against aphids, which may be explained with a different population resistance (Rabea, 2009; Halder et al., 2007). Although, it was believed that it is safe for humans, data in literature concerning its toxicity on target and non-target organisms is contradictory. Many reports in the recent years confirm that low concentrations of CPF are toxic for the aquatic organisms (discussed in Huang et al., 2020).

On the other side, significant results on animals and particularly rodents present the potential of CPF to induce oxidative damage, genotoxicity, hepatic dysfunction, immunological abnormalities, neuro-behavioral and testicular damage (Yahia and Ali, 2019; Ojha and Srivastava, 2014; Elsharkawy et al., 2014). Studies on human cells revealed that CPF induces oxidative stress and possesses genotoxic and DNA damaging properties (Gao et al., 2020; Zhao et al., 2019; Li et al., 2015). Contrary, lack of cell toxicity was observed in enterocytes, peripheral blood mononuclear cells and hepatic cells exposed to CPF concentrations up to 100 μM (Tirelli et al., 2007; Oostingh et al., 2009).

Further, although CPF is considered as a non- or low-toxic to plants, several studies reported that CPF possesses phytotoxic properties presented as changes in growth, number of pods, mito-inhibitory and genotoxic effect, oxidative stress and lipid concentration (Mercado & Bayona, 2019; Fernandes et al., 2018; Sinha & Kumar, 2014).

Thus, based on these data, several regulations exist. The Priority substances under the Water Framework Directive for the protection of aquatic ecosystems (Directive 2013/39/EU) lists CPF as a priority chemical pollutant. The US Environmental Protection Agency classifies it as a moderately toxic agent (Li et al. 2015). CPF as an ingredient of variety of insecticides is banned for use in China, Hawaii and California (Huang et al., 2020). Following the regulations of the European Commission, the insecticide has been forbidden for use and should be withdrawn from the markets since 10 January 2020 (EFSA, 2019). Although, many countries reduced the use of CPF, it still remains one of the most popular insecticides. Many kinds of research on various test organisms and endpoints have been performed, but they provide partial information for the mode of action of this insecticide.

Thus, there is a great need to develop set of tests with different resolution, which may provide fast and accurate information for the potential toxic effect of pesticides on different levels – organismal, cellular, sub-cellular, and molecular.

The present study aims to evaluate the reliability of the proposed by us set of test systems/endpoints for genotoxicity screening. One of the most commonly used pesticides – chlorpyrifos was chosen for our purpose. The proposed by us set of test systems includes target and non-target organisms.

Based on the contradictory data in literature concerning toxic effect of CPF on aphids, the green peach aphid *Myzus persicae* was chosen as a target organism due to their long-term pest control treatment with CPF. Studies on these aphids are important because of several reasons: it is considered as the most economically important aphid crop pest (Bass et al., 2015); these aphids are globally spread and cause direct and indirect damages on plants, which may lead to huge economic losses to the food production (Blackman & Eastop, 2000; 2006); they become resistant to almost all classes of insecticides; these aphids transmit various plant viruses such as the potato virus Y; the potato leafroll virus and several mosaic viruses (Bass et al., 2014; 2015).

Further studies were focused on the effect on weanling male Wistar rats. Tests performed in this work are recommended by the Organization for Economic Cooperation and Development (OECD) and widely applied in the environmental toxicology risk assessment providing relatively fast results.

Additionally, two unicellular organisms were also chosen – *Saccharomyces cerevisiae* and *Chlamydomonas reinhardtii*. These model systems have some benefits in genotoxicity testing such as fast and valuable results, inexpensive laboratory equipment and consumables (Chankova et al., 2014; Todorova et al., 2015a). *S. cerevisiae* is a suitable model for genotoxicological studies due to the high similarity with the mammals in different stress response pathways (discussed in Todorova et al., 2015a). *C. reinhardtii* is considered as a robust model for plant cell (Chankova et al., 2014), providing also evidence for genotoxicity of various pesticides in the water environment (Taylor et al., 2016).

Material and Methods

Organisms. The biological activity of CPF was tested on a target organism – laboratory reared aphids *M. persicae*. Tests for toxicity evaluation on non-target organisms were performed on *C. reinhardtii* (WT) and *S. cerevisiae* strain D7ts1.

Aphicidal activity. The aphid mortality was used as an endpoint. *Myzus persicae* (Sulz.) was laboratory reared and fed on radish plants (*Raphanus sativus*) for more than 30 generations without any pesticide exposure in a Growth Chamber GC400 at optimal 16/8 h light-dark pattern, temperature 23 °C ± 2 °C and 70% ± 5% relative humidity. *R. sativus* plants were grown at the same conditions as the aphids in the Growth Chamber GC 400. Concentration range of chlorpyrifos was: 5, 10, 25, 50 and 100 ppm based on preliminary data (unpublished data). Distilled water was used as a control. The insecticidal activity of chlorpyrifos on aphids was evaluated by „The dip leaf test method" (FAO) (FAO, 1979). The mortality was counted under magnification of stereo microscope "Zeis" 24 h hours later and the corrected mortality was recalculated by Abbott's formula (Abbott, 1925).

In vivo study

Animals and treatments. Weanling male Wistar rats (average body weight of 55±5 g) were obtained from the Animal Breeding House of the National Research Centre (NRC), Dokki, Giza, Egypt. Animals were given humane care, according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals." The Local Ethics Committee at the National Research Centre (NRC), Dokki, Giza, Egypt approved the experimental protocols and procedures.

After 7 days acclimatization to laboratory conditions, rats were randomly divided into two groups, each consisting of five rats. Group one - control, was given corn oil (1ml/kg b.wt) daily, via oral route for 28 consecutive days and adjusted weekly for body weight changes. Group 2 was given CPF at a dose 25.60 mg/kg b.wt. (1/25 LD_{50}) based on published LD_{50} (640 mg/kg b.wt.) (Tomlin, 2004).

Chromosome aberrations (CA) assay. Cytogenetic analysis was performed by the direct method of rinsing marrow of long bones, according to Adler (1984). CA was identified based on criteria established by the OECD Guideline 475, updated and adopted on 21 July 1997 (OECD, 1997a).

Mitotic index determination. Slides prepared for chromosomal aberration assay were used to calculate the mitotic index as the ratio of the number of dividing cells to the total number of cells, multiplied by 100.

The micronucleus assay. The method described by Schmid (1975) was used for analysis of micronuclei (MN) in polychromatic erythrocytes (PCEs) of rat bone marrow. The study was done in accordance with OECD Guideline 474, updated and adopted on 21 July 1997 (OECD, 1997b).

Treatment of the unicellular organisms. Cell cultures of *C. reinhardtii* (WT) with a density $1x10^6$ cells/ml and *S. cerevisiae* (strain D7ts1) with a density $1x10^6$ cells/ml in the end of exponential and the beginning of stationary phase were treated with various concentrations of chlorpyrifos (6.5-100 ppm for *C. reinhardtii* and 100-10000 ppm, for *S. cerevisiae* respectively) for 30 min. These concentrations were chosen based on our pilot, yet unpublished experiments.

Genotoxicity screening. Macro-colonies survival assay was performed to evaluate genotoxic potential of CPF. Survival fraction (SF) of *C. reinhardtii* colonies was calculated according to (Bryant, 1968).

Quantitative assay for superoxide anions in live cells. The experiments were performed as described in Stamenova et al. (2008). Briefly, after the treatment *S. cerevisiae* cells were incubated in 1xPBS with 125 µM XTT for 6 hours. The levels of superoxide anions were measured spectrophotometrically at wavelength 470 nm. The concentration of superoxide anions in live cells was calculated as described in Stamenova et al. (2008).

Measurement of DNA double-strand breaks (DSB) induction. Constant field gel electrophoresis (CFGE) was performed as described in Chankova and Bryant (2002), Chankova et al. (2007), Todorova et al. (2015b; 2019).

Test of "visible" mutant colonies was applied to evaluate mutagenic potential of chlorpyrifos on the unicellular algae. Changes in size, morphology and pigmentation of surviving colonies were analyzed (Shevchenko, 1979; Dimitrova et al., 2007).

Zimmermann's test for simultaneous detection of mitotic gene conversion at the trp-5 locus, reversion mutations in ilv1 locus and mitotic crossing-over. Zimmermann's test (Zimmermann et al., 1984) with *Saccharomyces cerevisiae* diploid strain D7ts1 (*MAT*a/*a ade2-119/ade2-40 trp5-27/trp5-12 ilv1-92/ilv1-92 ts1/ts1*) was applied as described before (Todorova et al., 2015a).

Statistical analysis. Data were analyzed using Graphpad Prism5 software (San Diego, USA) and the statistical analysis was done by one-way analysis of variances (ANOVA) followed by Bonferonni post-hoc multiple comparisons test. Linear correlation, using Pearson Product-Moment Correlation Coefficient analysis (PMCC, or r) and coefficient of determination (R^2) were determined.

Results and Discussion

Effect of chlorpyrifos on aphids

Data revealed that CPF is highly toxic to aphids *M. persicae*. Concentrations equal or higher than 100 ppm resulted in 100% mortality (Fig. 1). LC_{50} was calculated to be 31.5 ppm. Our data provide evidence for the high aphicidal activity of CPF. There is a contradictory data in literature concerning the toxic effect of CPF on *M. persicae*. Halder et al. (2007) reported relatively high aphids resistance with LC_{50} =1640 ppm in the Direct Spray method and 1060 ppm – in the Leaf residue method. Conversely, Rabea (2009) calculated LC_{50} to be 12.24 ppm. Such significant variation in the values could be explained with different population resistance. Bass et al. (2015) reported that *M. persicae* could be characterized with at least seven independent mechanisms of resistance by which they are able to avoid or overcome the toxic effect of insecticides. In our study, laboratory reared aphids were used so they were not in contact with any other insecticides.

Effect of chlorpyrifos on rats

Next experiments were performed on rats. The mitotic index was used in order to determine the rate of cell division. The status of mitotic index evaluated as a percentage of dividing cells revealed around 2-fold reduction, indicating cytotoxic potential of CPF (Fig. 2).

Such result corresponds well with the suggestion that the organophosphate pesticides inhibit mitosis probably by blocking mitotic cycle during interphase (Sinha and Kumar, 2014).

Chromosomal aberrations in rat bone marrow

Table 1 presents chromosomal aberrations induced in rat bone marrow cells after the treatment with CPF.

The results show that the tested CPF dose (25.60 ppm) can induce a statistically significant increase in the percentage of CA in bone marrow cells – around 7-fold.

Induction of micronuclei in rat bone marrow PCE

Around 4-fold statistically significant increase in the total number of bone-marrow micronucleated polychromatic erythrocytes (MnPCE) was calculated after the treatment with CPF (Table 2). These data show that the tested insecticide CPF possesses clastogenic capacity. Our results are in a good correspondence with the ones reported by Yahia & Ali (2019). Serpa et al. (2019) provided evidence that concentrations corresponding to 35 ppm are able to induce around 4-fold higher levels of micronuclei in human leukocytes. Some authors reported that the primary induced DNA damages by CPF may be due to the generation of oxidative stress (Mužinić et al., 2019; Ojha & Srivastava, 2014).

Effect of chlorpyrifos on Chlamydomonas reinhardtii – model for plant cell

Data revealed dose-dependent decrease in the cell survival (Fig. 3A). The lethal dose causing 50% mortality (LD_{50}) was calculated to be 36.56 ppm.

Data available in literature reveal that CPF cytotoxicity is high in microalgae when prolonged treatments are performed: *Chlorella pyrenoidosa* - 29.64 ppm (24h) to 11.46 ppm (72h) and from 27.80 ppm (24h) to 25.80 ppm (72h) in *Merismopedia* sp. (Chen et al., 2016). Our results provide new evidence for the acute toxicity within 30 min treatment.

Following the EU-Directive 93/67/EEC (Commission of the European Communities, 1996) concerning the different toxic classes according to their EC50-values, CPF fall into the class "10-100 mg/L (harmful to aquatic organisms)" for *C. reinhardtii*. Our data confirm that classification.

Next, in order to examine other damages related to the high genotoxicity, the mutagenic potential was evaluated. Data revealed no correspondence between high genotoxicity and the mutagenicity. Results obtained are in a support of concentrations dependent mutagenic effect of CPF – weak mutagenic potential of 50 ppm, and strong mutagenic activity of 100 ppm (Fig. 3B). No statistically significant mutagenic effect was obtained using doses lower than 50 ppm. Interestingly, small-sized or pigment mutant colonies were the only mutant type observed suggesting possible inhibition of cell division, damages in the chloroplast DNA and pigment content (Shevchenko, 1979).

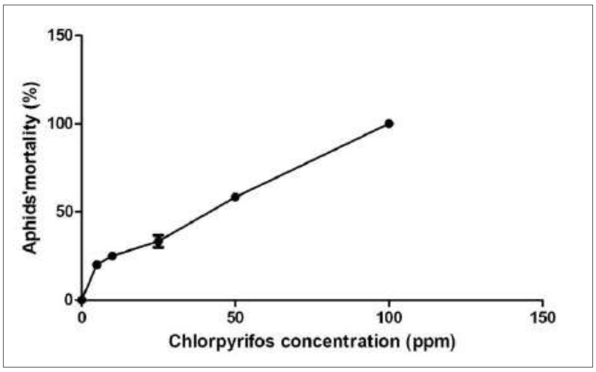

Fig. 1. Aphids' mortality after the treatment with various concentrations chlorpyrifos in the range of 5 – 100 ppm. Where no error bars are evident, they are equal or less than the symbols.

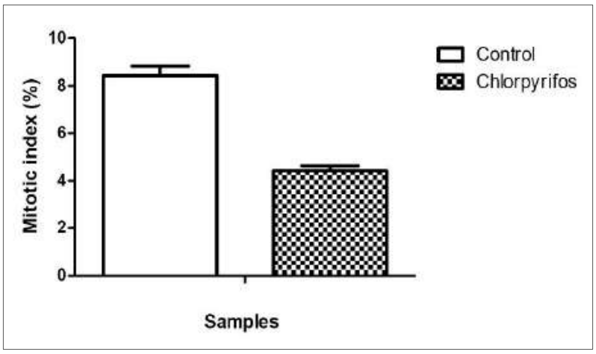

Fig. 2. Mitotic index calculated after the application of chlorpyrifos. Where no error bars are evident, they are equal or less than the symbols.

As CPF inhibit the cell division in rats, the small size mutant colonies obtained on *C. reinhardtii* confirm once again this mechanism of action. Further, Chen et al. (2016) discussed that although, organophosphorus insecticides are considered low toxic to plants, data exist that they can damage the photosynthetic pigments (Chen et al., 2016). Our data concerning increased level of pigment mutant colonies that are considered as a result of damages in both chloroplasts DNA and photosynthetic apparatus of *C. reinhardtii* are in agreement with this finding and could be serving as a suitable marker for an evaluation of the potential genotoxic effect of organophosphorus pesticides on plants.

DNA damaging capacity of CPF was evaluated based on the DSBs induction. DSB levels measured in treated samples were comparable with those in the control - untreated cells (Fig. 4). No correspondence between cell survival and DSB induction was found. Our finding might be due be to specific mode of action of CPF in plants. Chen et al. (2016) has suggested that CPF can damage chloroplast DNA.

Table 1. Effect of chlorpyrifos on chromosomal aberrations in rat bone marrow cells. *Legend:* Value is mean ± S.E.; n = 5 rats/group. Values are shared the same superscript letters not differ significantly at $p < 0.05$.

Group	Percent chromosome aberrations				Total number of aberrant cell (%)		No. of aberrations per cell	
	Gaps	Breaks and/or Fragment	Deletions	Multiple aberrations	Including gaps	Excluding gaps	Including gaps	Excluding gaps
Control	1.02±0.01	1.46±0.06	0.18±0.001	0.21±0.002	2.87±0.08^{b}	1.85±0.014^{b}	0.0287±0.0001^{b}	0.0185±0.0001^{b}
CPF	4.32±0.06	5.87±0.08	0.97±0.005	6.58±0.008	17.74±0.53^{a}	13.42±0.04^{a}	0.1774±0.020^{a}	0.1342±0.010^{a}

Table 2. Clastogenic potential of chlorpyrifos in the bone marrow measured as induction of micronuclei in bone-marrow micronucleated polychromatic erythrocytes. Legend: Value is mean ± S.E.; n = 5 rats/group. The number of the scored cells was 2000 cells/ animal. Mn: micronucleus, MnPCE: micronucleated polychromatic erythrocytes, PCE: polychromatic erythrocytes.

Group	No. of micronucleated polychromatic erythrocytes (MnPCE)			
	PCE with one Mn	PCE with two Mn	PCE with more than two Mn	Total MnPCE
Control	5.60±0.074	0.60 ± 0.001	0.20 ± 0.001	6.40 ± 0.14^{b}
CPF	17.4 ± 0.18	7.80 ± 0.19	3.20 ± 0.11	28.40 ± 1.76^{a}

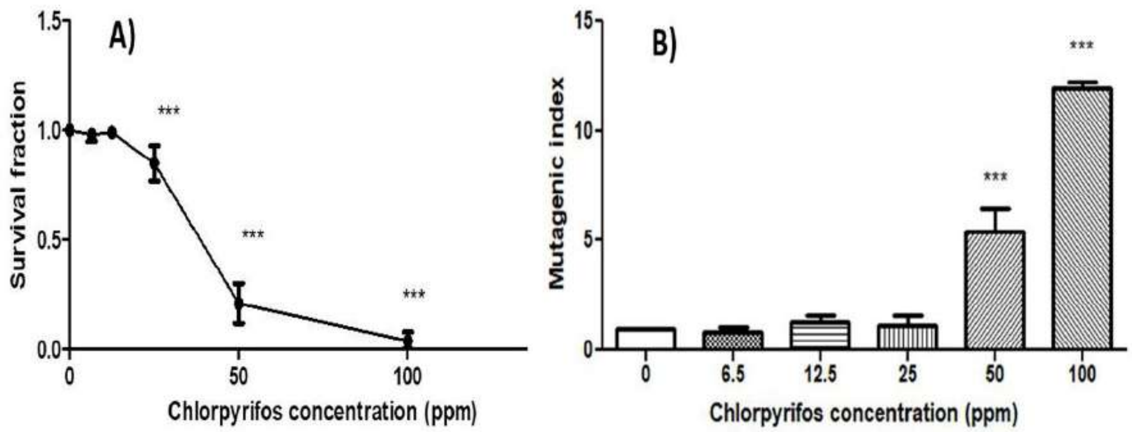

Fig. 3. Survival fraction (SF) of *C. reinhardtii* treated with chlorpyrifos at concentrations'

range from 6.5 to 100 ppm (A) and mutagenic activity of CPF presented as mutagenic index (B). Where no error bars are evident, they are equal or less than the symbols.

Effect of chlorpyrifos on Saccharomyces cerevisiae – model for animal cell

The effect of CPF was also evaluated on *S. cerevisiae*. Based on the results for the cell survival, LD_{50} was calculated to be 66.05 ppm (Table 3).

Further, the pro-oxidant potential was also evaluated. Data revealed induction of superoxide anions in yeast after the treatment with all the concentrations (Table 3). Statistically significant dose-dependent increase was calculated. Around 100-fold increase in the ROS levels was observed after the treatment with the highest tested concentration.

CPF at all the concentrations tested was also found to induce reverse mutations in *S. cerevisiae*, suggesting strong mutagenic effect. Interestingly, no effect of the concentration was observed after the treatment with concentrations 1000 and 10000 ppm suggesting possible reach of a threshold. Further, CPF was found to possess well-expressed recombinogenic effect increasing the mitotic gene conversion in a dose-dependent matter. On the other side significant increase in the percentage of total aberrants was observed after the treatment with the highest concentration tested – 10000 ppm (Table 3).

Next, the DSB induction was studied. Interestingly, all the concentrations tested resulted in around 2.5-fold increase of DSB levels (Fig. 5). No statistical differences among the concentration' effect was calculated. These results indicate that the DSB induction is not related to the dose range used by us for CPF.

The results reported in table 4 revealed that statistically significant positive correlation exist only among the increase in ROS levels with this in mitotic gene convertants ($P < 0.05$) and total aberrants ($P < 0.01$).

Table 3. Frequency of survival fraction, gene conversion in *trp5* locus, reversion in *ilv1-92* allele and mitotic crossing-over in *ade2* locus, induction of superoxide anions after the treatment of *S. cerevisiae* D7ts1 with various concentrations chlorpyrifos. *Legend:* Values are mean ± SD from at least three independent experiments.

CPF (ppm)	Survival fraction[1]	Convertants/ 10^5 cells[1]	Revertants/10^6 cells[1]	Aberrants (%)[1]	ROS pM/cell[1]
0	1 ± 0	0,20 ± 0,08	0,011 ± 0.01	0,023 ± 0,012	0.003 ± 0.001
100	0.515 ± 0.058***	0.81 ± 0,05^{ns}	0.042 ± 0.005^{ns}	0.018 ± 0.008^{ns}	0.024± 0.001***
1000	0.123 ± 0.028***	1.65 ± 0.41*	0.406 ± 0.083***	0.047 ± 0.013*	0.036± 0.002***
10000	0.072 ± 0.011***	5.26 ± 1.41***	0.433 ± 0.062***	0.287 ± 0.018***	0.297 ± 0.004***

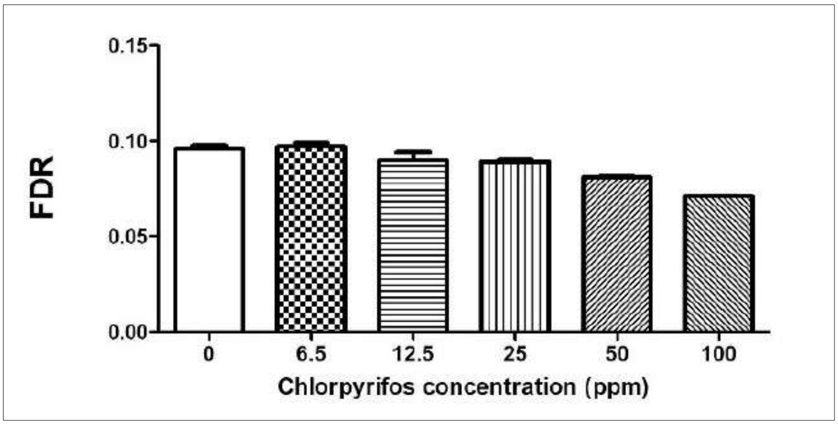

Fig. 4. Induction of double-strand breaks in *C. reinhardtii* treated with chlorpyrifos at concentrations' range from 6.5 to 100 ppm. Where no error bars are evident, they are equal or less than the symbols.

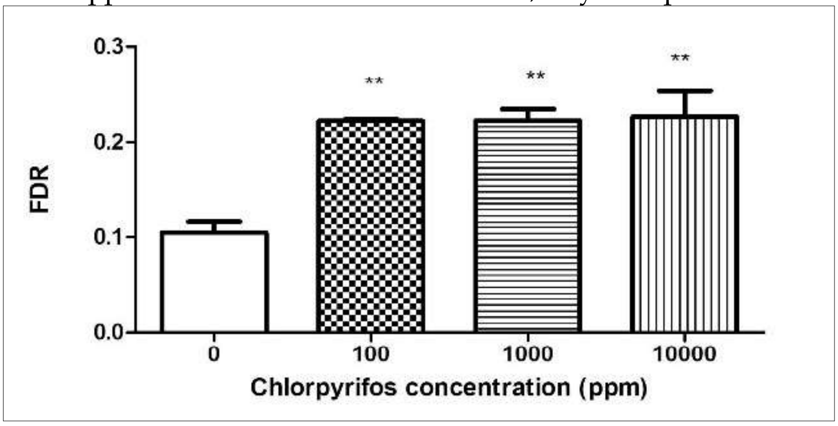

Fig. 5. Induction of double-strand breaks in *S. cerevisiae* treated with chlorpyrifos at concentrations' range from 100 to 10000 ppm. Where no error bars are evident, they are equal or less than the symbols.

Table 4. Correlation analysis of the genetic events induced after the treatment of *Saccharomyces cerevisiae* with various concentrations chlorpyrifos. *Legend:* Values represent the R^2 for linear correlation. Correlation coefficient (R) higher than 0.900 denotes a strong positive correlation. SF: survival fraction; MGC: mitotic gene conversion; TA: total aberrants; ROS: reactive oxygen species; RM: reverse mutations; DSB: double-strand breaks.

	SF	MGC	TA	ROS	RM	DSB
SF	–	-0.748	-0.609	-0.631	-0.912	-0.897
MGC	-0.748	–	0.982*	0.986*	0.787	0.552
TA	-0.609	0.982*	–	0.997**	0.687	0.396
ROS	-0.631	0.986*	0.997**	–	0.677	0.450
RM	-0.912	0.787	0.687	0.677	–	0.638
DSB	-0.897	0.552	0.396	0.450	0.638	–

The levels of DSBs remain similar despite the concentration. Additionally, concentrations equal or higher than 1000 ppm were not found to follow the dose response pattern. Thus, it could be speculated that the recombinogenic activity of CPF could be proportionally related to the well-expressed pro-oxidant activity. The cytotoxic, DNA damaging and mutagenic activity may be due to some direct actions most probably of the CPF metabolites or by reaching threshold levels of the CPF action.

Conclusions

The present study provides experimental evidence for the sensitivity of different organisms to the toxic action of chlorpyrifos. Aphids and *C. reinhardtii* are found to be very susceptible to CPF action suggesting aphicidal and phytotoxic effect. Large scale of effects was revealed: very pronounced clastogenic in rats, genotoxic and mutagenic in *C. reinhardtii* and *S. cerevisiae*, well-expressed pro-oxidative, recombinogenic and DNA damaging in *S. cerevisiae*. Additionally, mutagenic and damaging DNA effect was observed for concentrations lower than the recommended dose. New data are provided in a support of current knowledge that organophosphorus insecticides considered as low toxic to plants, can damage photosynthetic pigments and chloroplasts DNA inducing small sized and pigment mutant colonies in *C. reinhardtii.*

In short, using proposed by us set of test systems/endpoints wide spectrum of chlorpyrifos bioactivity was revealed - aphicidal, phytotoxic, genotoxic, mutagenic, recombinogenic, clastogenic and DNA damaging. From our understanding this set of test-systems/endpoints could be successfully used in a genotoxicity screening of traditional, new synthesized and natural products. This set could be successfully applied to obtain concise information concerning the genotoxicity of different pesticides and/or natural products.

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Sesquiterpene Patterns of the Leaves and Roots in Local Populations of Medicinal Plant Petasites hybridus (L.) from Bulgaria

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Abstract. Biosynthesis of biologically active substances, products of secondary plant metabolism is highly sensitive to various geographic, ecological and phenological factors. Clarification of these relationships is essential to define their impact on the efficacy and therapeutic potential of phytochemical preparations of medicinal plants. The aim of this study was to investigate the variation in the pattern of the main biologically active compounds of *Petasites hybridus* (L.) (Butterbur), classified as sesquiterpene esters of petasin and iso-petasin, in natural population from different habitats in Bulgaria. The results from TLC analysis confirmed the petasin chemo-type of Butterbur plants from the investigated areas. HPLC analysis revealed qualitative and quantitative differences of the six main sesquiterpene esters found in leaf and subterranean parts extracts. The investigated Bulgarian populations of *Petasites hybridus* have a relatively high content of petasin in the roots and are relatively poor in petasin content in the leaves. The present results indicate modulations in the profile and accumulation of secondary metabolites in *Petasites hybridus*, reflecting adaptive responses to specific environmental conditions in their natural habitats.

Key words: *Petasites hybridus*, methanol extracts, HPLC, sesquiterpene esters, TLC.

Introduction

The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant (Briskin, 2000). Plant secondary metabolites are a group of naturally occurring compounds known to play a major role in the adaptation of plants to their biotic and abiotic environment (Sun et al., 2007). Although medicinal plants are the subject of intensive research to identify specific secondary metabolites and to characterize the potential sites and modes of action of biologically active substances (BAS), many of the pathways for their biosynthesis and regulatory mechanisms remain unclear. It is well known that changes in the biosynthetic profile and the content of BAS are determined by the species, genotype, physiology, developmental stage and environmental factors (Kutchan, 2001; Gobbo-Neto & Lopes, 2007; Isah, 2019). Much information has been accumulated regarding the effects of biotic stress (e.g., pathogenic attacks or herbivore attacks) on the synthesis of secondary metabolites (Wink, 2010), but

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relevant information on how abiotic stress alters secondary metabolism is limited. Abiotic factors that determine plant habitats and inhibit the growth, photosynthesis and other processes of primary metabolism in stressful conditions, such as drought, salinization, high light intensity, UV radiation etc. can affect the profile, accumulation and antioxidant potential of secondary metabolites (Gobbo-Neto & Lopes, 2007; Fine et al., 2006).

The genus *Petasites* Mill (Asteraceae) is widely vegetated in Europe, Northwest Asia and North America and has a long history of use in alternative medicine. Taxonomic survey of *Petasites* reveals the existence of 18 species of this genus. Representatives of genus *Petasites* are shown to be potential sources of high levels of bioactive substances with very promising aspects of therapeutic utility (Aydın et al., 2012). In Bulgaria, three *Petasites* taxa can be naturally found: *P. hybridus* (L.) P. Gaertn., B. Mey. & Scherb (*P. officinalis* Moench), *P. albus* (L.) Gaertn. and *P. kablikianus* Tausch ex Bercht. The subject of the present study is *P. hybridus* (common Butterbur), the most common species and the main medicinal plant in *Petasites* genus, used in European phytotherapy. The plant grows most plentifully in humid thickets, marshy meadows, along the banks of streams, ravines and rivers, preferably in partial shade. The main active substances of *P. hybridus* include sesquiterpene esters (Debrunner et al., 1995). Plants also contains low amounts of pyrrolizidine alkaloids, mainly senecionine and intergerrimine, with hepatotoxic and carcinogenic effects, the amount of which does not depend on the content of sesquiterpenes (Wildi et al., 1998). The refined reparations of the extracts of *P. hybridus* root, free of pyrrolizidine alkaloids are finding an increasingly widespread pharmacological application for the prophylaxis and treatment of migraine, bronchial asthma and allergic rhinitis - diseases affecting a large number of people (Lipton et al., 2004). Extracts are also successfully used for the prevention of gastric ulcer, urinary tract irritation and respiratory problems (Ziolo & Samochowiec, 1998).

There are no systematic studies of the effects of ecological and phenological factors on the metabolic profile of the biologically active components of the Bulgarian populations of *Petasites hybridus*. This study aimed to investigate the variation in the pattern of the pharmacologically most relevant biologically active compounds of *Petasites hybridus* in natural population from some local habitats in Bulgaria, differing in altitude and specific climate conditions. Clarification of these relationships is essential to define their impact on the efficacy and therapeutic potential of phytochemical preparations of medicinal plants.

Material and Methods

Study area. Four natural locations of *Petasites*, in florogeographical regions, differing in altitude and soil-climatic conditions were selected (Table 1). Sampling was made in the middle of May 2019 after the flowering period and when water supply was non-limiting and temperature was moderately high. The environmental conditions during the measurement period were characterized by average minimum and maximum temperatures of 5 and 17 °C for Devil's bridge Kokaliane region and 6.2 and 17 °C for Rila Monastery Nature Park, respectively. For the plants growing in Sofia University Botanical Garden, St.St. Constantine and Helena, the corresponding temperature values in May was 15.0 and 23.0 °C and for Gorno Sahrane plants population 16 and 25 °C, respectively. There was non-limiting water supply (75.7 - 78 mm average monthly amount of precipitation) for all the investigated areas. The analysis of plant water status of investigated samples determined by measuring the hydration of leaves (gH_2O/gdw) show values of about

5.75 – 6.5. Similar values were obtained in preliminary studies in two-year period (2015-2016) (Yordanova et al., 2017). Averaged samples of leaves and subterranean plant parts (rhizomes, roots and runners) of 3 - 4 plants were collected.

Plant extraction and Quantitation. Dried and finely powdered leaves and subterranean parts were exhaustively extracted by ultrasonication with methanol at room temperature, according to Debrunner & Neuenschwander (1994). The extracts were concentrated under vacuum using a rotary evaporator and stored at -20 °C for later use. For the determination of the sesquiterpene type of *Petasites* plants we used a qualitative TLC method. 6 µL of the subterranean parts of the plant extracts were applied on a silica gel 60 F 254 HPLC plates (Merck, Germany) and the chromatogram was developed with toluene/ethyl acetate (93:7, v/v). Detection was performed with Vanillin/H_2SO_4 reagent and heating at 120 °C for 10 minutes. The spots containing separated petasines were visualized in UV light at 365 nm.

Chromatographic conditions. The HPLC analysis was performed on system Waters Alliance 2695 with 4 channel degasser, quaternary pump, autosampler with 100 µL loop, column thermostat and UV-VIS detector Waters 2998 PDA. The analysis was performed on column Venusil XBP C 18 (250 mm × 4.6 mm × 5 µm) (Agela Tech, USA) with particle characteristics: particle size 5 µm, pore size 150 Å and surface area 200 m^2/g. The isocratic mode was used with methanol, acetonitrile and water (32:31:37, v/v/v), as described by Wildi et al. (1998) at 30 °C temperature of column. The eluent flow rate was 1.0 mL/min in isocratic mode. A content of the six main sesquiterpene esters was quantified against an external petasin standard, measuring the peak area of the sesquiterpene esters at 235 nm. Software for system control, data acquisition and data processing Waters Empower 3 v7.20 00 00 was used.

Results and Discussion

Among many secondary metabolites of *Petasites* such as polyphenol acids, flavonoids, tannins and small concentrations of pyrrolizidine alkaloids, the pharmacological effects are mainly attributed to eremophilane (petasin) type sesquiterpenes (Debrunner & Neuenschwander, 1994; Wildi et al., 1998). A second chemotype which cannot be distinguished by morphological characteristics, furanopetasine type (with furanoeremofilanes) is also known (Novotný et al., 1966) and is considered as non-suitable for pharmaceutical purposes (Chizzola et al., 2006). The determination of the sesquiterpene type of the plants from the investigated areas was done by thin layer chromatography. The methanolic extracts from the subterranean plant parts show mostly blue and green-blue fluorescent zones from the start up to the solvent front, due to more than 20 sesquiterpenes (Wagner & Bladt, 1996). The sesquiterpenes petasin and iso-petasin are found as blue UV absorbing spots in the Rf range from 0.4 to 0.45 (Tzoneva et al., 2021-in press). Due to the limited number of sampling plants the existence of furanopetasine type plants in the investigated local populations can not be ruled out.

The main active substances of *P. hybridus* are esters of 3-isomeric sesquiterpene alcohols (petasole, iso-petasole and neopetasole) and include six main sesquiterpene esters: petasin and its isomers (i.e., iso-petasin and neopetasin) and s-petasin and its isomers, iso-s-petasin and neo-s-petasin (Debrunner & Neuenschwander, 1994). Among them, petasin has the highest antispasmodic activity (Debrunner & Meier, 1998). Differences in sesquiterpene profiles and the average content of petasin in different populations of the species have been established (Debrunner et al., 1995; Debrunner & Neuenschwander, 1994; Wildi et al., 1998) in dependence of geographical origin, seasons, altitude, diurnal cycles, the time of collection, parts of the plant, etc. (Ozarowski et al. 2013).

The comparative HPLC analysis of the methanol extracts from the subterranean parts of *P. hybridus* (Figure 1) show the presence of all six main sesquiterpene components, with petasin being dominating compound at all the investigated habitats. Gorno Sahrane is the richest population showing petasin values of 12.2 mg/g dried weight and relatively higher values of neo-petasin (2.58 mg/g) and iso-petasin (1.39 mg/g) but relatively lover contents of s-petasin and its isomers. The amount of iso-s-petasin is lower in all studied populations while the content of the other two components shows an upward increase in the order of neo-s-petasin, s-petasin, except for the population growing in Gorno Sahrane.

Table 1. Geographical coordinates of the studied areas.

Sampling sites	Locality	Altitude	Climate	Coordinates
Sofia University Botanical Garden, St.St. Constantine and Helena	Varna Municipality	58 m	Black Sea climate	N 43◦14″9′ E 28◦0″19′
Gorno Sahrane	StaraZogora	385 m	Transitional continental	N 42◦38″38′ E 25◦12″36′
Devil's Bridge, Kokalyane Region	Sofia Capital Municipality	667 m	Moderate continental	N 42◦33″37′ E 23◦25″18′
Rila Monastery, Nature Park	Rila Municipality	1710 m	Mountain	N 42◦8″27′ E 23◦21″6′

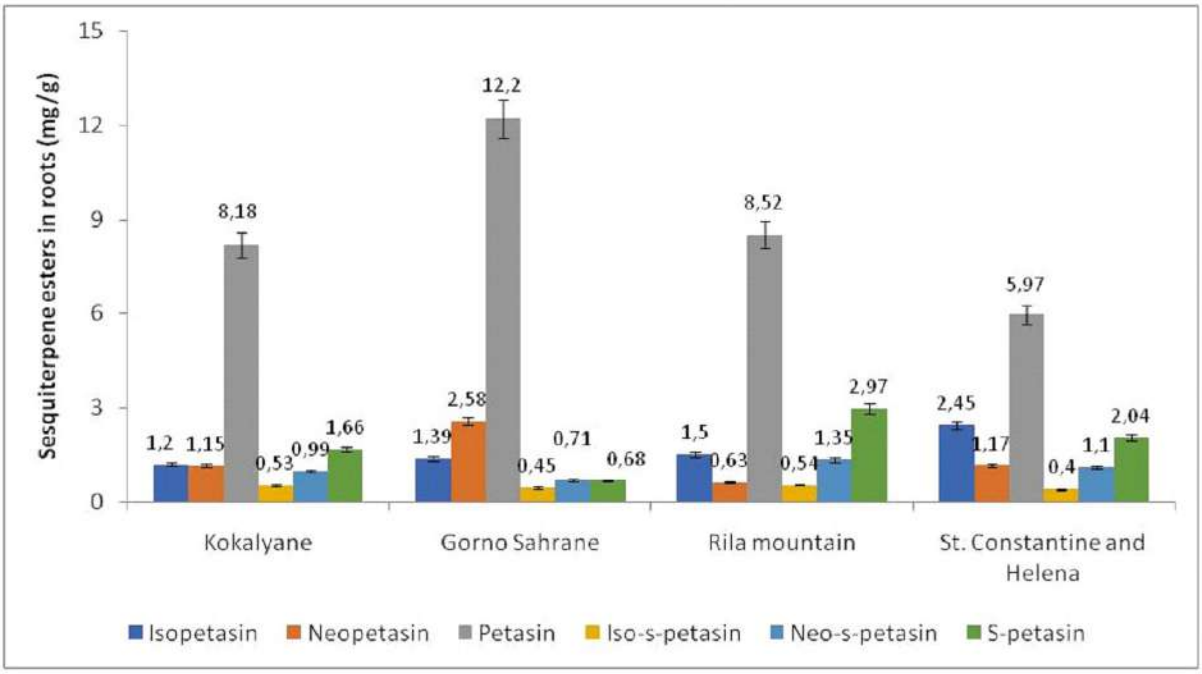

Fig. 1. Contents of the six main sesquiterpene esters (mg/g) found in the extracts from the subterranean parts of *P. hybridus* at different habitats by HPLC analysis.

HPLC-chromatograms of the leaf extracts from the plants growing in Gorno Sahrane and Kokaliane are comparable with that of subterranean parts in regard to sesquiterpene distribution but the content of petasin was considerably lover (Fig. 2). The contents of the other esters vary only slightly. The leaves of the plants from Black Sea area are very poor in petasines while the plants from highland population show unusual sesquiterpene pattern with very high contents of s-petasin. petasin was considerably lover (Figure 2). The contents of the other esters vary only slightly. The leaves of the plants from Black Sea area are very poor in petasines while the plants from highland population show unusual sesquiterpene pattern with very high contents of s-petasin.

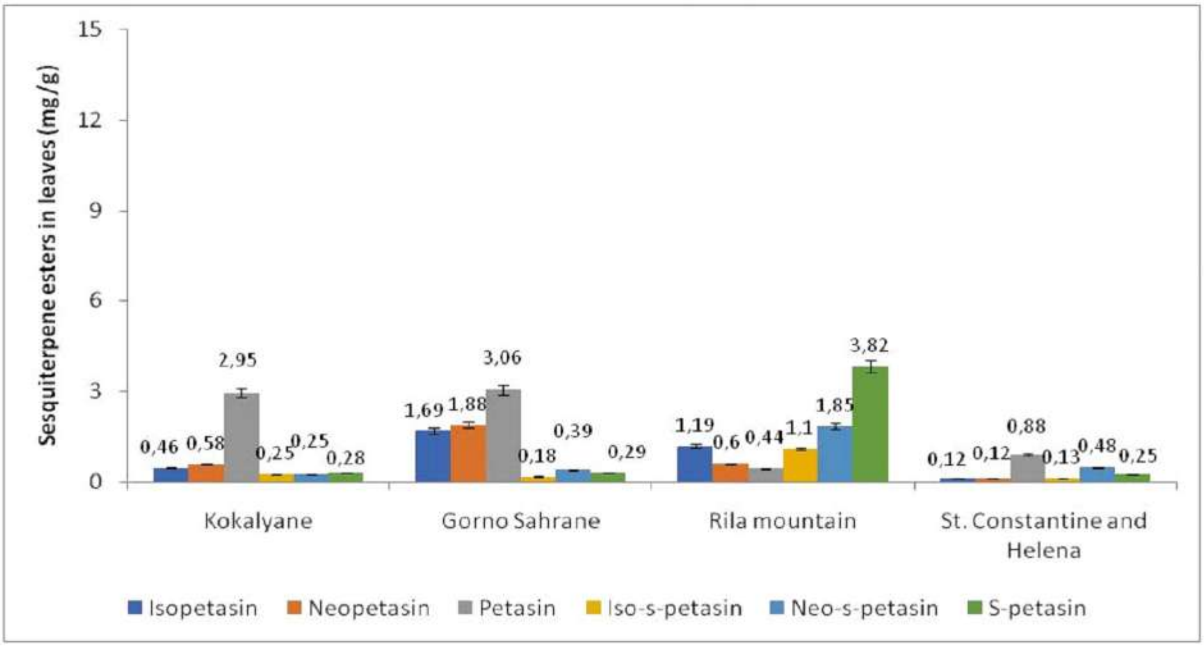

Fig. 2. Contents of the six main sesquiterpene esters in (mg/g) found in the extracts from the leaves of *P. hybridus* at different habitats by HPLC analysis.

Conclusions

The production of secondary metabolites by plants growing in natural populations is conditioned by environmental factors. Properties of photosynthetic apparatus may contribute to a great extent to plant habitat separation and adaptation to environmental factors. Our previous research (Yordanova et al., 2017), has shown a close relation of photosynthetic performance of *P. hybridus* plants to the specific climatic conditions in some natural habitats in Bulgaria, differing in altitude thus suggesting some changes in the accumulation and sesquiterpene profiles of this important Asteraceae medicinal species. We therefore continued our research by studying the peculiarities in the sesquiterpene patterns and contents in *Petasites* plants from the populations at the same florogeographical regions. Samples of leaves and subterranean plant parts (rhizomes, roots and runners) were collected on May, taking into account the data from the comprehensive research of Debrunner et al. (1995) over a complete vegetation period of *Petasites* showing the highest amounts of the six most important sesquiterpene esters in spring time. Our comparative studies have shown that the total amount of six main sesquiterpene esters in the subterranean parts of *P. hybridus* plants at different habitats varies from 13.22 mg/g to 17.33 mg/g, while in the leaves the differences are much more significant and the values vary from 1.98 mg/g to 9 mg/g. Given the data on the average content of petasin in subterranean parts of *P. hybridus* ranging from of 7.4 to 15.3 mg/g and from 3.3 to 11.4 mg/g in the leaves (Aydın et al., 2013) it could be concluded that the investigated Bulgarian populations of *Petasites hybridus* have a relatively high content of petasin in the roots and are relatively poor in petasin content in the leaves. The present results indicate modulations in the profile and accumulation of secondary metabolites in *Petasites hybridus*, reflecting specific adaptive responses to fluctuations in environmental factors in their natural habitats. Under field condition these responses can be synergistically or antagonistically modified by the superimpositions of different environmental constrains.

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First Implementation of Marine Strategy Framework Directive for Benthic Habitats Assessment in the Bulgarian Black Sea

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Abstract. Benthic habitats are ecosystem elements required to be assessed under Descriptors 1, 6 – "Biodiversity" and "Seabed integrity" of the Marine Strategy Framework Directive. This study represents the first environmental status assessment of the Bulgarian Back Sea benthic broad habitat types accomplished according to the criteria and methodological standards of Commission Decision (EU) 2017/848. Adverse effects on habitats condition from eutrophication and pollution were assessed using the ecological indices S, H', AMBI and M-AMBI(n), and dissolved oxygen in bottom water for which good status thresholds were established under the MSFD monitoring programmes. These indicators were also considered as indicative of adverse effects from physical disturbance of the seabed. The proportion of each benthic habitat area adversely affected according to the integrated abiotic and biotic indicators was estimated in six Marine Reporting Units of the Bulgarian Black Sea. Three of the coastal and both of the shelf marine areas did not achieve good environmental status as regards the broad habitat types present. The only marine area in good environmental status was "Emine-Maslen nos" coastal zone. The results are useful for fulfilling the reporting obligation of Bulgaria under the Marine Strategy Framework Directive.

Key words: Bulgarian Black Sea, benthic broad habitat types, environmental status, Marine Strategy Framework Directive.

Introduction

The contribution of seabed habitats and sea-floor integrity to the overall goal of achieving good environmental status (GES) of Europe's marine waters is addressed by Descriptors 1 and 6 of Directive 2008/56/EC (EC., 2008) establishing a framework for community action in the field of marine environmental policy (hereafter Marine Strategy Framework Directive or MSFD).

Descriptor 1: Biological diversity is maintained. The quality and occurrence of habitats and the distribution and abundance of species are in line with prevailing physiographic, geographic and climatic conditions.

Descriptor 6: Sea-floor integrity is at a level that ensures that the structure and functions of the ecosystems are safeguarded and benthic ecosystems, in particular, are not adversely affected.

In Commission Decision (EU) 2017/848, these two aspects of GES have been brought together via a set of five criteria for the determination of GES in relation to a set of broad habitat types: D6C1 Physical loss; D6C2 Physical disturbance; D6C3 Adverse effects of physical disturbance on habitats; D6C4 Extent of habitat loss; D6C5 Extent of adverse effects on the condition of a habitat. D6C5 shall integrate the adverse effects from all relevant pressures addressed by Descriptors: D2 "Non-indigenous species", C3 "Commercially exploited species", D5 "Nutrient enrichment", D6 "Physical loss and physical disturbance", D7 "Hydrographical conditions", and D8 "Contaminants".

The relevant ecosystem elements of the seabed assessed under MSFD Descriptors 1 and 6 are the benthic broad habitat types. These are listed by Commission Decision (EU) 2017/ 848 Annex, Part II - Table 2 and equate to one or more habitat types at hierarchical level 2 of the "EUNIS marine habitat classification, 2019".

The study objective is to evaluate the environmental status of seabed habitats in the Bulgarian Black Sea using Commission Decision (EU) 2017/848 assessment framework.

In order to assess the state of a habitat across its distribution it is necessary to determine the extent of the habitat which is considered to be in a good condition or, conversely, not in good condition (adversely affected). These two aspects - quality and extent of quality - are reflected in the criterion D6C5, which requires the setting of threshold values for adverse effects and the maximum allowable extent of those adverse effects.

Material and Methods

The Bulgarian Black Sea is subdivided into eight Marine Reporting Units (MRUs) which represent areas with distinctive physio-geographical characteristics: five coastal, two shelf and one open sea area. In this study four of the coastal and both shelf MRUs were assessed as regards benthic habitats of the seabed sediments and dissolved oxygen in bottom water (Fig. 1).

Sampling for macrozoobenthos was carried out in October 2017 at 107 points. Altogether 238 qualitative samples for macrozoobenthos were collected by means of Van Veen Grab (0,1 m^2) (Fig. 1). Species composition and abundance were determined in the laboratory following the procedures of Todorova & Konsulova (2005).

Bottom seawater was also sampled in October 2017 at 174 stations by Niskin bottles (5 l) Rosette System attached to SEABIRD CTD probe. Dissolved oxygen measurements were performed by Winkler titration method (Grasshoff et al., 1999).

Brey-Curtis similarity (Bray-Curtis, 1957) on 4^{th} root transformed biomass and hierarchical classification analysis (Clarke et al., 2014) were employed to differentiate macrozoobenthic communities and associate them with the broad habitat types.

M-AMBI(n) (Sigovini et al., 2013) was used to assess adverse effects on benthic macroinvertebrates. The method combines AMBI (Borja et al., 2000), a biotic index based on species sensitivity/tolerance to pressures, with Shannon-Wiener diversity index (H') and species richness (S) as an arithmetic mean of their minimum-maximum normalized values. Thresholds for good status were developed by Todorova (2017) using the ecological quality ratio method from reference conditions in line with the Water Framework Directive (WFD) methodological approach for intercallibration of the biological quality element macrozoobenthos (Todorova et al., 2018). The thresholds and EQRs, given in Table 1 were established for several benthic habitat sub-types on sandy bottom ("*MSFD Monitoring Programmes*" (BSBD, 2020)). Due to data deficiency on reference conditions for muddy bottom habitats, their status was assessed relative to the current best conditions using $EQR_{M\text{-}AMBI(n)}$=0.68 as a good status threshold.

The good status threshold for dissolved oxygen in bottom water was set at 75 % saturation in the coastal waters (Regulation № H-4 of 14.09.2012) and 4.69 mg/l for the shelf area (HELCOM, 2013). The extent threshold for good status of dissolved oxygen was set at 90 % of the total extent of each MRU.

Assessments of seabed habitats require the use of maps of habitat types as a prerequisite to estimate the extent of each habitat which is adversely affected. A predictive map of seabed habitats, covering all MSFD regions, including the Black Sea, is provided by the European project EMODnet (2020) Seabed Habitats according to the EUNIS typology, and also aggregated to MSFD broad habitat types (Fig. 1).

The extent to which good environmental status is achieved is expressed as an estimate of the proportion of adverse effects per habitat type and whether this has achieved the extent threshold value set at 20 % in the Bulgarian Black Sea (Todorova, 2017).

The extent of each habitat in good or not good status was estimated using GIS. Inverse Distance Weighted interpolation of $EQR_{M\text{-}AMBI(n)}$ point values at equal resolution of 500 m was employed to create raster. The squares with interpolated values were converted to "good" and "not good" classes in relation to the threshold value $EQR_{M\text{-}AMBI(n)}$=0.68. Dissolved oxygen maps were created using similar approach. The final maps of overall adverse effects were created by integration of the maps for M-AMBI(n) and dissolved oxygen according to the rule one-out-all-out for each square (Fig. 2). The resultant map was intersected with the EMODnet map of benthic broad habitats types. The proportion from the total extent of each habitat in good or not good status in each MRU was calculated using Zonal Statistic function in GIS. Finally, the proportion of habitats in good status from total number of habitats present in each MRU was estimated and compared to overall GES threshold of 80 % (e.g. MRUs achieve GES if 80 % of the present habitats are in good condition).

Results

Dissolved oxygen saturation in the coastal bottom water varied between 63 - 112 %. Only 7 values under the threshold of 75 % saturation were recorded across the coastal areas, two of which were found in the northernmost MRU "Sivriburun-Kaliakra".

Oxygen concentration in shelf bottom water varied between 1,3 - 11,5 mg/l with 34 % of the samples below the threshold of 4,69 mg/l (23,3 % in the "Northern shelf" and only 10,7 % in the "Southern shelf").

The extent (as proportion of total area) of each MRU with values of bottom water oxygen above the thresholds for good status is given in Table 2. Four MRUs achieved GES with more than 90 % of their extent having values of dissolved oxygen above the respective thresholds for coastal and shelf areas. In two MRUs - northern coastal area "Sivriburun-Kaliakra" and "Northern shelf" area - less than 90 % of the area was in good condition of oxygen, therefore these areas were not in GES.

The taxonomic composition of the macrofauna encompassed 146 species and higher taxa - 50 polychaetes, 28 bivalves, 9 gastropods, 41 crustaceans and 18 miscellaneous (sponges, anemones, nemerteans, turbellarians, oligochaetes, echinoderms and ascidians).

Six biotopes with characteristic communities were differentiated based on Brey-Curtis similarity classification which were allocated to 5 broad habitat types (Table 3).

Habitat condition according to the average $EQR_{M\text{-}AMBI(n)}$ was above the threshold for good status at 63 monitoring points and below the threshold at 44 monitoring points (Fig.1).

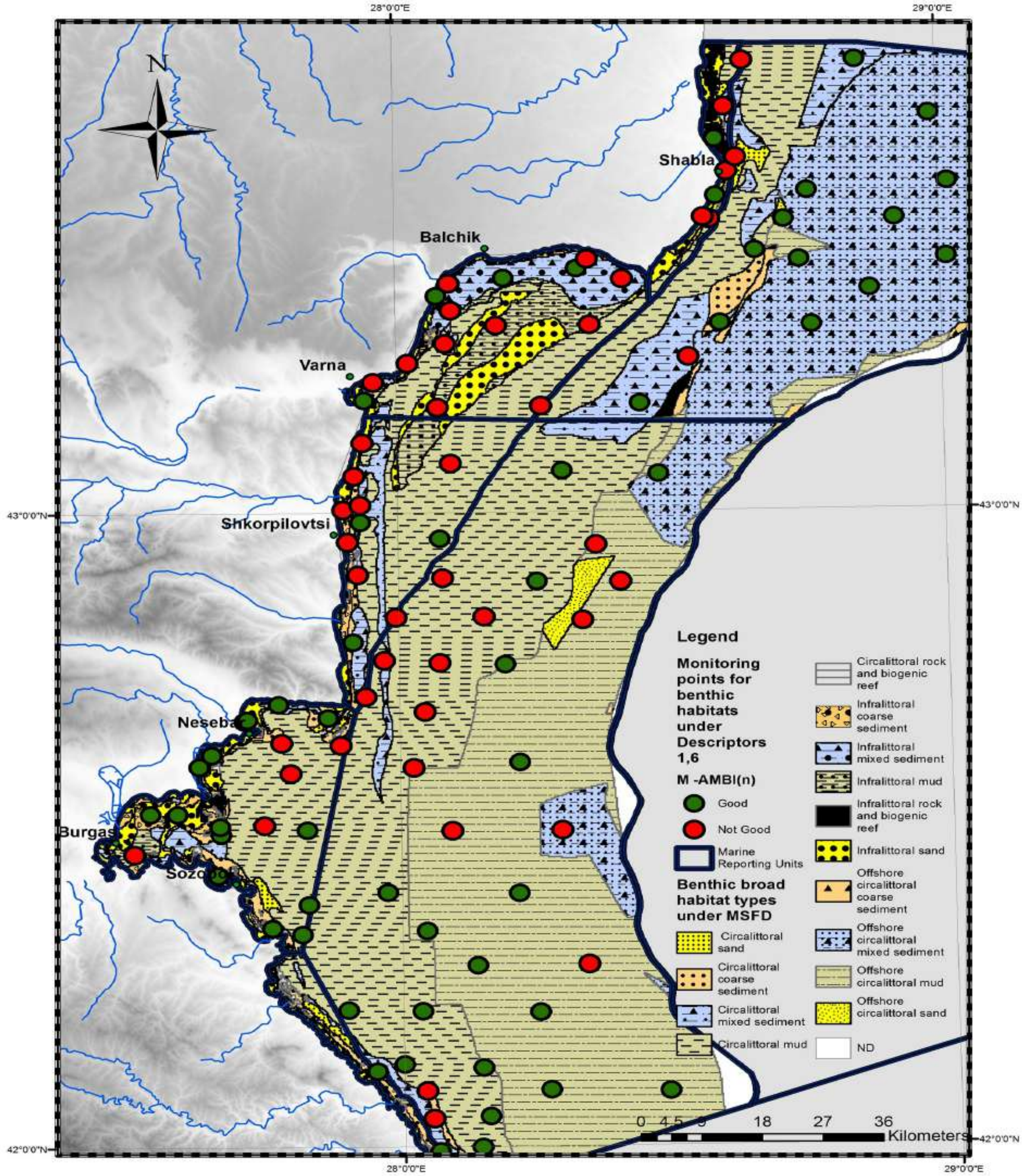

Fig. 1. Map of the study area in the Bulgarian Black Sea with outlined Marine Reporting Units, MSFD benthic broad habitat types and habitat condition according to $EQR_{M\text{-}AMBI(n)}$ at sampling points for macrozoobenthos.

Table1. Reference conditions and good status thresholds for ecological indices for some benthic habitat sub-types in the Bulgarian Black Sea.

Index	EQR	AMBI	H'	S	M-AMBI(n)
Habitat	Upper infralittoral meadium and fine sands dominated by *Donax trunculus*				
Reference conditions	1	0.5	3.1	18	0.91
Good status	0.68	2.26	2.11	12	0.62
Habitat	Infralittoral fine and medium sands dominated by *Chamelea gallina, Lentidium mediterraneum, Tellina tenuis*				
Reference conditions	1	0.3	3.4	30	0.87
Good status	0.68	2.12	2.31	20	0.59
Habitat	Infralittoral coarse and medium sands dominated by *Upogebia pusilla*				
Reference conditions	1	2.5	3.4	35	0.96
Good status	0.68	3.62	2.31	24	0.65
Habitat	Circalittoral shelly sands and gravel with diverse variable fauna				
Reference conditions	1	1.9	3.8	42	0.94
Good status	0.68	3.28	2.58	29	0.64

Table 2. Extent (% proportion of the total area) of MRUs that achieved good status thresholds for dissolved oxygen in bottom water.

MRU	Extent above thresholds, %	Status
Sivriburun-Kaliakra	70.31	Not in GES
Kaliakra-Galata	96.90	In GES
Galata-Emine	98.98	In GES
Emine-Maslen Nos	97.62	In GES
Norther shelf	84.43	Not in GES
Southern Shelf	99.87	In GES

Table 3. Benthic broad habitat types and biotopes identified in the study area.

Broad habitat type	Biotopes
Infralittoral sand	Upper-infralittoral (1 - 7 m) medium and fine sand dominated by *Donax trunculus*
	Infralittoral (5-15 m) fine and medium sand, dominated by *Chamelea gallina*
Circalittoral coarse sediment	Shallow circalittoral (17-35 m) shelly gravel and coarse sand with varied infauna (*Modiolus adriaticus, Gouldia minima*)
Circalittoral mud	Circalittoral mud with *Pitar rudis и Spisula subtruncata*
Offshore circalittoral mud	Offshore circalittoral mud with *Terebellides stroemi*
Circalittoral mixed sediments	Circalittoral mixed sediments with *Modiolula phaseolina*

The distribution of good and not good habitats condition over the habitats extent in the Bulgarian Black Sea is shown on Fig.2 which integrates the interpolated results for dissolved oxygen and $EQR_{M\text{-}AMBI(n)}$. Generally, the habitats condition of the coastal marine areas between the northern border and c. Emine and the central part of the shelf is not good as made evident on the figure.

Summary of benthic habitats status in the Bulgarian Black Sea in 2017 is presented in Table 4. Five from six examined MRUs did not achieve GES as regards benthic habitats. In three of the coastal MRUs none of the habitats present was in good status. MRU "Emine-Maslen nos" was singular coastal area in GES with 80 % (seven from eight) of the present habitats in good status, each of the habitats with less than 20 % of their total extent adversely affected. The "Northern shelf", although not in GES, achieved higher proportion - 62.5 % (five from eight) of the benthic habitats present in good condition as compared to the Southern shelf. The "Southern shelf" was not in GES with only 14.3 % (one from seven) of the habitats present in good condition.

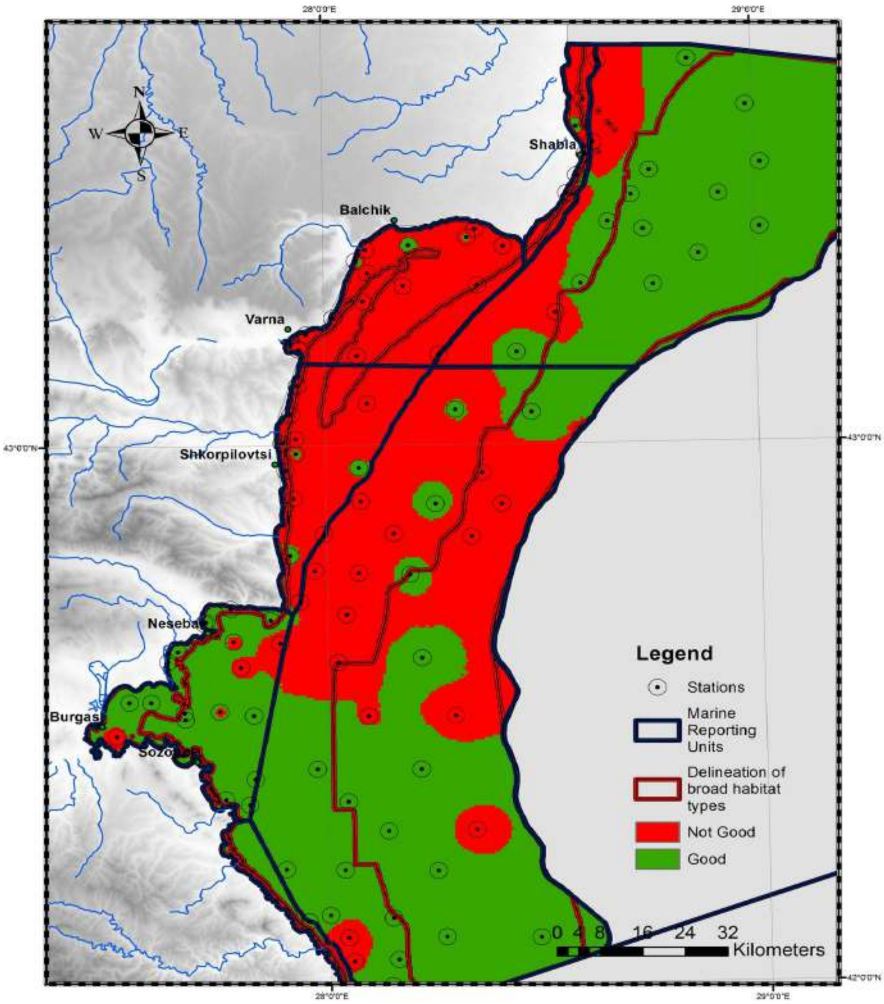

Fig. 2. Map of the study area in the Bulgarian Black Sea with Marine Reporting Units and extent of good and not good habitats status according to $EQR_{M\text{-}AMBI(n)}$ and dissolved O_2.

Table 4. Extent of adverse effects in broad habitat types (np-not present) as proportion (%) of total habitat area, proportion (%) of habitats in good status from overall number of habitats and general conclusion on environmental status in MRUs.

MRU	Sivri-burun-Kaliakra	Kaliakra-Galata	Galata-Emine	Emine-Maslen nos	Northern shelf	Southern shelf
Broad benthic habitat types	**Extent of adverse effects (% proportion of total habitat area)**					
Infra-littoral sand	87.8	99.8	84.0	1.6	np	Np
Infra-littoral mud	np	100	100	45.8	np	Np
Infra-littoral mixed sediment	100	100	81.6	2.6	np	Np
Infra-littoral coarse sediment	100	94.2	100	0	np	Np
Circa-littoral sand	82.5	100	25.0	0	97.7	Np
Circa-littoral mud	98.1	100	96.8	16.9	76.1	54.4
Circa-littoral mixed sediment	100	100	76.1	12.5	0.0	58.3
Circa-littoral coarse sediment	100	100	95.4	0	44.5	66.5
Offshore circa-littoral sand	np	np	np	Np	0	6.0
Offshore circa-littoral mud	np	np	np	Np	0,0	30.6
Offshore circa-littoral coarse sediment	np	np	np	Np	7.8	35.7
Offshore circa-littoral mixed sediment	np	np	np	Np	1.3	41.7
	Proportion (%) of benthic broad habitat types in good status in MRUs					
	0	0	0	80	62.5	14.3
GES achieved in MRU	Not in GES	Not in GES	Not in GES	In GES	Not in GES	Not in GES

Discussion

Pressures on seabed habitats can have physical, biological and/or chemical effects on the habitat affected, therefore, habitat status is assessed through the changes in its abiotic and biotic characteristics. Bottom-water oxygen supply is a key abiotic factor governing the biogeochemistry of marine sediments and of vital importance to the communities of benthic invertebrates. Reduced dissolved oxygen concentration is indicative of increased biological oxygen demand from elevated levels of nutrients and organic matter and it has negative impact on benthic invertebrates ranging from physiological effects to mass mortality during anoxic events (Diaz & Rosenberg, 1995; 2008). Consequently, dissolved oxygen in bottom water was established as one of the primary criteria for assessing eutrophication adverse effects under MSFD. Permanent water stratification in the Black

Sea due to vertical salinity gradient results in decreasing dissolved oxygen with increasing depth, while summer thermocline further reduces the ventilation of bottom water from the atmosphere thus creating conditions for hypoxia over the shelf (Stanev et al., 2014; Friedrich et al., 2014). It is therefore essential to distinguish the natural from the anthropogenic changes in dissolved oxygen and establish specific thresholds for biological zones of different depth range in the Black Sea. The results for the extent of MRUs in good condition as regards dissolved oxygen suggest small deviations in oxygen levels caused by natural or anthropogenic factors. Altogether well oxiginated bottom waters during the sampling season were possibly related to isothermal conditions in the water column and stormy weather in October 2017 that enhanced vertical mixing. Noticeable exception was observed in the northernmost coastal MRU "Sivriburin – Kaliakra" with nearly 30 % of its area bellow threshold and the "Northern shelf" area with 15 % (Table 2). These areas were possibly affected by the Danube transboundary influence, as it has been demonstrated that Danube water discharge to the Black Sea has a major impact on the Roumanian and Bulgarian shelf nutrient budget and oxygen regime (Velikova et al., 2005). Khrischev et al. (1998) and Panin & Jip (2002) uphold the view of significant Danube sediment drift influence on the biogenic carbonate sediments in the Bulgarian shelf to the north of c. Kaliakra due to high bottom current velocity that carries away the fine grains and thus forms massive shell deposits.

Regardless of the type of pressure, the assessment of what constitutes an adverse effect on a natural habitat should be based firstly on changes to the species composition and their relative abundance within the community compared to an unimpacted or less impacted state. The multi-metric index M-AMBI(n) reflects the changes in species richness and diversity, and the relative abundance of five ecological groups of species: sensitive, indifferent, tolerant, secondary and primary opportunists. The assessment concept is based on the Pearson & Rosenberg (1978) successional model according to which disturbance–sensitive taxa decrease, while tolerant and opportunistic species increase along the increasing pressure gradient, coupled with decrease in species richness and evenness of distribution. In the Bulgarian Black Sea M-AMBI(n) is validated in the WFD intercallibration exercise against the predominant pressures in the coastal waters including point sources of pollutants: loads for BOD, suspended solids (SS), total heavy metals (HMET), detergents (DET), phenols (PHE), total petroleum hydrocarbons sources and defuse sources of pollutants from urbanization, tourism, and navigation (Todorova et al., 2018). As far as the shelf is affected by diffuse (navigation), transboundary (currents) and air-born pollution, M-AMBI(n) presumably indicates the negative effects on the benthic invertebrates.

Another key human activity that causes significant physical disturbance to the seabed in EU waters, including the Black Sea, are fisheries with mobile bottom contacting gears (ICES, 2019). Although a relationship was demonstrated between AMBI and physical disturbance from dredging and sand extraction (Muxica et al., 2005), M-AMBI(n) is not validated against the predominant physical disturbance from fisheries on the Bulgarian Black Sea shelf. It is therefore important to establish the distribution and intensity of fisheries associated abrasion on the seafloor and then validate the conventional and novel ecological metrics as suitable indicators for adverse effects from the specific pressure.

The current assessment involves several sources of uncertainty such as the unknown pressure-response relationship of M-AMBI(n) with physical disturbance on the seafloor and unestablished good status thresholds for some benthic habitat types. Moreover, the adverse effects from other pressures, e.g. non-indigenous species and commercial exploitation were not examined in this study. The extent of adverse effects threshold shall be harmonized at regional and Union level, therefore the national threshold established at 20 % may be revised.

Regardless of the outlined deficits, this pilot study provided valuable experience in implementing the Commission Decision (EU) 2017/848 conceptual framework for assessing the environmental status of benthic habitats in the Bulgarian Black Sea. Using EMODnet seabed habitat maps enabled evaluating the extent of adverse effects on the habitats condition. The results are indicative of the benthic habitats status in the Bulgarian Black Sea in 2017 and provide useful information for fulfilling the reporting obligation of Bulgaria under MSFD.

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Synopsis

Therapeutic Potential and Biotechnological Utilization of the Indigenous Biosynthetic Capacity of Artemisia alba Turra: A Review

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Abstract. Genus *Artemisia* is one of the largest in the Asteraceae family, consisting of ca. 500 representatives, known under the common names "mugwort", "wormwood" and "sagebrush". Traditionally many *Artemisia* species have been utilized as spices, aromatic agents, as well as for their medicinal and ornamental properties. The present review presents a summary of the pharmacological activity, based on secondary metabolites identified in different representatives of the *Artemisia* genus. The effect of plant extraction on the type of biologically active compounds has also been discussed. Special accent has been given to distribution of *Artemisia* species throughout Southeast Europe and essential oil bearing of *A. alba* Turra which is also characteristic for the flora of Bulgaria. Experience of our team on *A. alba* Turra as a model system for biotechnological development of different classes of secondary metabolites has been summarized. The established flexibility of plant secondary metabolism in relation to morphogenesis in this species might serve as a useful tool for obtaining plant material with desired secondary metabolite profile through modeling plant growth, development and morphogenesis and without the use of genetic modifications *in vitro*.

Key words: *Artemisia genus, Artemisia alba* Turra *in vitro,* essential oils, phenolic and flavonoid compounds, endogenous cytokinins.

General characteristics of the Artemisia genus

The *Artemisia* genus is one of the largest in the tribe Anthemideae of the Asteraceae family, comprising of ca. 500 species (Watson et al., 2002; Zhen et al., 2010 and references cited within). Its representatives are widely distributed in the Northern Hemisphere and rare in the Southern Hemisphere. Many of the representatives of the genus find ornamental, medicinal or economic application (Zhen et al., 2010 and references cited within).

The central Asian region is considered to be one of the centers of origin and specification of the genus, with over 180 taxa, 40 of which endemic (Kapustina et al., 2001). There are 174 *Artemisia* species in Russia, 150 - in Asia and China, about 50 in Japan

(Kurşat et al., 2015 and references cited within) and 35 in Iran (Nigam et al., 2019). According to Tutin & Persson (1976), there are 57 representatives of the genus in Europe. About 30 species are reported in Italy (Nigam et al., 2019). The flora of Turkey contains 22 species of the genus (Davis, 1975).

The region of South Eastern Europe represents a rich pool of *Artemisia* species with a great potential for investigations, with 37 *Artemisia* taxons on a subspecies level (Table 1).

Pharmacological application and phytochemical basis of the biological activities of representatives of the Artemisia genus

Probably the most widely studied species in the genus is *A. annua* L. (Sweet Wormwood) with the antimalarial sesquiterpene lactone artemisinin. The first mention of the plant in Traditional Chinese Medicinal records dates as far as 281–340 B.C. It was made by Hong Ge in the "Handbook of Prescriptions for Emergency Treatment" (Hou Bei Ji Fang) as a remedy to treat fever and chills (Efferth, 2017). Further official records are found in the "Compedium of Materia Medica" (Ben Cao Gang Mu) by Li Shizen in 1596 (Efferth, 2017).

A. annua L. and its main active constituent artemisin have been re-discovered for up-to-date medicinal application as a result of the secret 523 Project of the Chinese government launched on May 23, 1967. The project was initiated by the Chinese chairman Mao Zedong in response to the request of the Vietnamese government to cope with chloroquine-resistant malaria (Su & Miller, 2015). In January 1969, Professor Youyou Tu from the China Academy of Traditional Chinese Medicine was involved and assigned in the leadership of the project (NobelPrize, 2015). Upon her research within over 2000 ancient Chinese prescriptions for treatment of fever, she came upon *A. annua* as the most frequently occurring species. Then after variation of the plant part and extraction method used, finally on October 4, 1971, the biologically active preparation designated as "sample #191" was obtained. It was capable of inhibition of rodent and monkey malaria with 100% activity (Su & Miller, 2015). The results were announced on March 8, 1972 and followed by a broad research on structure elucidation of the active principle underlying this activity. Artemisinin was identified as an endoperoxide sesquiterpene lactone, in 1972, with its structure determined by X-ray analysis in 1979 (Dhingra et al., 2000). In 2015 professor Tu was assigned the Nobel Prize in Physiology or Medicine for the discovery of the team led by her (NobelPrize, 2015).

In addition to *A. annua* L. as the main artemisinin source, the compound has also been identified in low concentration in *A. apiacea* and *A. lancea* (Ferreira et al., 2010).

The years of intensive research on artemisinin have led to the identification of also other activities such as the prominent anti-inflammatory and immunoregulatory properties of this compound (Kim et al., 2019b). These activities have led to the clinical application of artemisinin in inflammatory and autoimmune conditions such as alcohol-induced liver damage, tubulointerstitial nephritis, and rheumatoid arthritis (Kim et al., 2019b and references cited within). The ability of artemisinin to alleviate inflammatory response is due to blocking pro-inflammatory cytokines (tumor necrosis factor-α, interleukin-1β (IL-1b), and IL-17) expression. The mechanisms underlying this are through reactive oxygen species generation, regulation of mitogen activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) signaling pathways, and pathogenic Th17 responses (Li et al., 2006, Wang et al., 2008, and references cited within).

The mechanism of the anti-inflammatory effect of artemisinin in uric acid induced inflammation was through suppression of the interaction between NEK7 and the NLRP3 inflammasome in uric acid-induced inflammation. Thus, artemisinin was shown to suppress foot and ankle swelling in MSU crystal-induced arthritis mice (Kim et al., 2019b).

In addition the anti-viral, anti-parasitic, anti-fungal, anti-inflammatory and anticancer activities of artemisinin, as well as its derivatives were established (Ho et al., 2014).

Noteworthy, research has shown that besides the well-studied artemisinin, marked *in vitro* and *in vivo* anticancer activity was established for *A. annua* extract containing no detectable artemisinin (limit of detection=0.2 ng/mg). It was established that the main chemical constituents responsible for this activity were 6,7-dimethoxycoumarin, chrysosplenol D, casticin, arteanniun B, arteannuic acid (Table 1 and 2, Lang et al., 2019).

Prominent antihepatofibrotic, anti-inflammatory, choleretic, and hepatoprotective activities have been reported also for other biologically active compounds isolated from *Artemisia* species. Thus, *p*-hydroxyacetophenone, *β*-sitosterol, scoparone, cirsimaritin, quercetin, arcapillin, capillin, 6,7-dimethylesculetin, 6,7-dimethoxycoumarin, capillone, capillarin, 4′-methyl capillarisin, cirsilineol, cirsimaritin, and capillarisin, occurring in *A. capillaris,* as reviewed in Jang et al. (2015).

Table 2 and 3 contain a non-exclusive survey of literature of investigations performed in the last decade by different working groups on the pharmacological activities and identified phytochemical compounds in some *Artemisia* species.

Table 1. Distribution of *Artemisia* species throughout the region of Southeast Europe based on the online database of the Information resource for Euro-Mediterranean plant diversity (EuroMed PlantBase, 2020). *Legend:* Al – Albania, BH - Bosnia and Herzegovina, BG – Bulgaria, Cr – Croatia, Gr – Greece, Md - Moldova; Mn – Montenegro, NM – North Macedonia, RO – Romania, SKV – Serbia including Kosovo and Vojvodina, Sl – Slovenia, TEU - Turkey in Europe incl. Gökçeaga.

Taxa	**Al**	**BH**	**BG**	**Cr**	**Gr**	**Md**	**Mn**	**NM**	**RO**	**SKV**	**Sl**	**TEU**
A. abrotanum L.	+	+	+	+		+			+		+	
A. absinthium L.	+	+	+	+	+	+	+	+	+	+	+	+
A. alba Turra L.	+	+	+	+	+		+	+	+	+	+	
A. alpina Willd.			+						+			
A. annua L.	+	+	+	+	+	+	+	+	+	+	+	+
A. arborescens (Vaill.) L.				+	+		+					
A. argyi Lév. & Vaniot									+			
A. atrata Lam											+	
A. austriaca Jacq.		+	+	+		+			+	+		
A. caerulescens L.	+	+		+			+				+	
A. caerulescens L. ssp. *caerulescens*	+			+								
A. campestris L.	+	+	+	+	+	+	+	+	+	+	+	+
A. campestris ssp. *alpina* (DC.) Arcang.									+			
A. *campestris* L. ssp. *campestris*			+	+		+		+	+			+
A. *campestris* ssp. *inodora* Nyman			+			+			+			
A. *campestris* ssp. *lednicensis* (Spreng.) Greuter & Raab-Straube										+		+
A. chamaemelifolia Vill.			+									
A. codonocephala Diels									+			
A. dracunculus L.		+	+			+			+	+	+	
A. dzevanovskyi Leonova									+			

A. genipi Stechm.											+	
A. lancea Vaniot									+			
A. lerchiana Stechm			+		+				+			
A. nitida Bertol.											+	
A. pancicii Danihelka & Marhold										+		
A. pedemontana Balb.			+									
A. pontica L.			+			+			+	+	+	
A. santonicum L.			+	+	+	+		+	+	+		+
A. santonicum ssp. *patens* K. M. Perss.									+	+		
A. santonicum L. ssp. *santonicum*			+		+	+			+	+		+
A. scoparia Waldst. & Kit.	+	+	+	+	+	+	+	+	+	+	+	+
A. siversiana Willd.						+						
A. trautvetteriana Besser						+			+			
A. umbelliformis Lam.	+	+	+		+		+	+	+	+		
A. umbelliformis ssp. *eriantha* (Ten.) Vallès-Xirau & Oliva Brañas	+	+	+		+		+	+	+	+		
A. verlotiorum Lamotte				+	+						+	
A. vulgaris L.	+	+	+	+	+	+	+	+	+	+	+	+

Table 2. Pharmacological activities established for different preparations of some representatives of the *Artemisia* genus.

Artemisia species	Pharmacological activity	Parts used/method of preparation	References
A. absinthium L.	Antifungal (*Fusarium moniliforme*, *F. oxysporum fs. lycopersici* (Scheldt) and *F. solani*) and antiparasitic (*Leishmania infantum* PB75 strain) activities	Essential oils obtained by Clevenger distillation	Bailen et al., 2013
A. absinthium L.	Neuroprotective against disturbances induced by cerebral ischemia and reperfusion injury in rats	Methanol extract of the aerial parts	Bora & Sharma, 2010
A. absinthium L.	*In vitro* and *in vivo* alleviation of acute inflammation, induced by *Montivipera xanthine* viper venom	Methanol extract of the aerial parts	Nalbantsoy et al., 2013
A. annua L.	*In vivo* and *in vitro* anticancer activity	Commercial extract, MoMundo GmbH capsules (Bad Emstal, Germany)	Lang et al., 2019
A. capillaris Thunb.	Antioxidant, antisteatotic, anti-inflammatory, antiviral, choleretic, antifibrotic, antitumor activities in diverse *in vitro* and *in vivo* hepatic experimental models.	Aqueous extracts of the aerial parts	Jang et al., 2015
A. capillaris Thunb., *A. iwayomogi* Kitam. and *A. annua* L.	Antioxidant and anti-fibrotic activity in Hepatic stellate T6 (HSC-T6) cells	Aqueous extracts of the aerial parts	Saravanakumar et al., 2019
A. iwayomogi Kitam.	Lowering of gene expression of pro-inflammatory cytokines, reduction of visceral fat accumulation and obesity-related biomarkers in mice fed on a high-fat diet	Ethanol extract	Choi et al., 2013

A. iwayomogi Kitam.	Protective effect of against hypertriglyceridemia induced by a high-fat diet (HFD) in mice	Ethanol extract	Lee et al., 2017
A. iwayomogi Kitam.	DPPH and superoxide radical scavenging; anti-age potential due to tyrosinase, elastase and collagenase inhibition activity	Ethyl acetate and water fractions of the ethanol extract	Kim et al., 2019a
A. rutifolia Spreng	Antioxidant and antimicrobial (*Escherichia coli*, *Pasteurella multocida*, *Bacillus subtilis*, *Staphylococcus aureus*, *Aspergillus flavus*, *Aspergillus niger*, *Fusarium solani* and *Rhizoctonia solani*)	Comparative study of the hexane, chloroform and methanol extract of the aerial parts	Ashraf et al., 2017
A. anethifolia Poljakov, *A. commutate* Besser, *A. desertorum* Spreng, *A. integrifolia* Richards, *A. latifolia* Ledeb, *A. leucophylla* Turcz. ex Besser, *A. macrocephala* Jacquem. ex Besser, *A. messerschmidtiana* Besser, *A. palustris* L., *A. sericea* Weber ex Stehm., *A. tanacetifolia* L., *A. umbrosa* (Besser) Pamp.	*α*-glucosidase and *α*-amylase inhibitory activity.	SPE fractionation of 60 % Ethanol extract	Olennikov et al., 2018
A. vulgaris L.	Concentration-dependent genotoxicity (in human peripheral blood lymphocytes, PBLs) when applied alone; concentration dependent genoprotective effect upon mitomycin C co-treatment. No cytotoxicicty in individual application. Moderate cytotoxic effect in co-treatment with mitomycin C after long-term exposure (against SW-480 human colon cancer cells). Low cytotoxicity in control human periodontal ligament stem cells (PDLSCs).	Methanol extract of the aerial parts	Jakovljević et al., 2020

Table 3. Biologically active chemical constituents identified in some representatives of the *Artemisia* genus.

***Artemisia* species**	**Phytochemical constituents identified**	**Parts used/method of preparation**	**References**
A. annua L.	Arteannuin B, casticin, chrysosplenol D, arteannuic acid; 6,7-dimethoxycoumarin	Momundo *Artemisia annua* acetonitrile extract, MoMundo GmbH commercial capsules (Bad Emstal, Germany)	Lang et al., 2019
A. iwayomogi Kitam.	Major components scopolin, acetophenone glycoside and	Ethanol extract	Choi et al., 2013

	scopoletin		
A. iwayomogi Kitam.	3,5-dicaffeoylquinic, 5-O-caffeoylquinic and 3,4-dicaffeoylquinic acids; scopolin, scopoletin and patuletin-3-O-glucoside	Ethanol extract	Lee et al., 2017
A. iwayomogi Kitam.	Scopolin , 2,4-dihydroxy-6-methoxy-acetophenone-4-O-ß-D-glucopyranoside, scopoletin, kaempferol-3-O-methyl ether and luteolin	Ethyl acetate and water fractions of the ethanol extract	Kim et al., 2019a
A. rutifolia Spreng	Gallic acid, caffeic acid, chlorogenic acid, syringic acid, sinapic acid, p-coumaric acid, m-coumaric acid, ferulic acid, vanillic acid, myricetin, and quercetin	Methanol extract of the aerial parts	Ashraf et al., 2017
A. anethifolia Poljakov, *A. commutate* Besser, *A. desertorum* Spreng, *A. integrifolia* Richards, *A. latifolia* Ledeb, *A. leucophylla* Turcz. ex Besser, *A. macrocephala* Jacquem. ex Besser, *A. messerschmidtiana* Besser, *A. palustris* L., *A. sericea* Weber ex Stehm., *A. tanacetifolia* L., *A. umbrosa* (Besser) Pamp.	Caffeoylquinic acids, Flavonoids,	SPE fractionation of 60 % Ethanol extract	Olennikov et al., 2018
A. vulgaris L.	Domination of chlorogenic acid, quercetin-3-O-glucopyranoside, syringic, trans-cinnamic and p-coumaric acids, presence of fisetin and syringing.	Methanol extract of the aerial parts	Jakovljević et al., 2020

An impressive number of works have been performed regarding the phytochemical richness of the genus. Studies have shown the presence of triterpenes, steroids, hydrocarbons, polyacetylenes, flavonoids, coumarins, mono and sesquiterpenoids with a wide range of biological activities such as antimalarial, cytotoxic, antihepatotoxic, anti-bacterial, antifungal and antioxidant properties (Maggio et al., 2012 and references cited within).

The broad research on pharmacological activity of *Artemisia* species is based on the long-lived knowledge and practices of the indigenous population in many regions of the world. Thus, the traditional utilization of representatives of the genus for treatment of malaria, hepatitis, cancer, inflammation, as well as against fungi, bacteria, and viruses has been documented (Willcox, 2009; Abad et al., 2012; Nigam et al., 2019). Thus, *A. abrotanum* L. ("southernwood") has been traditionally used in the treatment of upper respiratory tract diseases and applied nowadays for culinary and cosmetic purposes (Abad et al., 2012). *A. absinthium* L. ("wormwood") is distributed throughout Europe and Siberia and utilized as antiparasitic and for treatment of anorexia and as a digestive. Its properties are the reason for its broad inclusion in digestive herbal preparations, dietary supplements and it is especially known as an additive in alcoholic beverages, such as the Absinth, known all over the world (Lachenmeier, 2010). *A. iwayomogi* Kitamura ("hanin-jin" or "dowijigi" in Korean) growing in Korea has been known for its traditional application in treating liver ailments, includingly hepatitis (Park, 1999). *A. vulgaris* L. ("mugwort") occurring in Asia, Europe and North America is widely used in the Philippines (locally known as "herbaka") for its antihypertensive properties. Its traditional application as anti-inflammatory, antispasmodic, carminative and anthelmintic remedy has also been reported, as well as its effect in the treatment of painful menstruation and in the induction of labour or miscarriage (Quisumbing, 1978).

Phytochemical research has shown the scientific basis of the activities recorded for many of the *Artemisia* species. As seen by the works surveyed in Tables 2. and 3, in addition to the sesquiterpene lactones, responsible for the characteristic bitterness of *Artemisia* species, terpenoids in their essential oils, as well as phenolic compounds in their extractable preparations attribute for the wide array of pharmacological activities in the different species.

Artemisia alba Turra – general outline and phytochemicals with therapeutic potential

A. alba Turra is a fragrant shrub species with an Euro-Mediterranean distribution widespread in the South Eastern parts of Europe.

According to some literature sources, its morphological variability attributes to its botanical placement to different subgenera of the *Artemisia* genus (Maggio et al., 2012). Throughout the years it has been placed in subgenera *Absinthium* (Fiori, 1927), *Abrotanum* (Greger et al., 1982) or *Artemisia* (Tutin & Perron, 1976). Due to this complexity, for example the Sicilian population has been classified both as *A. camphorata* Vill. var. *subcanescens* Ten. and *A. alba* var. *incanescens* (Jord.) Fiori (Maggio et al., 2012 and references cited within).

According to other sources, the species could be placed in the *Artemisia* subgenus *Seriphidium* Less. Heterotypic synonyms of the species name are *A. achilleifolia* Ten., *A. alba* ssp. *camphorata* (Vill.) P. Fourn., *A. alba* ssp. *canescens* Priszter & Soó, *A. alba* ssp. *lobelia* (All.) Hegi, *A. alba* ssp. *saxatilis* (Willd.) P. Fourn., *A. alba* ssp. *suavis* (Jord.) P. Fourn., *A. biasolettiana* Vis., *A. camphorata* Vill., *A. camphorata* var. *canescens* DC., *A. fruticosa* Asso, *A. humilis* Wulfen, *A. incanescens* Godr., *A. lobelia* All., *A. saxatilis* Willd., *A. suavis* Jord. and *A. subcanescens* Willd. (Greuter, 2006).

Traditionally, the decoction of the plant has been used in the Mediterranean region as a stomach digestive (Rigat et al., 2007). Research, been performed on the biological activity and secondary metabolites of the species are summarized in Tables 4 and 5.

A key factor in cancer genesis is the process of chronic and repeated inflammation, affecting tissue homeostasis and repair (Rakoff-Nahoum, 2006). The functional relationship between inflammation and cancer is not new. In 1863, Virchow hypothesized that the origin of cancer was at sites of chronic inflammation, in part based on his hypothesis that some classes of irritants, together with the tissue injury and ensuing inflammation they cause, enhance cell proliferation (Balkwill & Mantovani 2001). Thus it has been considered a challenging perspective in anti-cancer therapy to normalize the inflammatory network in order to recover the normal host response of the patient which would lead to decreasing the tumour-promoting properties of the infiltrating cells (i.e. pro-inflammatory cytokines) while increasing their tumour-suppressing properties, such as anti-inflammatory cytokines (Coussens & Werb, 2002). The established results of the significant toxicity on SW-480 colon cancer cells when applied alone, and the significant increase of the antiproliferative activity and decreased the required concentration (IC50) of the chemotherapeutic agent Mitomycin C (MMC), together with the obtained results of the anti-radical and anti-inflammatory effect of preparations of the species demonstrate the potential of *A. alba* as a dietary food supplement or as a supplement to chemotherapy (references summarized in Table 4).

Literature sources of volatile and non-volatile phytochemical constituents of preparations of the species are summarized in Table 5.

Noteworthy are the results obtained in the literature on the marked variability of the terpenoid profile of the essential oil of the species, as well as the effect of external factors on their mono : sesquiterpenoids ratio (Table 5).

This motivated our further research to develop biotechnologically the species in order to study the factors affecting *in vitro* production of essential oils and polyphenolics in *A. alba* Turra.

Biotechnological development of the species

Based on the obtained variability of essential oil production discussed in literature, as well as the phytotherapeutic potential of the phenolic compounds established in the species, biotechnological development of *A. alba* Turra was performed. The effect of modification of *in vitro* morphogenesis on the terpenoid profile of essential oils as well as total phenolic and flavonoid compounds was performed. Dependencies were established with the endogenous cytokinin pools of the plant.

Shoot cultures of *A. alba* Turra were initiated from the surface sterilized stem explants of the aerial parts of the plant kindly provided by Assoc. Prof. Ljuba Evstatieva from the collection of the Institute of Biodiversity and Ecosystem Research-BAS (Danova et al., 2012). Stock shoots were maintained in plant growth regulators (PGR)-free culture medium. In order to modify plant growth and development, auxin (indole-3-butyric acid, IBA) and benzyl adenine (BA) treatments were applied.

Two main *in vitro* morphotypes of the plant were obtained (Fig. 1). The PGR-free control, as well as 0.5 mg/l IBA and 1.0 mg/l IBA treated plants (designated as GAIP_0, GAIP_1 and GAIP_2, respectively), were characterized by the formation of both aerial and root parts (Danova et al., 2012). Plants, treated with auxin and cytokinin combination - 0.5 and 1.0 mg/l IBA together with 0.2 mg/l BA (GAIP_3 and GAIP_4, respectively) were characterized with inhibition of root development. Extensive callusogenesis at the plantlets' base was recorded instead, with only up to 15 % of indirect rooting through callus.

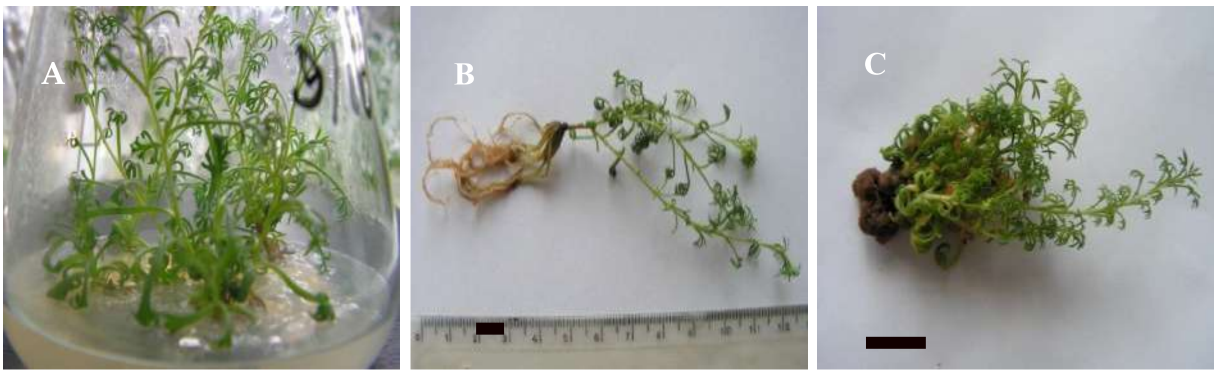

Fig. 1. Morphoregulatory effect of PGR on *A. alba* shoot cultures. Stock shoots cultivated in PGR-free medium (A); rooting moprhotype, illustrated by *A. alba* Turra cultivated in GAIP_1 - 0.5 mg/l IBA supplemented medium (B); non-rooting morphotype, illustrated by the plant grown in GAIP_3 - 0.5 mg/l IBA + 0.2 mg/l BA medium (C). *Space bar = 1 cm.*

Table 4. Investigations on biological activity established for different preparations of *A. alba* Turra.

Parts used/method of preparation	Pharmacological activity	References
Lyophilized ethanol extract	Anti-inflammatory effect through inhibition of NF-κB and AP-1 transcription factors in human hepatoma (HepG2) and human umbilical vein endothelial cells (HUVEC).	Stalińska et al., 2005
Ethanol extract	Anti-inflammatory through suppression of nitric oxide and TNFα (tumor necrosis factor α) synthesis in cells of monocyte origin	Strzelecka et al., 2005
Essential oil of the aerials of the plant (under the name of *A. lobelii* All.	Antimicrobial activity against *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*	Stojanovic et al., 2000
Essential oil of the aerial parts of *A. alba* Turra	Antioxidant activity (FRAP assay 0.023 ± 0.00 mmol Fe2 +/g; DPPH assay, EC_{50} = 14.08 ± 1.09 mg/ml). Antimicrobial activity (Gram (+) bacteria: *Micrococcus luteus*, *Micrococcus flavus*, *Staphylococcus aureus*, *S. epidermidis*, *Enterococcus faecalis* and *Bacillus subtilis*; Gram (-) bacteria: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*; two standard strains of the *Candida albicans* yeast).	Dordević et al., 2013
70 % Ethanol extract of the aerial parts of *A. alba* Turra	Antioxidant activity (FRAP assay 1.6 ± 0.08 mmol $^{Fe2+}$/g; DPPH assay IC_{50} = 0.032 ± 0.002 mg/ml). Antimicrobial activity (Gram (+) bacteria: *Micrococcus luteus*, *Micrococcus flavus*, *Staphylococcus aureus*, *S. epidermidis*, *Enterococcus faecalis* and *Bacillus subtilis*; Gram (-) bacteria: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*; two standard strains of the *Candida albicans* yeast). Activity of the extract showed to be higher than the one of the essential oil.	Dordević et al., 2013
Ethyl acetate extract of the aerial parts	Induction of early stage apoptosis in SW-480 colon cancer cells.	Jakovljević et al., 2019
Methanol extract of the aerial parts of *A. alba* Turra	Concentration-dependent genotoxicity (in human peripheral blood lymphocytes (PBLs)) when applied alone; concentration dependent genoprotective effect upon mitomycin C co-treatment. Significant cytotoxic effect against SW-480 human colon cancer cells. Low cytotoxicity in human periodontal ligament stem cells (PDLSCs) as normal control.	Jakovljević et al., 2020

Table 5. Volatile and non-volatile phytochemical constituents identified in different *A. alba* Turra preparations.

Parts used/method of preparation	Phytochemical constituents identified	References
	Volatile constituents	
Aerial parts of *A. alba* of Calabrian origin	Five oxygenated nerolidol derivatives	Appendino et al., 1985
Essential oil from inflorescences of *A. alba* Turra collected at different location of the Adriatic area of NE Italy	Thermophytic plant communities - domination of sesquiterpene hydrocarbons; mesophytic plant communities - domination of oxygenated monoterpenes	Coassini Lokar et al., 1987
Essential oil of the aerials of the plant (under the name of *A. lobelii* All.	Major constituents camphor (33.2-36.8%), 1,8–cineole (15.2-21.1%) and artemisia ketone (6.0-24.2%)	Stojanovic et al., 2000
Essential oil of the aerials of the plant originating from contrasting serpentine (*A. alba* var. *saxatillis*) and calcareous (*A. alba*) habitats in Serbia	Germacrene D (38.3%) - major constituent in the accession of the serpentine habitat; spathulenol (11.8%), artemisia ketone (10.1%), camphor (7.5%) and 1,8-cineole (7.4%) in the samples of the calcareous habitat. Overall ca. 73 % sesquiterpenes and ca. 60 % monoterpens, in the first and latter samples, respectively. Four times lower yield in the *saxatillis* var.	Radulović & Blagojević, 2010
Essential oil of the aerial parts of *A. alba* Turra, obtained by Clevenger distillation	Major constituents: camphor (23.7%), artemisia ketone (15.2 %) 1,8-cineole (14.1%) and trans-chrysanthenol (8.6%), followed by camphene (3.2%), silphiperfol-5-en-3-one A (6.4%) and α-bisabolol (6.1%). Mono- towards sesquiterpenoids ratio 3:1.	Dordević et al., 2013
Volatile fraction obtained from 70 % Ethanol extract of the aerial parts of *A. alba* Turra	49 components, among which scopoletin (14.0%), corymbolone (10.3%), trans-chrysanthenol (7.4%), umbelliferone (4.9%), camphor (4.3%), 1,8-cineole (5.9%) and artemisia ketone (2.9%)	Dordević et al., 2013
Comparative analysis of the essential oils of *A. alba* Turra from four different locations in Italy, obtained by hydrodistillation	Variability in the oil yield (from 0.03% up to 1.5 % w/w). Generally high levels of camphor and isopinocamphone. Domination of sesquiterpenes (60 %) in one of the oils, comparable levels of mono- and sesquiterpenes in the remaining three samples (with mono- towards sesquiterpenes ratio varying from 0.38 up to 0.95).	Maggio et al., 2012
Hydrodistillation from the aerial parts of *A. lobelia* All. from Serbia	Dominating components: camphor (41.94 %), 1,8-cineole (13.8 %), syn-anti-antiHelifolen-12-al A(10.2 %), camphene (8.89 %), borneol (3.38 %). Domination of oxygenated monoterpenes (61.4 %) with mono-sesquiterpenoid ratio 3.08.	Janaćković et al., 2019
	Non-volatile constituents	
Dichloromethane extract of the aerial parts of *A. alba* Turra from Sicily	Artalbic acid - sesquiterpene with unusual skeleton.	Maggio et al., 2011
70 % Ethanol extract of the aerial parts of *A. alba* Turra	In the non-volatile fraction of the extract: kaempferol 3-O-(6''-O-malonylglucoside)-	Dordević et al., 2013

	7-O-rhamnoside, chlorogenic acid and rutin (16.1%, 11.4% and 9.5%, respectively), followed by kaempferol 3,7-O-diglucoside (8.4 %), luteolin 5-O-(6″-O-malonylglucoside) (7.7 %), apigenin (3.8 %), luteolin (3.4 %), genkwanin 5-O-glucoside, kaempferol 7-O-rhamnoside (1.9 %), luteolin 5-O-glucoside (1.2 %) and gallic acid (0.3 %).	
Chloroform extract of the aerial parts of *A. alba* Turra	11 highly oxygenated sesquiterpenoids: 2β,5α,12-trihydroxygermacra-1(10),4(15),11(13)-triene, 12-acetoxy-1α,10β-epoxy-5α-hydroperoxy-2β-hydroxygermacra-4(15),11(13)-diene, 1β,10β-epoxy-2β,5α,12-trihydroxygermacra-4(15),11(13)-diene, 5α,10α-Epoxy-1α,2β,12-trihydroxygermacra-4(15),11(13)-diene, 12-acetoxy-5α,10α-epoxy-1α,2β-dihydroxygermacra-4(15),11(13)-diene, 9β-Acetoxy-1β,3β,4β-trihydroxygermacra-5,10(14)-diene, 9β-Acetoxy-1β-hydroperoxy -3β,4β-dihydroxygermacra -5,10(14)-diene, 1α,2β,12-trihydroxyeudesma-4(15),11(13)-diene, 12-acetoxy-2α,4α,10α-trihydroxyguai-11(13)-ene, 7β-acetoxy-2β-hydroxyoplopenone, 7β-acetoxy-2α-hydroxyoplopenone, and 7-hydroxycadin-4-en-3-one 2 flavonoids: centaureidin and axillarin	Todorova et al., 2015
Methanol extract of the aerial parts of *A. alba* Turra	chlorogenic acid, 3,5- and 4,5- dicaffeoylquinic acids, scopoletin, umbeliferone, luteolin, nepetin, quercetin 3-methyl ether, axillarin, apigenin, hispidulin, diosmetin, chrysoeriol, desmethoxycentaureidin, jaceosidin, quercetin-3,3′-dimethylether, centaureidin and rutin were isolated from the methanol extract. UHPLC-PDA-MS confirmed the presence of additional 11 compounds : phenolic acids and flavonoids	Trendafilova et al., 2018
Ethyl acetate extract of the aerial parts of *A. alba* Turra	Phenolic acids (gallic, p-coumaric, vanillic, and ferulic acids) and flavonoids (rutin, myricetin, luteolin, quercetin, and apigenin)	Jakovljević et al., 2019
Methanol extract of the aerial parts of *A. alba* Turra	Domination of chlorogenic acid, quercetin-3-O-glucopyranoside, 2,5-dihydroxybenzoic, trans-cinnamic and p-coumaric acids. Presence of ferulic acid, quercetin, 3,5-dihydroxybenzoic acid, rutin, syringin, protocatechuic acid, vanillic acid and fisetin.	Jakovljević et al., 2020

The essential oil of the plantlets was obtained by micro steam distillation-extraction of the fresh shoots of the in vitro grown plants in a modified Lickens-Nickerson apparatus for 2.5 h using diethyl ether as a solvent (Sandra & Bicchi,1987). The essential oil of the five *A. alba* Turra treatments was characterized based on up to 34 terpenoid components. The mono- towards sesquiterpenoids (M/S) ratio of the essential oils was shown to be dependent on the morphotype of *A. alba* Turra *in vitro.* Thus, while its value was 5.2 for the rooting morphotype, its value dropped to 2 - 2.5 for the non-rooting *A. alba* Turra (Danova et al., 2012). Interestingly, the reverse dependencies were obtained from the underground parts of the plants. Thus, while in the rooting morphotype the M/S ratio of the oils of the underground parts was 0.01 -

0.02, and for the non-rooting morphotype it was 0.09 - 0.17, showing the generally strong predominance of sesquiterpenoids in the underground samples (Krumova et al., 2013). The qualitative analysis of the oils showed that the chemical composition of the sesquiterpenods in the aerial and underground samples differed, excluding the option of root-to-shoot translocation as a factor affection the terpenoid profile in the two morphotypes (Danova et al., 2018).

Rooting is a key feature in cytokinin biogenesis and translocation. The biosynthesis of free cytokinins occurs mainly in root apical meristems (Sakakibara, 2006). In order to establish the possible dependencies, endogenous cytokinin pools of the *A. alba* Turra samples were studied by HPLC coupled to hybrid triple quadrupole/linear ion trap mass spectrometer with internal standards (Djilianov et al., 2013, Žižková et al., 2015).

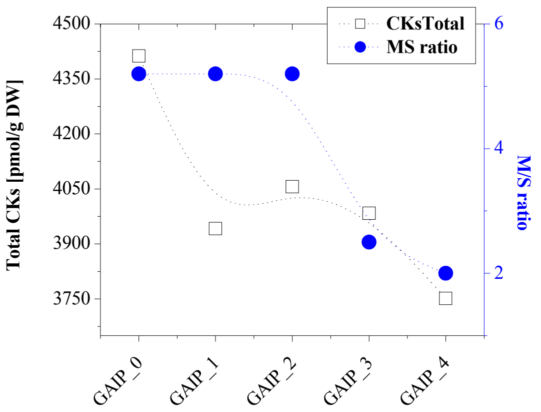

Fig. 2. Comparative representation of the content of total cytokinins and the mono- towards sesquiterpenoids (M/S) ratio in the different *A. alba* Turra PGR treatments *in vitro*.

The graphical representation of the dependencies obtained is given in Figs. 2 and 3. The relations between the M/S ratio and the total cytokinins, and bioactive cytokinins (representing the sums of cytokinin free bases and ribosides), Figs. 2 and 3, respectively showed that rooting of the plant influenced by the exogenous PGR treatments plays a major role in both terpenoid biogenesis and status of endogenous cytokinin pools of the plant.

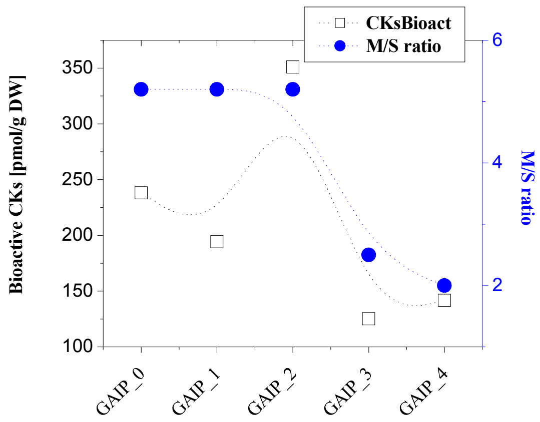

Fig. 3. Comparative representation of the content of bioactive cytokinins (sum of free bases and ribosides) and the mono- towards sesquiterpenoids (M/S) ratio in the different *A. alba* Turra PGR treatments *in vitro*.

The levels of total phenolic and flavonoid compounds were also studied spectrophotometrically (Koleva et al., 2015).

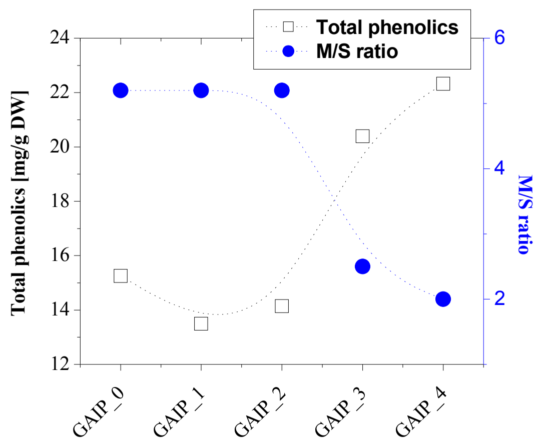

Fig. 4. Comparative representation of the content of total phenolic compounds and the mono- towards sesquiterpenoids (M/S) ratio in the different *A. alba* Turra PGR treatments *in vitro*.

It was established that the levels of both phenolic (Fig. 4) and flavonoid (Fig. 5) compounds were related to the *A. alba* Turra morphotypes obtained.

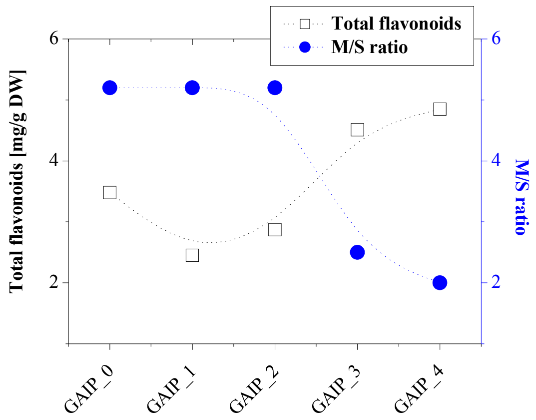

Fig. 5. Comparative representation of the content of total flavonoid compounds and the mono- towards sesquiterpenoids (M/S) ratio in the different *A. alba* Turra PGR treatments *in vitro*.

The biotechnological development of *A. alba* Turra and the dependencies obtained by this experimental design provide clues of the possibilities to target terpenoid and phenolic compounds biogenesis by means of inducing morphological changes of the *in vitro* cultivated plants without performing genetic manipulations.

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Synopsis

Organic Cultivation of New Medicinal and Aromatic Plants as a Source for Pharmaceutical Industries

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Abstract. Medicinal and aromatic plants continue to be the subject of novel and straight forward applications as a source of active constituents for pharmaceutical industries. Fertilizers are important factor in modern-day agriculture as they increase crops yield and allow crops to be planted in nutrient deficient soils. Although chemical fertilizers are important tool for higher production of different crops, the extensive and intensive use of them resulted in the accumulation of chemicals. Some organic fertilizers as Farmyard manure (FYM) and compost at different rates and different combinations could be used instead of chemical fertilizers to meet the demand of plants from NPK and some micronutrients to improve the quantity and quality of medicinal plants. The present review introduces some researches performed to elaborate the benefits of organic cultivation of some important medicinal and aromatic plants such as *Coleus forskohlii, Satureja hortensis L., Amaranth* species etc.

Key words: organic fertilizers, medicinal plants, microorganisms, phytohormones.

Introduction

Human being has been used medicinal and aromatic plants from ancient time and scientists are constantly brings to light additional information on the relationship between plants and traditional medicines. 80% of global population use medicinal plants to cover all or part of their health care needs (WHO, 2008). Thousands of higher plants have been reported to be of high medicinal value and constitute a major source of raw material for pharmaceuticals, cosmetics, and drug industries. Many of these plants synthesize substances that are useful to the maintenance of health in humans and other animals. These include aromatic substances, most of which are phenols or their oxygen-substituted derivatives such as tannins while others contain alkaloids, glycosides, saponins and many secondary metabolites (Naguib, 2011).

Organic fertilizers are obtained from animal sources such as animal manure or plant sources like green manure. Continuous usage of inorganic fertilizer affects soil structure. Hence, organic manures can serve as alternative to mineral fertilizers for improving soil structure (Dauda *et al.*, 2008) and microbial biomass (Suresh et al., 2004). Composting is a biological process in which organic biodegradable wastes are converted into compost for use as a soil conditioner and an organic fertilizer (Popkin, 1995). Vermicomposting is a simple biotechnological process of composting, in which certain species of earthworms are used to enhance the process of waste conversion and produce a better end product (Gandhi *et al*., 1997). These are also used to provide biological control against various plant pathogens (Hoitink & Grebus, 1994). The addition of municipal solid waste compost to agricultural soils has beneficial effects on crop development and yields by improving soil physical and biological properties (Zheljazkov & Warman, 2004).

Biofertilizers are microbial inoculants consisting of living cells of micro-organism like bacteria, algae and fungi alone or combination which may help in increasing crop productivity. Biological activities are markedly enhanced by microbial interactions in the rhizosphere of plants (Tilak & Reddy, 2006). The plant growth promoting rhizobacteria (PGPRs) can influence plant growth directly through the production of phytohormones and indirectly through nitrogen fixation and production of bio-control agents against soil-borne phytopathogens (Glick, 2003). *Azospirillum* species are nitrogen-fixing organisms, capable of forming an associative relationship with the roots of several economically important crops (Vande Broek & Vanderleyden, 1995).

Most of the cultivators of medicinal and aromatic crops have know the importance of cultivation of crop under organic condition and were better equipped to take a more informed decision about expanding its cultivation under organic system of production (Malik, 2014). The decision on area allocation by a cultivator for a given crop, more so for a new crop, is influenced by several factors about some of which the cultivator may have had little or no knowledge when he first started its cultivation. Having once decided to cultivate the crop, the decision on its cultivation under organic farming system is influenced by the experience the cultivator and availability of resources and more deterministic information about the other complementary factors (Afaq et al., 2013).

This review has mainly been focused on some information about organic fertilizer and contemplating and prospecting the work done so far to know the impact of organic farming system on growth and yield of some economical medicinal and aromatic crops.

What are organic fertilizers made of?

Organic fertilizers are made from mined rock minerals as well as natural plant and animal materials. They include ingredients like manure, guano, dried and powdered blood, ground bone, crushed shells, finely pulverized fish, phosphate rock, and wood. While inorganic or synthetic, fertilizers may contain some organic ingredients. The main difference is that they act quickly to simply feed the plant without actually enriching the soil, and may contribute to a toxic build up of salts in the soil when over applied.

What types of organic fertilizers are there?

- *Plant-Based Fertilizers:* Plant-based fertilizers are usually high in nitrogen and sometimes potassium. Some crops are grown specifically to be made into organic fertilizer, while others, such as cottonseed meal, are by-products of another industry.
- *Alfalfa Meal:* This fertilizer is also available in pellets. It has a moderate amount of nitrogen (2 to 3 %) and contains some trace minerals.
- *Corn Gluten:* This by-product of the corn-processing industry contains 10% of nitrogen in a form that's quick to break down. It also has the unique ability to inhibit germination of seeds and is sold as an organic pre-emergent herbicide to control crabgrass in lawns.
- *Cottonseed Meal:* This by-product of the cotton industry is made from the remains of cotton seed after the oil is pressed out. It's a slow-release fertilizer, moderately high in nitrogen (6%) with some phosphorous. It can acidify the soil. Since cotton is such a heavily sprayed crop, there are concerns about pesticides on the seed and in the meal. It's

preferable to use low-residue or pesticide-free cottonseed meal.

• *Seaweed:* Extracts of seaweed and kelp are found in meal and liquid forms. They are good sources of minerals, with some potassium and nitrogen. They also enhance the microbial activity in the soil. Liquid versions of seaweed can be sprayed directly on plants as a foliar fertilizer.

• *Soybean Meal:* This high-nitrogen fertilizer (7%) is similar to alfalfa meal and contains more nitrogen than cottonseed meal.

• *Animal-Based Fertilizers:* By-products from the dairy and meat processing industries produce a bevy of organic fertilizer products.

• *Blood Meal:* A by-product of the slaughtering industry which is a rich source of nitrogen (14%) but the smell may attract dogs and wild animals to the garden.

• *Bonemeal:* Another by-product of the slaughtering industry, bonemeal is a rich source of phosphorous (10%) and calcium (22%) and it supplies some nitrogen. "Steamed" bonemeal has less nitrogen but somewhat faster nutrient availability than "raw" bonemeal.

• *Fish Products:* By-products from fish industry are used in organic fertilizer products such as fish emulsion and fish meal, high in nitrogen (up to 10%) and quickly available to plants.

• *Compost:* Compost is considered the Cadillac of organic fertilizers. It can be made from plant-, animal-, and mineral-based materials. Finished compost has a low but good balance of nutrients while being high in organic matter that helps feed the soil's microorganisms. Composts are available commercially or can be made in field from various kinds of animal manures and lawn and garden wastes. It can be used with other fertilizers. Making compost is a way to deal with yard waste.

• *Animal Manures:* Manures can be derived from a variety of animals and even insects. Most are available bagged, composted, and sometimes sterilized. The nutrient composition of animal manures varies based on animal, bedding and method of manure storage. Aged manure is better than fresh, and cow is better than horse (high in weed seeds) but any manure will give worthwhile advantages. Cow manure is the manure most commonly found bagged in garden centers while nutrient content is low, the plants can absorb them moderately quickly. On the other hand, manure from sea birds, chickens, and bats is rich in nutrients, especially nitrogen.

Benefits of Organic Fertilizers:

The use of organic fertilizers could open an array of opportunities/ benefits related to the agronomic, economic and social domains;

A) Agronomic benefits:

1. Enhancing soil biological activity through favoring the root colonization by mycorrhizal fungi or by rhizosphere bacteria, these microorganisms can improve N, P and K supply and microelements by mobilization of low-soluble nutrient.

2. Improving the soil physical properties, thus ameliorating soil structure and water holding capacity.

3. Supplying nutrients in a balanced way, which increases plant growth and prolongs the plant health status by suppressing certain soil borne diseases and parasite.

4. The new organic sourced and processed fertilizers (often marketable products) make it possible to be applied according to the nutrient demand of the plant and soil status. This opportunity is mainly relevant to regions with a high soil P content, where P has become the limiting factor of fertilization by organic fertilizers.

5. Environmental benefits due to keeping/ enhancing microbial diversity, reducing soil acidification or alkalization and by reduction of decomposition of toxic substances (Yara, 2010).

B) Economic benefits:

1. Reduces needs in chemical fertilizers and soil management practices.

2. Reduces costs of externalities (i.e. those linked to the whole production and utilization chains).

C) Social benefits:

1. Enhances recycling of organic materials, with benefit for the environment with developing new industries/ production processes.

2. Facilitates the contact between local farms for exchange of organic sources which should allow choosing a product that is the most efficient for a particular crop.

Relationship between organic fertilizers and medicinal & aromatic plants

Medicinal and aromatic plants constitute a major segment of the flora, which provides raw materials for pharmaceuticals, cosmetics and drug industries. Looking into the important role of medicinal plants in different industries, it is great significance to increase production of biomass without using harmful chemical.

The use of organic fertilizers and microbial symbiosis with medicinal and aromatic plants helps in improving the yield and quality. However, the debate on the relative benefits of conventional and organic farming systems has in recent time gained significant interest. Additionally, global agricultural development has focused on increasing productivity rather than on a holistic natural resource management for food security. Thus, developing more sustainable farming practices on a large scale is of utmost importance. Many studies have shown positive results by organic and biofertilization practices. For example, application of rock phosphate and bio-fertilizers produced the maximum herb fresh weight and volatile oil production of *Mentha longifolia* plants (El-Gohary et al., 2013) while fertilizing *Thymus vulgaris* plants with nitropin+compost produced a significant increase in vegetative growth parameters and oil production (Nejatzadeh-Barandozi & Pourmaleknejad, 2014). In addition, using mycorrhiza, azotobacter and vermicompost to fertilize basil plants led to an increase in essential oil yield (Shirzadie et al., 2015).

In the following part we will focus on some new medicinal plants as *Coleus forskohlii, Satureja hortensis L., Amaranth* species etc.

1 - Coleus forskohlii Briq.:

Coleus (*Coleus forskohlii Briq.*), belonging to the family Lamiaceae, is an originated Indian medicinal herb (Valdes et al., 1987). It grows in the subtropical temperate climates of India, Nepal, Burma, Sri Lanka and Thailand. It is also found on the dry and barren hills (Anon, 1950). Apparently, it has been distributed to Egypt, Arabia, Ethiopia, tropical East Africa and Brazil (Willemse, 1985). It is the most important species of genus Coleus popularly known as 'garmar'. It is cultivated for the tuberous roots which are pickled and eaten (Anonymous, 1950). It is also used for the medicinal purposes mentioned in the Hindu and Ayurvedic schools of medicines (Ammon and Muller, 1985) as it is the source of forskolin (De Souza & Shah, 1988).

All plant parts are found to have traces of forskolin while roots are the main source (0.5-1%) and are commercially preferred for its extraction (Valdes et al., 1987). The tuber attachment region contains maximum amount (1-3 times higher forskolin), Yanagihara (1995). Forskolin is used in the treatment of congestive cardiomyopathy, hypertension and glaucoma (Seamon, 1984). The indiscriminate collection of *C. forskohlii* from the wild has made the species vulnerable and it has been included in the list of endangered species (Vishwakarma et al., 1988).

1-1-Influence of organic and inorganic fertilizers on growth and tuber yield of coleus (Coleus forskohlii Briq.) under northern dry zone of Karnataka

The field experiment conducted on sandy loam soil under irrigated conditions in Karnataka, India, showed that the application of 75% of RDF (Recommended dose of fertilizer) with 10t FYM + vermicompost 5 t/hectare significantly increased plant height (66.49cm), number of branches/ plant (85.95), leaf area index (7.49) at harvest, absolute growth rate(3.39g/plant/ day), crop growth rate (0.943 g/m^2/day) and relative growth rate (0.046g/g/ week) were recorded at 120-160 days old (Sadashiv et al., 2014).

1-2-Effect of potential bioinoculants and organic manures on root-rot and wilt, growth, yield and quality of organically grown Coleus forskohlii in a semiarid tropical region of Bangalore, India (Table 1).

Another 2-year field experiment was conducted with five bioinoculants and neem cake under organic field conditions (with vermicompost as a nutritional supplement) for evaluation of their potential to control root-rot and wilt (a complex problem involving *Fusarium chlamydosporum* and *Ralstonia solanacearum*) of the medicinal plant *Coleus forskohlii*. Plants treated with *Arbuscular mycorrhizal* fungus, *Glomus fasciculatum*, neem cake or *Pseudomonas fluorescens* showed significant increment in plant height (15- 31%), plant spread (25- 33%), number of branches (63-67%) and dry root yields (129-200%) with reduction of disease incidence (47-50%) compared to controls. Increases in yields were accompanied by increases in N (51- 81%), P (17- 76%) and K (44- 74%) uptake. The forskolin content of the roots was found not to be affected by any of the bioinoculants, but calculated forskolin yield was increased significantly by the treatment of *G .fasciculatum* (227%), neem cake (222%) and *P. fluorescens* (159%), Singh et al. (2012) (see Table 2).

1-3-Effect of organic manures, biofertilizers and inorganic fertilizers on productivity, biochemical parameters and active ingredient of Coleus forskholii.

An autumn season experiment was carried out during 2006 in India for determining the effect of *Azotobacter chroococcum* (N fixing) and *Pseudomonas striata* (phosphorus solubilizing bacteria, PSB) along with organic manures farmyard manure (FYM), vermicompost and chemical fertilizers (NPK, 50: 50: 30 kg/ ha) on the productivity, biochemical parameters and active ingredient of *Coleus forskohlii* with nine treatment combinations. The results showed that the maximum dry root yield (16.52 q/ ha) and fresh root yield (168.10 q/ha) were obtained upon treatment with chemical fertilizers (NPK at 50:50:30 kg/ ha) with maximum biochemical changes in roots, N, P, K contents (1.15%, 4.97%, 106.67ppm), protein (7.20%) and fat contents (7.24%) were recorded upon treatment with the chemical fertilizer. Whereas FYM at 5t/ha with vermicompost at 2.5 t/ ha increased carbohydrate (10.28%) and fiber content (13.30%) in roots. finally, Forskolin was maximum (17.29%) with the NPK treatment (Jyoti et al., 2008).

1-4-Organic Cultivation of Medicinal Plants: Influence of Composted Coir Pith on the Growth and Yield of Coleus forskohlii (willd.) Briq

In another study, composted coir pith (CCP) was used as an organic fertilizer and its effect on the growth, primary metabolite and secondary metabolite (forskolin) of *C. forskohlii* were studied. The CCP was amended to the soil in the plots at three rates, 5 t/h (T1), 10 t/ h (T2), and 15 t/ h (T3). It was found that CCP increased the growth and development of *C. forskohlii* in fields amended with 15 t/ h (T3). The activity of the three prime soil enzymes, namely, urease, phosphatase, and dehydrogenase in the rhizosphere soil of *C. forskohlii* also increased with an increase in the quantum of CCP amended to the soil (Padmadevi et al., 2016).

2 - Savory (Satureja hortensis L.)

Satureja species belongs to Lamiaceae family. Summer Savory (*Satureja hortensis L*) is an annual herbaceous plant with small erect hairy stems, grows about 30 cm in height. The branches are pinkish, leaves dark green, petiolate, leathery about 1 cm long. Lilac, pink or white flowers appear in small spikes in the leaf axils, during late summer. In folk medicine, summer savory is currently believed to benefit the entire digestive system. According to its believers, savory acts as a carminative, an anti-flatulent, an appetite stimulant, and works in diarrhea. The herbal tea is considered beneficial as an expectorant and cough remedy. One very interesting use of the tea in Europe is for excessive thirst in diabetics. The tincture can be used in small amounts for alleviating symptoms in cases of rachitic children and for rheumatoid afflictions followed by high fever. Many other therapeutic applications are listed by various herbalists. Savory (*Satureja hortensis L.*) Different forms of dried and processed products of savory (Rezaei et al., 2020).

2-1-Effect of Different levels of Seaweed Fertilizer on Growth Parameters, Yield and Essential Oil Content of Summer Savory (Satureja hortensis L.)

A complete randomized block design experiment was carried out for evaluation of studying the effect of different levels of seaweed fertilizer on the growth characteristics, plant material yield and essential oil percentage. Treatments were foliar application of 0 (control), 2.5, 5 and 10 ml/liter seaweed fertilizer in three times during growing season. It was mentioned that different concentrations of seaweed produced significant increments in all growth and yield parameters. The highest recorded values were those of 10 ml/liter seaweed fertilizer including branches number (35.44), shoot dry weight, root fresh and dry weight (15.17 and 6.42 g), leaf width (8.07 mm), plant height (54.66 cm), shoot fresh and dry weight (181.01 and 37.69 g), essential oil percentage and yield (2.51% and 6.28 g/m^2) (Rezaei et al., 2020).

2-2-Effect of Organic Fertilizers on Nutrients Content and Essential Oil Composition of Savory (Satureja hortensis L.)

Vermicompost as well as washed and unwashed mushroom compost in five levels (10, 20, 30, 40 and 50% v/v) were applied to savory. These produced significant induction of savory growth with macronutrient accumulation. The highest N content (6.3%) and P (0.98%) in savory shoot was obtained with 40% vermicompost application whereas higher potassium (3.19%) and calcium (2.48%) content was found in plants grown in the media containing 30% of vermicompost and 50% of washed spent mushroom compost (SMC) as compared to control plants. The highest essential oil percentage was produced by application of 30 % of vermicompost, the main components of essential oil were carvacrol and gamma-trepenine, while the highest level of carvacrol (62.10%) and gamma-trepenine (32.05%) were obtained in plants in substrates containing 40 and 20% of washed spent mushroom (Behrooz et al., 2018).

3 - Chervil (Anthriscus sylvestris. L) Hoffm.

Chervil (garden chervil, *Anthriscus cerefolium*, *Apiaceae*) is an essential oil bearing plant, related to parsley. It is commonly used to season mild flavored dishes and is a constituent of the French herb mixture fine herbs. It is native to the Caucasus and was spread by the Romans through most of Europe, where it is now naturalized (Vaughan et al., 1997). It is an excellent source of antioxidants that stabilize cell membranes and reduce inflammation associated with headache, sinusitis, peptic ulcer, and infections. Its essential oil contains estragole (as tarragon and basil), plus anethole. Leaves contain a fixed oil, high concentrations of potassium and calcium and apiin-α- glycoside. Flavonoids extract from herb and lignans from root showed strong free radical quenching activity, while the volatile oil obtained from herb was less effective. The identification of the constituents of the extracts indicated that apiin is the main flavonoid, deoxy podophyllo is the major lignan, and methyl chavicol is predominant constituent of essential oil (Feijes et al., 2003).

3-1-Improvement of growth parameters and essential oil productivity of Anthricus cerefolium L. by planting distances and fertilization treatments

Previously, it was established that the main constituents of essential oil of the plant cultivated in Egypt are strongly dependent on the application of nitrogen (N) and/or potassium (K) fertilizers. In order to broaden the possibility to enhance the productivity of the plant in this presented work in 2019, the effect of planting distances (15 cm – 30 cm – 45 cm), NPK feedings (0, 25%, 50%, 75% and 100%) as well as combination treatments between compost (10, 15 and 20 m^3/fed) and NPK were experimented. It was established that the increment of sowing distance from 15 cm to 45 cm increased gradually vegetative growth parameters (see Table 3, 4, 5).

The highest mean values of oil percentage were resulted from sowing distance at 45 cm followed by 30 cm. Compost at 20 t/fed produced the highest values of growth characters and essential oil (%). NPK fertilizer levels caused very noticeable effect on different growth parameters compared with untreated plants. Thus, 100% of NPK gave the maximum values of traits parameters and essential oil (%). The combination treatments between NPK and compost had a great effect on growth traits and essential oil (%). The combination treatment between compost at 20 t/fed and NPK at 100% produced the greatest values of growth characters and essential oil%. Essential oil constituents were identified with GC-MS. The main constituents were methyleugenol and estragole (Amer et al., 2019) (see Table 6 and 7).

Table 1. Fresh and dry tuber yield as influenced by organic manures and inorganic fertilizers in *C. forskohlii*. Data tabulated in this table indicate that T3 (75 per cent RDF + 10 t FYM + vermi compost 5.0 t/ ha) gave the maximum mean values of fresh and dry weight of tuber followed by T2 (75 per cent RDF + 10 t FYM + vermicompost 2.5 t/ha) while T8 (10 t FYM + vermicompost 2.5 t/ ha) resulted in the lowest values.

Treatments	**Fresh tuber yield**			**Dry tuber yield (q/ha)**
	g/plant	**q/ ha**	**g/plant**	
T_1 : 100 per cent RDF + 10 t FYM/ ha (control)	165.46	183.84	21.6	24.08
T_2 : 75 per cent RDF + 10 t FYM + vermicompost 2.5 t/ ha	202.13	224.58	26.47	29.42
T_3 : 75 per cent RDF + 10 t FYM + vermicompost 5.0 t/ ha	225.47	250.52	29.53	32.81
T_4:50 per cent RDF+10t FYM+vermicompost 2.5t/ ha	181.32	201.46	23.75	26.39
T_5:50 per cent RDF+10t FYM+vermicompost5.0t/ha	184.10	204.56	24.11	26.79
T_6:25per cent RDF+10t FYM+vermicompost2.5t/ ha	160.02	177.80	20.96	23.29
T_7:25per cent RDF+10t FYM+vermicompost 5.0t/ha	162.39	180.43	21.27	23.63
T_8: 10 t FYM + vermicompost 2.5 t/ ha	153.70	170.78	20.13	22.37
T_9: 10 t FYM + vermicompost 5.0 t/ ha	160.38	178.20	21.01	23.34
Mean	177.22	196.91	23.21	25.79
S.E.	3.307	6.102	0.719	0.799
C.D. (P=0.05)	9.914	18.29	2.16	2.39

Table 2. Effect of bioinoculants and neem cake on growth and yield parameters of *Coleus forskohlii* at harvesting in field conditions. *Legend:* TV: *Trichoderma viride*; BS: *Bacillus subtilis*; AZ: *Azotobacter chroococcum*; GF: *Glomus fasciculatum*; PF: *Pseudomonas fluorescens*; NC: neem cake; VC: vermicompost.

Treatment	**Plant height (cm)**	**Plant spread (cm)**	**Number of branches**	**Dry shoot yield (t ha^{-1})**	**Dry root yield (t ha^{-1})**	**Forskolin yield (kg ha^{-1})**
TV	41.70ab	43.70ab	20.30a	1.34a	0.18a	1.10a
BS	40.0ab	46.70b	19.30a	1.36a	0.17a	1.02a
AZ	40.20ab	41.30ab	18.30a	1.49a	0.22a	1.32ab
GF	49.60c	49.30b	28.30b	2.58b	0.41c	2.71c
PF	43.60b	47.10b	28.00b	2.01a	0.32bc	2.15bc
NC	48.20c	46.30b	27.70b	2.64b	0.42c	2.67c
VC (Control)	38.00a	37.10a	17.00a	1.33a	0.14a	0.83a
LSD (P< 0.05)	4.10	7.00	6.08	0.80	0.1	0.84

Table 3. Effect of compost on growth characters of chervil plants (mean values of two successive seasons). Means followed by similar letter(s) within the same column are not significantly different at P ≤ 0.05 according to Duncan's multiple range test.

Compost	Plant height		Fresh weight g/plant		Dry weight g/plant	
	1st cut	2nd cut	1st cut	2nd cut	1st cut	2nd cut
10	19.42c	18.41b	142.95c	111.53c	39.06c	30.47c
15	24.85b	18.99b	174.16b	129.84b	47.59b	35.48b
20	33.77a	24.81a	208.54a	142.68a	56.98a	38.98a
LSD at 5%	0.71	0.81	2.45	2.07	0.79	0.71

Table 4. Effect of compost on essential oil percentage and yield (ml/plant) (mean values of two successive seasons). Means followed by similar letter(s) within the same column are not significantly different at P ≤ 0.05 according to Duncan's multiple range test.

Compost	Essential oil %		Essential Oil Yield (ml/plant)	
	1st cut	2nd cut	1st cut	2nd cut
10	0.076c	0.084b	0.110c	0.093c
15	0.090b	0.087b	0.160b	0.118b
20	0.110c	0.110a	0.234a	0.159a
LSD at 5%	0.007	0.006	0.020	0.006

Table 5. Effect of the combination treatments between compost and NPK on growth characters (mean values of two successive seasons). Data are presented as means. Means followed by similar letter(s) within the same column are not significantly different at P ≤ 0.05 according to Duncan's multiple range test.

Compost	NPK	Plant height (cm)		Fresh Weight g/ pot		Dry Weight g/ pot	
		1st cut	2nd cut	1st cut	2nd cut	1st cut	2nd cut
10 m³ compost	0 NPK	17.14g	15.06e	96.61k	99.50f	26.40j	27.19e
	25 % NPK	18.55g	17.20e	133.43i	102.20f	36.46h	27.22hi
	50 % NPK	20.20ef	19.96d	145.45h	104.50f	39.74g	28.55gh
	75 % NPK	20.43ef	19.83d	168.44g	120.50e	46.02f	32.92f
	100 % NPK	20.79ef	20.00d	170.84g	130.97d	46.68f	35.78e
15 m³ compost	0 NPK	20.43ef	16.50e	124.59j	100.93f	34.04i	27.58hi
	25 % NPK	22.20de	16.19e	167.86g	103.87f	45.86f	28.38ghi
	50 % NPK	23.65cd	17.20e	178.18f	132.13d	48.68e	36.10e
	75 % NPK	25.12c	22.03cd	195.02e	149.10c	53.28d	40.74d
	100 % NPK	32.83b	23.05bc	205.17d	163.18b	56.06c	44.58c
20 m³ compost	0 NPK	32.59b	22.08cd	131.83i	104.07f	36.02h	28.43ghi
	25 % NPK	32.40b	24.70ab	181.29f	107.47f	49.53e	29.36g
	50 % NPK	32.61b	25.06ab	209.35c	151.70c	57.20c	41.45d
	75 % NPK	35.40a	25.23ab	255.02b	166.96b	69.68b	45.62b
	100 % NPK	35.83a	26.99a	265.20a	183.18a	72.46a	50.05a
LSD at 5%		1.59	1.81	3.83	5.30	1.65	0.88

Table 6. Effect of the combination treatments between compost and NPK on essential oil percentage and yield (mean values of two successive seasons). The presented data are means. Means followed by similar letter(s) within the same column are not significantly different at P ≤ 0.05 according to Duncan's multiple range test.

Compost	NPK	Essential oil %		Essential Oil Yield (ml / plant)	
		1st Cut	2nd Cut	1st Cut	2nd Cut
10 m^3 compost	0 NPK	0.070^{e}	0.100^{abcd}	0.068^{f}	0.100^{e}
	25 % NPK	0.070^{e}	0.080^{cd}	0.093^{ef}	0.082^{g}
	50 % NPK	0.080^{de}	0.080^{cd}	0.116^{def}	0.084^{fg}
	75 % NPK	0.080^{de}	0.080^{cd}	0.135^{cdef}	0.096^{e}
	100 % NPK	0.080^{de}	0.080^{cd}	0.137^{cdef}	0.105^{e}
15 m^3 compost	0 NPK	0.070^{e}	0.070^{d}	0.087^{ef}	0.071^{h}
	25 % NPK	0.090^{cd}	0.080^{cd}	0.151^{cde}	0.083^{fg}
	50 % NPK	0.090^{cd}	0.080^{cd}	0.160^{cde}	0.106^{e}
	75 % NPK	0.080^{de}	0.087^{cd}	0.156^{cde}	0.134^{c}
	100 % NPK	0.120^{b}	0.120^{ab}	0.246^{b}	0.196^{a}
20 m^3 compost	0 NPK	0.100^{c}	0.090^{bcd}	0.132^{cdef}	0.094^{ef}
	25 % NPK	0.100^{c}	0.110^{abc}	0.181^{cd}	0.118^{d}
	50 % NPK	0.100^{c}	0.130^{a}	0.209^{bc}	0.197^{a}
	75 % NPK	0.130^{a}	0.120^{ab}	0.332^{a}	0.200^{a}
	100 % NPK	0.120^{b}	0.100^{abcd}	0.318^{a}	0.183^{b}
LSD at 5%		0.008	0.021	0.051	0.009

Table 7. Effect of Fertilizers on the relative percentage of the main constituents of the essential oil of chervil plant.

No	RT	Constituents	KI*	Compost 10			Compost 15			Compost 20		
				0	50	100	0	50	100	0	50	100
1	3.90	α-Pinene	1109	t	t	t	0.62	0.40	t	0.28	0.2	0.44
2	5.28	(-)-β-Pinene	1190	1.00	0.36	1.12	0.85	0.97	0.21	0.58	0.19	0.77
3	7.38	D-Limonene	1278	1.17	0.92	1.33	1.37	1.23	0.99	0.92	0.36	1.15
4	7.71	Eucalyptol	1291	0.26	t	0.5	2.31	0.97	t	1.14	0.39	1.45
5	8.64	γ-Terpinene	1324	0.21	1.1	0.33	2.24	0.76	t	0.97	0.26	1.25
6	9.43	o-Cymene	1351	t	0.31	t	0.7	0.28	t	0.33	t	0.44
7	12.26	3-Nonanone	1444	0.57	0.29	0.48	0.28	0.52	0.6	0.24	0.3	0.44
8	14.74	1-Nonene	1524	2.71	2.19	2.24	1.49	1.96	2.84	2.18	2.16	2.2
9	15.39	Isomenthone	1544	0.5	0.53	0.48	1.58	0.59	0.35	1.09	0.4	1.22
10	15.81	Copaene	1558	0.19	0.2	t	t	t	0.22	t	0.26	t
11	16.06	Non-3-Enyl Acetate	1566	0.53	0.36	0.35	0.21	0.29	0.54	0.34	0.38	0.42
12	16.34	p-Menthan-3-onecis	1575	0.15	t	0.21	0.90	0.29	t	0.59	t	0.6
13	16.58	3-Nonanol	1582	0.32	0.25	0.35	0.3	0.31	0.25	0.26	0.2	0.28
14	17.03	Pentadecane	1597	0,9	0,66	0,66	0,59	0,67	0,33	0,64	0,97	0,84
15	17.82	2-Nonenal, (E)-	1623	t	0.21	0.35	0.19	0.20	0.15	0.36	0.23	0.25
16	17.90	1-Hexadecanol	1625	0.54	0.42	0.41	0.26	0.46	0.23	0.34	0.62	0.48

17	18.32	1-Nonen-3-ol	1639	1.01	0.51	0.77	0.63	0.79	0.75	0.49	0.49	0.63
18	19.03	Caryophyllene	1663	0.17	0.18	t	t	t	0.24	t	0.24	0.19
19	20.64	3-Octen-2-ol, (Z)-	1716	1.24	0.73	0.69	0.52	0.72	0.32	0.61	0.76	0.67
20	21.05	Pulegone	1731	0.62	1.36	0.55	3.2	1.00	t	1.96	0.43	2.37
21	21.82	Estragole	1757	21.14	19.26	24.29	20.6	25.33	26.43	20.49	23	22.63
22	22.39	Germacrene D	1777	1.54	1.76	1.03	1.04	0.94	1.46	1.32	1.42	1.16
23	22.86	(-)-Zingiberene	1793	3.46	4.32	2.44	2.72	2.73	4.10	3.28	4.36	3.36
24	23.00	β-Bisabolene	1798	0.43	0.55	0.27	0.31	0.29	0.39	0.42	0.53	0.40
25	23.83	α-Farnesene	1827	0.5	0.54	0.34	0.33	0.46	0.17	0.4	0.6	0.47
26	24.21	B-Sesquiphellandrene	1840	0.33	0.34	t	0.21	0.21	0.28	0.27	0.36	0.26
27	24.39	Curcumene	1847	0.42	0.56	0.27	0.3	0.23	0.28	0.44	0.46	0.38
28	28.14	Geranyl propionate	1983	0.72	0.5	0.34	0.35	0.37	0.13	0.4	0.49	0.40
29	28.75	Geranylisovalerate	2006	0.97	0.61	0.44	0.5	0.5	0.26	0.62	0.73	0.59
30	30.33	2-Allyl-1,4-dimethoxybenzene	2068	8.71	7.99	8.43	7.25	7.83	9.48	8.33	8.41	8.34
31	30.87	Methyleugenol	2090	44.67	46.11	44.41	42.39	43.33	43.87	40.37	41.55	40.37
32	31.57	Trans-MethylIso-Eugenol	2115	1.45	0.93	0.96	0.99	0.96	1.35	0.93	1.27	1.11
33	49.18	1-Octadecanol	2546	0.86	1.97	1.62	1.18	1.46	0.70	3.76	2.94	1.45
Oxygenated compounds				83.55	81.57	84.97	83.05	85.25	85.63	82.32	82.85	83.70
Hydrocarbons				12.84	13.79	10.03	12.77	11.13	10.96	11.39	11.14	12.47
Total identified compounds%				96.39	95.36	95.0	95.82	96.38	96.59	93.71	93.99	96.17

4-Amaranthus

Amaranthus genus, belonging to *Amaranthaceae* family, includes more than 60 species (Pisarikova et al., 2006). It is a worldwide distributed although most species are found in the warm temperate and tropical regions of the world (Sauer, 1993). Amaranth was cultivated by early civilizations over 2000 years ago, and continues to be used essentially worldwide, even to the present day (Liu & Stützel, 2004). Amaranth can be considered a multipurpose crop. Several *Amaranthus* species are cultivated as ornamentals, pseudo-cereals with high nutritive value (amaranth grain), leaf-vegetables, potherbs and for fodder (Sauer, 1967 & Mallory et al., 2008).

Amaranthus species are classified into three subgenera; the most economically important one is the subgenus *Amaranthus proper*, which includes the three species domesticated for grain production; *Amaranthus hypochondriacus* L., *Amaranthus cruentus* L, and *Amaranthus caudatus* L. (Trucco & Tranel, 2011). The high nutritional value of both seeds and leaves as well as recent interest of crop may be regarding its high-quality protein ،unsaturated oil, and various other valuable constituents - support the use of species from this genus as oilseed crops for oils production, all over the world (Kauffman, 1992; Venskutonis & Kraujalis, 2013).

4-1-Effect of organic root plus (bio-stimulant) on the growth, nutrient content and yield of Amaranthus

The effectiveness of organic root plus (bio-stimulant) was compared with conventional fertilizer on the growth and yield of Amaranthus in a glass house study. The treatments consisted of control, full rate each of bio-stimulant and fertilizer, and combination of fertilizer with bio-stimulant at full and half rates. The urea, single super-phosphate and potash were applied at 100kg N, 60kg P_2O_5 and 30kg K/ ha (fertilizer full rate). Results showed that the use of organic bio-stimulant alone was not as effective as that of fertilizer alone in most determined increases of number of leaves, plant height and leaf area in the first cycle and regeneration. Complimentary application of bio-stimulant with mineral fertilizer promoted the vegetative growth, nutrient composition, root development and yield of *Amaranthus*. When full rate of bio-stimulant was combined with full rate of mineral fertilizers, the number of leaves, plant height, leaf area and shoot yield were increased over the mineral fertilizer. In conclusion, the combinations of the two materials at various ratios were also effective (Akande, 2006).

4-2-Effect of Inorganic and Organic Fertilizers on the Performance and Profitability of Grain Amaranth (Amaranthus caudatus L.) in Western Kenya

Protein malnutrition is a major cause of morbidity and mortality in developing countries where the cost and availability of animal protein remain prohibitive. Grain amaranth (*Amaranthus caudatus L*) has the potential to substitute expensive animal protein. Nitrogen is a key limiting element in grain amaranth production. A study investigated the effects of different rates of inorganic nitrogen and cattle manure on the growth and yield of grain amaranth over a period of two years. Inorganic fertilizer at the rate of 100 kg N/ha significantly delayed flowering. Grain yield showed a linear response to inorganic and organic N application. Regression analysis projected the optimum inorganic fertilizer and manure application rates (87.5 kg N/ha and 9 t/ha, respectively) with yield of 1.84 t/ha as shown in Table 9. The highest profitability was achieved at the optimum manure and fertilizer rates (Richard et al., 2012). Moreover, Dry matter yield increased gradually as inorganic or organic fertilizer increased.

4-3-Influence of organic, mineral and organo-mineral fertilizers on growth, yield, and soil properties in grain amaranth (Amaranthus cruentus. L)(see Table 8)

A pot trial was conducted in the screen-house, Nigeria, during 2014 to examine the influence of organic, mineral and organo-mineral fertilizers on growth, yield, and soil properties in grain amaranth (*Amaranthus cruentus*) as well as residual effects. The treatments comprised of Aleshinloye Grade A (Organo-mineral fertilizer), Aleshinloye Grade B (Un-amended compost), Sunshine Grade A (Organo-mineral fertilizer), Sunshine Grade B (Un-amended compost), NPK and control. The experiment was a completely randomized design (CRD) with four replicates. All the treatments (except the control of no soil additive) were applied at the rate of 90 kg N ha^{-1}. Pre and post cropping analysis of soils used in screen house were done.

The treatments were:

1. Aleshinloye Grade A (compost amended with mineral fertilizer)2. Aleshinloye Grade B (un-amended compost)

3. Sunshine Grade A (compost amended with mineral fertilizer).

4. Sunshine Grade B (un-amended compost).

5. Mineral fertilizer (NPK at rate of 15:15:15).

6. Control.

The results show that the assessed parameters were significantly influenced (P≤0.05) by the applied fertilizer types. Dry shoot weight values were 2.3 and 2.1g, respectively, with Sunshine Grade A and Aleshinloye Grade A and these were significantly higher than that of NPK treatment after the first cropping. Residual effect of Amaranth fresh shoot weight values obtained from Sunshine Grade A and Aleshinloye Grade A were also significantly higher than that of the NPK treatment. Sunshine Grade A and Aleshinloye Grade A had a significant and additive effect on soil nutrients after harvesting of *Amaranthus cruentus* when compared with NPK in the first and second cropping. Thus organic fertilizers fortified with mineral fertilizer have great potential in the production of amaranth and could also be used effectively in increasing soil fertility for amaranth production (Olowoake, 2014**)**.

4-4-Effect of Organic and Inorganic Fertilizer on Growth and Yield of Amaranthus Caudatus L. in Northern Guinea Savanna of Nigeria

Two field trials were conducted during the wet seasons of 2009 and 2010 in the northern Guinea savanna ecological zone to study the effect of organic and inorganic fertilizer on the growth and edible yield of *Amaranthus caudatus L.* The treatments consisted of three levels of farmyard manure organic fertilizer (FYM) 0, 5 and 10 t ha-1 and four levels of inorganic fertilizer (Compound fertilizer NPK 20:10:10) 0, 150, 300 and 450 Kg ha^{-1} arranged in a randomized complete block design. Most of the crop parameters were maximized with the application of 300 kg NPK ha^{-1}. The regression of edible yield per hectare to NPK level indicates a strong linear response up to 450kg NPK ha^{-1} and the difference between this rate and 30 kg ha^{-1} was not significant. The rate of 5 t ha^{-1} FYM also significantly increased all the growth attributes. No significant interaction was observed between NPK and FYM, on almost all the characters accessed. Application of 300 kg ha^{-1} NPK and 5 t ha^{-1} FYM gave the best edible yield of vegetable amaranth (Joseph et al., 2012) (see Table 10).

5- Foliar application of selenium and humic acid changes yield, essential oil, and chemical composition of Plectranthus amboinicus (Lour.) plant and its antimicrobial effects

Plectranthus amboinicus is an indigenous vegetable that can be freshly eaten. This plant is used for medicine to cure common illnesses such as cough, stomachache, headache, and skin infection. This study was conducted to study the effect of both selenium (2, 4, 8, 12, and 16 g/ l) and humic acid (1.5 and 3.00 g/ l), in addition to control, which was sprayed with water. Generally, mass production of P. amboinicus (Lour.) plants has significantly increased as a result of application of different levels of selenium and humic acid treatments, compared with the control treatment. Essential oil percentage and yield (ml/plant) increased significantly as a result of selenium and humic acid treatments compared with control (S0H0). For essential oil constituents, the results clear that carvacrol (5.96–15.45%) is the first main compound followed by γ-Terpinene (6.74–11.80 %). The third main component is Limonene (3.23–11.32%), whereas the fourth one is α-Muurolene. Moreover, these treatments had a positive effect on selenium, total carbohydrates, photosynthetic pigments, and total phenolic content. Based on scavenging the stable ATBS [2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] radical, all treatments increased significantly inhibition % especially S4H2 compared with untreated plants. Antibacterial and antifungal activities of *P. amboinicus* were studied. The results indicated that *P. amboinicus* had a great antifungal and antibacterial effect (El-Gohary et al., 2020) (see Table 11).

6- Effect of Rock Phosphate vs. Biofertilizer on Growth, Yield, and Essential Oil Content of Mentha longifolia subsp schimperi Grey

This study was conducted during two successive seasons 2012 and 2013 to determine the influence of phosphate fertilizer namely rock phosphate at the rates of 150, 300, 450 and 600 Kg/ Fed, bio-fertilizer (*Azotobacter chroococcun and Bacillus megaterium var. phosphaticum*) and their interaction on growth characters, essential oil content and essential oil composition (EL Gohary et al., 2013). The obtained data cleared that plant height, branches number/ plant, herb fresh weight (g/ plant), essential oil percentage and essential oil yield (ml/ plant) were augmented due to phosphate fertilizer treatment. The highest values of these previous parameters were observed when receiving the plants rock phosphate at the highest rate.

In regard to bio-fertilizer treatment, this treatment led to increase the growth and essential oil. The best results in terms of these characters were obtained as a result of rock phosphate at 600 Kg / Fed + bio-fertilizer. The main essential oil constituent were carvon, menthone and 1, 8-cineol. All fertilizers treatments had a pronounced effect on essential oil composition.

7- Comparative Effect of Organic Fertilizers on Growth and Chemical Constituents of Plantago Ovata Plant

This study was conducted in Experimental Farm of National Research Centre, during two successive seasons to evaluate the influence of different kinds and levels of organic fertilization (compost, compost tea and humic acid) on the growth, production and chemical constituents of *Plantago Ovata* plant (Hussein et al., 2012). Generally, all fertilizer treatments produced a pronounce increment in all growth parameters of *Plantago ovate.* The maximum mean values of fixed oil content (%) was recorded as a result of foliar application with humic acid at level of 0.06%. The superior stimulation for mucilage accumulation was observed with the highest level of compost tea followed with the highest humic acid level. The uptake of the nutrients N, P, K, Fe, Zn and Mn, in general, was significantly increased in response to supplying various sources of organic fertilizers. The highest mean value of total carbohydrate content (%) was observed with the highest humic acid level.

In conclusion, the highest level of humic acid and compost tea caused the maximum values of soluble and non-soluble sugars.

8- Influence of fertilization on growth, yield and chemical constituents of Lallemantia iberica plant

This investigation was carried out to study the influence of fertilizers on growth, yield and chemical constituents of *Lallemantia iberica* (Bieb) (El-Sherbeny et al., 2015). The field experiments were carried out during two successive seasons 2010/2011 and 2011/2012 at Sekem farm (50 km from Cairo North East Governorate, Egypt) to study the effect of mineral nitrogen fertilizer (140 and 200kg N/ ha) or compost as organic fertilizer (12 and 18 tons/ha) alone or in combination with bio fertilizer (rhizobacterin). Growth characters, nutrients content, total carbohydrates (%), mucilage (%), fixed and essential oil were estimated.

The data revealed that various fertilizer levels improved the different growth characteristics as well as the amount of studied chemical constituent's content. The highest values regarding plant height, number of branches, total fresh and dry weights, nutrients content, as well as total carbohydrates and mucilage were recorded as a result of application of combined fertilizer of compost at 7.5 Ton + 90 Kg N/ Fed and rhizobacterin. The accumulation of fixed and essential oils were also promoted by the various fertilizer treatments.

9- Effect of Foliar Organic Fertilization on the Growth, Yield and Oil Content of Mentha piperita var. citrata

This study was carried out to evaluate the response of *Mentha piperita var. citrata* (Eau de Cologne mint) to foliar fertilization under Egyptian conditions. *Mentha piperita var. citrata* was cultivated at Sekem Experimental farm (Hendawy et al., 2015). Fresh, healthy, insect and disease free suckers were transplanted in furrow at a depth of 4-5 cm as per the treatments. Three weeks later after transplanting, the plants were sprayed with aqueous solution of the test nutrient compounds humic acid (0. 2.5 and 5 g/L) and amino spot (0, 1 and 1.5 mL/L). The crop was harvested in mid-May (First cutting) and mid-August (Second cut). Growth and yield characters were measured. The essential oil percentage was determined in both cuts from fresh herb and was analyzed by GC/Mass. It was evident from results that, humic acid and/or amino spot fertilizer (Algae extract) had a significant effect on growth characters during both cuts. Increasing amino spot doses increased growth characters (plant height, herb fresh and dry weight) at all doses in the two cuts. The interaction effect was significant in both cuts, the highest values of plant height, herb fresh and dry weight (g/plant) were produced from the treatment sprayed with humic acid at 5 g/L+amino spot at 1.5 mL/L.

The second effective level was the treatment sprayed with 2.5 g/ L humic acid+1.5 mL/ L amino spot at the two cuts. During the 1st cut, humic acid or amino spot fertilizer had a significant effect on essential oil percentage and yield (mL/plant). All treatments produced significant effect on oil percentage and oil yield (mL/plant) except the interaction treatments, which had no significant effect on essential oil percentage during second cut. Linalool and linalyl acetate were the main constituents of essential oil of this plant. All treatments or cuttings had a pronounced effect on essential oil constituents. Based on the experimental results it is recommended to treat *Mentha piperita* var. citrata (Eau de Cologne mint) plants with humic acid at 5 g/L+ amino spot at 1.5 mL/L to produce high mass production and oil yield.

Table 8. Effect of bio-stimulant and/or fertilizer treatments on root development and yield of *Amaranthus*. The presented data are means. Means having the same letter within a column are not significantly different (P=0.05) according to DMRT.NK: N and K fertilizer.

Treatments	Root length (cm)	Root fineness (cm/ g)	Fresh root Weight (g)	Dry root weight (g)	Fresh shoot yield (g)	
					1st cycle	Regeneration
Control	21.6[c]	0.53[d]	27.7[c]	5.43[d]	355[d]	64[d]
Bio-stimulant (BS)	32.1[ab]	1.19[ab]	35.0[b]	11.07[c]	463[cd]	95[c]
Fertilizer (Fert)	33.3[a]	1.07[b]	46.7[a]	18.79[a]	780[b]	260[ab]
BS + ½ Fert	31.6[ab]	0.96[bc]	36.0[b]	11.86[c]	798[b]	288[a]
½ BS + Fert	33.9[a]	0.97[bc]	46.7[a]	15.49[b]	650[c]	153[bc]
½ BS + ½ Fert	32.5[ab]	1.76[a]	36.3[b]	18.18[a]	711[bc]	300[a]
BS + Fert	34.9[a]	0.85[c]	43.0[ab]	15.99[b]	899[a]	158[bc]
BS+NK	28.8[b]	1.03[b]	32.3[bc]	17.05[ab]	845[a]	208[b]

Table 9. Effect of inorganic fertilizer and manure on yield and harvest index of grain amaranth in Western Kenya in 2008 and 2009 growing seasons. *Legend:* NS: Non significant.

Treatments		2008 season			2009 season		
		Grain yield (t/ ha)	Dry matter yield (t/ ha)	Harvest index	Grain yield (t/ ha)	Dry matter yield (t/ ha)	Harvest index
Inorganic fertilizer	0 kg N/ha	0.29	0.74	0.28	0.23	0.65	0.26
	30 kg N/ha	0.90	2.31	0.28	0.76	2.10	0.27
	60 kg N/ha	1.55	3.61	0.30	1.47	3.71	0.28
	100 kg N/ha	2.10	5.14	0.29	1.94	4.98	0.28
LSD (5%)		0.573	1.220	NS	0.347	1.012	0.059
Manure	0 t/ha	0.01	0.024	0.29	0.01	0.025	0.29
	0.5 t/ha	0.05	0.120	0.29	0.05	0.122	0.29
	1 t/ha	0.11	0.280	0.28	0.13	0.300	0.30
	2 t/ha	0.25	0.560	0.31	0.39	0.890	0.30
	3 t/ha	0.67	1.560	0.30	0.79	1.810	0.30
LSD (5%)		0.093	0.179	0.051	0.210	0.531	NS

Table 10. Effect of mineral and organic fertilizers on yield of *Amaranthus Caudatus L.*

Treatments	Shoot dry wt g/plant			Days to 50% anthesis	Edible yield kg ha^{-1}
	20	30	40		
NPK-Fertilizer, Kg ha^{-1}					
0	2.4b	5.8b	10.5c	40.3	37c
150	3.2ab	9.2b	17.1b	38.3	59b
300	3.6a	9.9b	22.2a	39.3	78a
450	3.1ab	15.3a	19.8ab	39.3	86a
±SE	0.33	1.77	1.47	0.91	4.33
FYM t ha^{-1}					
0	2.6b	6.5b	12.2c	41.0a	49b
5	2.9ab	10.4ab	17.9b	38.8a	69a
10	3.7a	13.6a	22.2a	38.2b	78a
±SE	0.28	1.53	1.27	0.79	3.76
Interaction					
N×F	NS	NS	NS		NS

Table 11. Herb fresh and dry weights of *Plectranthus amboinicus* (Lour.) at different levels of selenium and humic acid (mean values of two successive seasons). *Legend:* CV%, coefficient of variation %. Means with the same letters in each column indicate no significant difference between treatments at 5% level of probability.

Treatments	First cut		Second cut		Total herb weight	
	Herb fresh weight (g/ plant)	Herb dry weight (g/ plant)	Herb fresh weight (g/ plant)	Herb dry weight (g/ plant)	Fresh weight (g/ plant)	Dry weight (g/ plant)
S0H0	553.00^{f}	55.54^{d}	514.68^{h}	54.04^{h}	1067.68^{j}	109.58^{h}
S1H1	709.25^{e}	72.34^{c}	612.05^{g}	62.43^{g}	1321.3^{i}	134.77^{g}
S1H2	793.55^{c}	79.67^{b}	712.33^{d}	80.49^{c}	1505.88^{f}	160.16^{d}
S2H1	784.40^{d}	78.44^{b}	638.63^{f}	63.86^{g}	1423.03^{h}	142.3^{f}
S2H2	796.80^{c}	79.68^{b}	733.08^{c}	73.31^{e}	1529.88^{e}	152.99^{e}
S3H1	797.70^{c}	81.98^{b}	679.75^{e}	69.86^{f}	1477.45^{g}	151.51^{e}
S3H2	826.50^{a}	87.32^{a}	790.00^{b}	83.97^{a}	1616.5^{b}	176.29^{a}
S4H1	822.00^{b}	87.95^{a}	719.20^{d}	76.95^{d}	1541.2^{d}	164.90^{c}
S4H2	829.40^{a}	88.05^{a}	803.00^{a}	88.33^{a}	1632.4^{a}	176.38^{a}
S5H1	821.80^{b}	89.24^{a}	738.00^{c}	81.18^{c}	1559.8^{c}	170.42^{b}
S5H2	818.75^{b}	88.98^{a}	809.00^{a}	84.91^{b}	1627.75^{a}	173.88^{b}
CV%	0.268	3.127	0.978	2.487	0.44	1.61

Summary

- Medicinal and aromatic plants continue to be the subject of novel and straight forward applications as a source of active constituents for pharmaceutical industries.
- Fertilizers are an important factor in modern-day agriculture as they increase crops yield and allow crops to be planted in nutrient deficient soils. Although chemical fertilizers are important tool for higher production of different crops, the extensive

and intensive use of them resulted in accumulation of chemicals.

• Some organic fertilizers as Farmyard manure (FYM) and compost at different rates and different combination could be used instead of chemical fertilizers to meet the demand of plants from NPK and some micronutrients to improve the quantity and quality of medicinal plants.

• The present review introduces some researches performed to elaborate the benefits of organic cultivation of some important medicinal and aromatic plants such as *Coleus forskohlii, Satureja hortensis L., Amaranth* species, etc.

Conclusions

• The review of literature has clearly revealed that medicinal and aromatic crops respond positively to organic cultivation practices.

• It is possible to move in the direction of integrated nutrient and pest management practices from the current chemical cultivation techniques.

• Medicinal and aromatic plants can be shift totally to organic farming in a phased manner to keep plants free from any chemical residues and save our ecosystem.

• However, lots of research work needs to be carried out for this transformation to take place and to scientifically validate some of the current practices.

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Synopsis

Carbon Sequestration – a Research Subject of a Present Importance

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Abstract. The current study investigates published data, concerning carbon sequestration on a global scale. The investigation is based on the use of the search engine of Nature Research Journal in order to acquire information about the studies, concerning carbon sequestration – the latter words were used as key words. Two main periods are examined, covering the years 1845-1999 and 2000-2019. During the first period - 1845-1999 29 manuscripts, regarding carbon sequestration were published. The second period saw an unprecedented boom when a total count of 513 results came into being. Some of the most important among them are a part of several scientific journals, including *Scientific Reports*, *Nature Communications*, *Nature Journal*, *Nature Sustainability* and *Nature Climate Change* with *Nature Journal* having an impact factor of *43.070* in 2018. This can serve as a proof of the quality of the scientific research. The results also show a division by country for several of these scientific works. The author stress on the need of such an overview study in order to reveal the present day importance of this subject.

Key words: scientific articles, Nature Research Journal, impact factor.

Introduction

Carbon sequestration is among the most discussed subjects in present day scientific investigations. Many consider this process as being vital for climate change mitigation. In its essence it represents a long-term capture and storage of atmospheric carbon dioxide in plants, soils, ocean, etc. via a series of chemical, biological and physical processes. Then carbon becomes part of stocks that are keeping it away from the atmosphere, thus weakening its influence on any planetary warming processes. Carbon dioxide is regarded as one of the most potent greenhouse gases, therefore its removal from the atmosphere adds weight to climate regulation. Earth's systems may be considered as carbon sources and carbon sinks. Anthropogenic activities lead to the release of carbon by burning of fossil fuels (coal, petroleum, natural gas), for example. Another part of the carbon cycle is its release via decomposition of biogenic material. Among the most prominent carbon sinks are forests, the edaphic sphere and major water bodies. Terms, such as, blue carbon, green carbon and black carbon are gaining more popularity throughout the society and are linked with carbon sequestration. Blue carbon refers to that part of carbon, mainly captured by mangroves, salt marshes and sea grasses, thus it is the carbon of ocean ecosystems. Green carbon is captured by vegetation and soil of natural ecosystems, while black carbon is regarded as a particulate matter component, forming through the combustion of fossil fuels and biomass.

Scholars are becoming more and more aware of the importance of carbon sequestration and this is proved by the increase of scientific studies, focused on this process. Major effort is being put on studying carbon sequestration from a global point of view and there are several examples of such studies. Freibauer et al. (2004) studied agricultural soils. Scharlemann et al. (2014) investigated the terrestrial carbon pool and Achat et al. (2015) carried a global forest analysis, aimed at carbon dynamics. Schuur et al. (2015) focused their study on climate change. Corbeels et al. (2016) investigated limited carbon contents. Zomer et al. (2017) conducted a research on global sequestration potential of soils in agricultural territories and it is among the most significant investigations on this subject up-to-date. Piñeiro et al. (2017) focused their efforts on conducting a world assessment about fine root biomass and soil carbon levels. Gosling et al. (2017), Hagemann et al. (2017), Kroeger et al (2017), Macreadie et al. (2017), Sanderman et al. (2017) and Tang et al. (2017) provided more insight about carbon storages. Bulgarian scientists also acknowledged carbon sequestration importance. In 2017 Zhiyanski et al. (2017) and Bratanova-Doncheva et al. (2017) worked on a methodology for assessment and mapping of ecosystems. Yaneva et al. (2018) assessed and mapped areas in Central Balkan National Park, focusing on carbon sequestration in the context of ecosystem services. At the same year Di et al. (2018), Espenberg et al. (2018), Hodgkins et al. (2018) and Leifeld & Menichetti (2018) provided additional data regarding carbon sequestration. Adamczyk et al. (2019) and Bhardwaj et al. (2019) also provided manuscripts, based on organic carbon. The research papers of Iizumi & Wagai (2019), Kravchenko et al. (2019), Ogle et al. (2019) and Sayer et al. (2019) added even more data about carbon pools.

The current paper presents an informative insight of the scientific studies, focused on carbon sequestration, allowing for making assumptions. It represents an attempt of an overview study about the significance of this matter in the scientific world.

Materials and methods

An attempt was made for summarization of the number of studies, connected to carbon storage investigation. A specific search engine was used in order to fulfill the main aim. The authors acknowledge it as being one of the most up-to-date engines, providing an insight about that specific issue. It is also clear that it cannot provide the fullest list of articles, based on carbon sequestration. However, the engine gives an opportunity to search for scientific journals with a very high impact factor, thus it can be regarded as thorough enough. The carried search aimed at publications that are part of Nature Research Journal. It was based on the two key words - "carbon sequestration" (www.nature.com/search?q=carbon+sequestration). Generally, the search refinement allows for "article type choice", "journal" and "date choice". Database searches were performed in English. The first option differentiates research, reviews, news and views, comments and opinion, correspondence, special features, books and arts, etc. The current investigation focuses only on those studies that are a part of the "research" section. The advanced journal search gives an option to choose between several major journals and the date search allows for a search by years. The advanced search provides opportunity for a search by "authors", "terms" or "title" and exactly here "carbon sequestration" was applied. The current investigation covers two major periods: from 1845 to 1999 and from 2000 to 2019. The start of the new millennium marks the beginning of the new investigated period. It was chosen because it serves the role of a major baseline in chronological terms. Moreover, it marks a beginning of the acknowledgement of the importance of carbon sequestration as an important climate change mitigation tool. The adoption of the current methodology provided a basis for the revelation of interesting results.

Results and Discussion

The outcomes are summarized in the following lines. The examination of the two periods displays a significant imbalance of the total number of articles. During the first or the old period, spanning from 1845 to 1999, a total count of 29 manuscripts were published in Nature Research Journal and the other scientific journals, included in the Nature Research engine. These scientific papers are issued about carbon sequestration or are at least mentioning it several times. The analysis points out that they are focused on carbon storage as a chemical component in ecosystems, but are not aiming directly at climate change issues and this is rather interesting. It may be said that climate change obsession had not started yet during that period, explaining the lack of such focus in scientific papers.

Since the start of the new millennium a major boom in carbon sequestration articles occurred. Studies, concerning carbon sequestration, emerged with an immense power. A total count of 513 results (397 research manuscripts) were found in the Nature Research engine. They were published from 2000 till the end of 2019 in a wide array of scientific journals. The number of those that were published in *Scientific Reports*, *Nature Communications*, *Nature Journal*, *Nature Sustainability* and *Nature Climate Change* is 315. The total breakdown of the results includes: 67 reviews, 9 news, 11 news and views, 16 comments and opinion, 5 research highlights, 3 correspondences, etc. These numbers are neglected in the current analysis, which is focused on research articles and they serve only as an illustration of the immensity of published research, based on carbon sequestration. Their yearly distribution is displayed on Fig. 2. The aforementioned five scientific journals are well acknowledged in the current study due to their high impact factor ratings. There are 181 research works, published in *Scientific Reports* (impact factor 4.011 for 2018) - an open-access, multidisciplinary journal from Nature Research, accounting for 35% of the research articles for the period. This journal is not taking the first place, regarding impact factor ratings; however the large number of studies published in it speaks for themselves. Research articles in *Nature Communications* (impact factor 11.880 for 2018) were at the count of 69 or 13.5% of all. This journal represents an open access journal that publishes high-quality research from all areas of the natural sciences. It has a higher impact factor rating than the previous one and that may be regarded as a reason for the lower count of published investigations – it is more difficult to earn a place in this scientific platform. Research works in *Nature Sustainability* (with a five-year impact factor of 12.092) were at the count of 19 or almost 3.7 %. Research articles in *Nature Climate Change* (impact factor 19.181 in 2017) - a monthly journal dedicated to publishing the most significant and cutting-edge research on the nature, underlying causes or impacts of global climate change, was accounting for 3.1% of all articles, equaling 16 articles. These two scientific journals are having increased impact factor ratings, which normally leads to a decrease of the published articles. Moreover, the specificity of their scientific area – including climate change processes may have led to a restricted number of published investigations. The weekly international publishing source *Nature Journal* (impact factor 43.070 in 2018) included 30 articles, equaling to 5.8 % of the total count. This result is in conflict with the already observed principle – the higher the impact factor rating, the lower count of published articles. One possible reason is that authors are putting their best efforts to publish in a journal with such a sky high impact factor (Fig. 1).

An interesting discussion topic is the way research articles increase during the second period, peaking in 2019. The number from 2000 to 2019 growed more than 20 times, which is significant enough. Once again significant efforts are put into the revelation of the mechanisms for climate change mitigation.

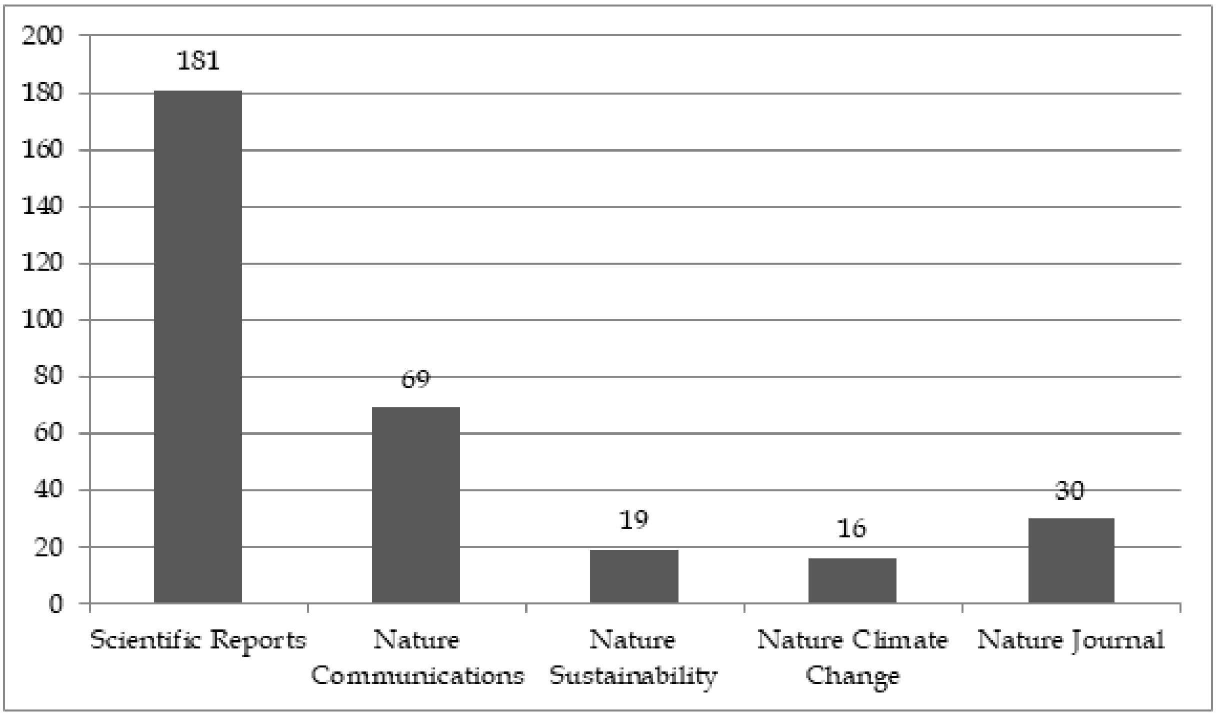

Fig. 1. Count of research articles (2000-2019) in five significant scientific journals.

Research centers are investing more and more finances in this subject and the results of the current study present indisputable proof of this. A large difference is observed from 2013 till 2019. The exponential growth is showing almost seven times more research in the end of the period. The explanation for this at least, regarding EU territory, may be hidden in the document, regarding the adoption of EU Strategy on adaptation to climate change, discussed in Brussels at 16.4.2013. Along with the expectation of the adoption in 2014 of the 5th Assessment Report of the IPCC, this document acknowledges the urgent need for quick measures, regarding climate change adaptation. Moreover, the Multiannual Financial Framework (MFF) draft 2014-2020 discusses a proposed raise of expenditure, regarding climate issues to be at least 20% of the EU budget. Obviously, the Commission started to acknowledge the importance of taking climate change measures by proposing relevant finance initiatives.

An interesting outcome of the investigation focuses on the distribution of research articles by countries. Only data, concerning soil investigation, will be discussed as soils are representing the largest terrestrial reservoir of organic carbon. They also possess the ability to act as a carbon pool, playing a central role in the mitigation of climate change. The information is summarized in Fig. 2 where several countries stand out. Along with the incontestable leader – China, the other leading countries here include the USA, Australia, Brazil and the UK. They may have been chosen by research teams, as their soils are representing a significant unit in carbon sequestration around the world. Moreover, scientific teams aimed at placing them on the global carbon sequestration map. Climate change adaptation and mitigation measures are adopted in each of the aforementioned countries, resulting in a rise of scientific research. An essential programme that deserves attention is the one, implemented by the Chinese government. In 1999 officials adopted the so-called "Grain for Green" (GFG) programme in order to battle over-cultivation, erosion and deforestation. This programme allowed for immense financial injections, including those for research studies, regarding this issue. The results of these efforts are apparent, as it can be seen from the provided data in Fig. 2.

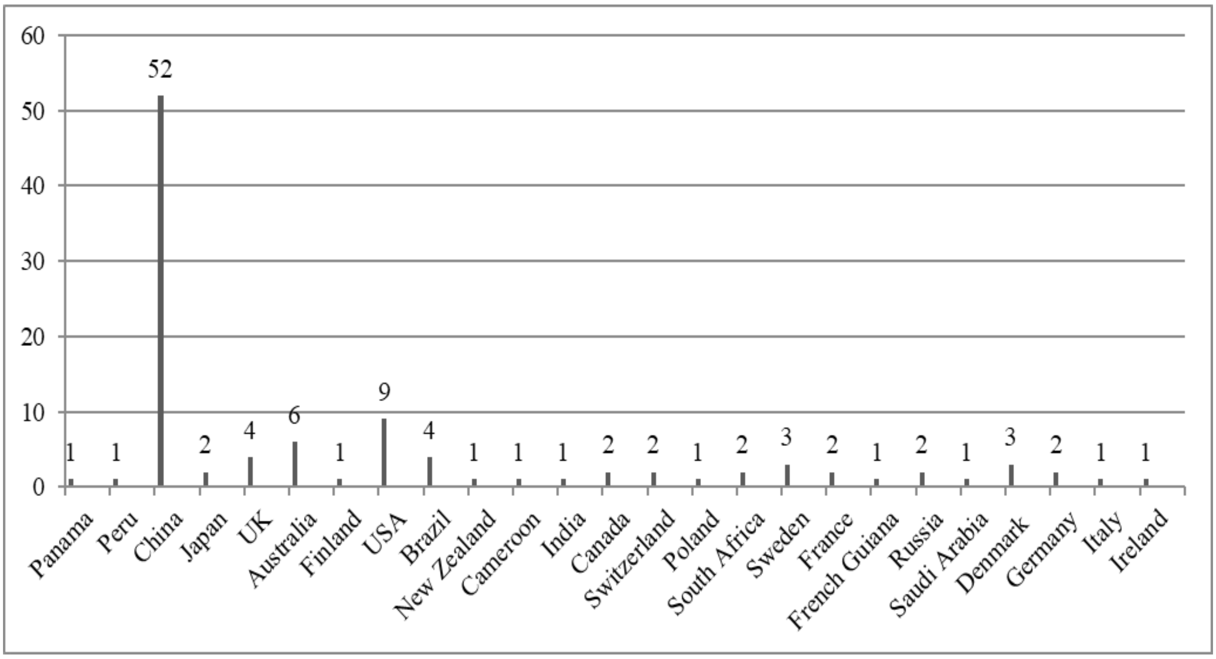

Fig. 2. Distribution of the conducted research by countries.

Conclusions

The current study was focused on carbon sequestration as one of the most discussed processes by scientists in recent years. It plays a major role for climate change mitigation and its significance is being more and more acknowledged throughout the scientific world. The results of the present study show a boom of published research. A special focus is put on five major scientific journals (*Scientific Reports*, *Nature Communications*, *Nature Journal*, *Nature Sustainability* and *Nature Climate Change*), because of their wide recognition among scientists. It can be concluded that the impact factor plays an essential role when it comes to addressing your article. Generally speaking, the higher the impact factor, the lower the number of published manuscripts. However, when it comes to N*ature Journal*, there is a conflict with the established principle. This may be explained by the efforts and desire of the authors to publish their data in the highest ranked journal.

When it comes to research articles in general, there is a high increase, especially after 2013. This is a proof for the importance that scientists are rendering to carbon sequestration investigations. It also became clear that when the investigation of carbon sequestration becomes a major part of countries' programmes then research is steadily increasing. When it comes to a differentiation by countries, then China and the USA are the leaders in scientific studies.

The authors of the present investigation are stressing on its successful outcomes and evaluate it as being thorough enough. Yet, efforts should be put at the investigation of more scientific journals' data, regarding carbon sequestration in order to increase the comprehensiveness of such overview studies.

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ECOLOGIA BALKANICA - INSTRUCTIONS FOR AUTHORS (2020)

General information

Submissions to "*Ecologia Balkanica*" can be original studies dealing with all fields of ecology, including ecology and conservation of microorganisms, plants, animals, physiological ecology, behavioral ecology, population ecology, population genetics, community ecology, ecosystem ecology, parasitology, animal evolution, ecotoxicology, ecological monitoring and bioindication, landscape and urban ecology, conservation ecology, as well as new methodical contributions in ecology. **The journal is dedicated to publish studies conducted on the Balkans and Europe.** Studies conducted anywhere else in the world maybe accepted only as an exception after decision of the Editorial Board and the Editor-In-Chief. ***The Editorial Board of "Ecologia Balkanica" reserves its right to reject publication of any manuscript which does not fit the aim and scope or does not comply with these instructions.***

Manuscript submission

The following types of manuscripts are accepted: *short research notes* (up to 4 pages), *research articles* (4 to 10 pages) and *review papers* (10 to 20 pages). *Short research notes* are shorter submissions of a preliminary nature or those including new records or observed phenomenon, etc. *Research articles* should present significant original research in the various fields of ecology, mentioned above. *Review papers* should deal with topics of general interest or of contemporary importance, being synthetic rather than comprehensive in emphasis. Authors of review papers should consult with the Editor before submission. The Editor may also invite review articles concerning recent developments in particular areas of interest. The Editor reserves the right to decide if a manuscript should be treated as a short note or research article. In general, studies that are purely descriptive, mathematical, documentary, and/or natural history will not be considered for publication.

Manuscripts must conform strictly with the instructions for authors and sent to the Editor. **All manuscripts must be accompanied with a cover letter, signed by ALL authors,** which can be downloaded from here. All fields from the cover letter form must be filled out and the cover letter must be sent along with the full text of the manuscript to the journal's e-mail. Incoming manuscripts are initially judged by the Editor. *Manuscripts may be rejected without peer review if they do not comply with the instructions to authors or are beyond the scope of the journal.* If the manuscript is acceptable, it will be forwarded to referees for evaluation. All manuscripts are peer-reviewed by 2 or 3 independent reviewers. After final edition and approval by the Editorial Board, the manuscript will be accepted for publication. The Editor reserves the right to make editorial changes. The authors agree, after the manuscript's acceptance, with the transfer of copyright to the publisher.

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Language
The manuscripts must be written in English. *Contributors who are not native English speakers are strongly advised to ensure that a colleague fluent in the English language*, if none of the authors is so, *has reviewed their manuscript.* Spelling should be British or American English and should be consistent throughout the text. All abbreviations and acronyms should be defined at first mention. To facilitate reader comprehension, abbreviations should be used sparingly.

Technical information
Submissions must be in **electronic version only**, as well as the original figures and tables, implemented in the text. Figures must be sent as separate files as well (see more information below). The manuscript text should be **prepared in rich text format (.rtf)**, justified, font size 11, font "Book Antiqua", without footnotes, column or page breaks, single spaced (about 60 lines per page), on A4 (210 x 297 mm) paper, with margins of exactly 2.5 cm on each side. Pages and lines should not be numbered.

The manuscripts should conform to the following format:
Title: Provide a title that is concise, but also an informative synthesis of the study. Where appropriate, include family or higher taxon.
Author(s): Full first name(s), middle initials and surname(s) in ***bold italic.*** The corresponding author should be marked with the *-symbol.
Affiliation(s) with postal address: As complete as possible. Affiliation should be given in the following order – University (Institute), Faculty, Department, postal address, city, COUNTRY (in capital letters). E-mail address is given only for the corresponding author!
Abstract: Maximum length 250 words. The abstract should state briefly the objective of the research, the primary results and major conclusions, with no description of methods, discussions, references and abbreviations.
Key words: Usually 3–10 words suitable for information-retrieval system.

The standard order of sections should be: Abstract, Key words, Introduction, Material and Methods, Results, Discussion (or Results and Discussion), Conclusions (optional), Acknowledgements (optional) and References.

The *Introduction* has to explain the actuality of the researched problem and give the aim of the study.
Materials and Methods have to provide sufficient information to permit repetition of the experiment and/or fieldwork. The technical description of study methods should be given only if such methods are new; otherwise a short presentation is enough.
The *Results* section must be a concise presentation of the finding of the study. **Avoid presentation of the same information as text and/or figure and/or table!**
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The *Conclusions* should shortly describe the main contributions and recommendations of the study without including citations and statistics.
In the *Acknowledgements* section all persons and organizations that helped during the study in various ways, as well as the organization that financed the study must be listed.

Short Notes (generally less than four-five manuscript pages) should be produced as continuous text, preceded by an abstract of no more than 150 words.

Tables: The tables must not repeat information already presented in the figures or in the text. Each table must be self-explanatory and as simple as possible. Avoid large landscape oriented tables! Tables must be numbered consecutively. **They should be placed within the text at the desired position by the author(s).** An explanatory caption, located on the top of the table, should be provided.

Example:

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Example:

Fig. 1. Indicative map of the study area.

All tables and figures must be referred to in the text!

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