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Този сборник съдържа доклади, изнесени на „Семинар по Екология - 2016“, с международно участие, проведен на 21-22 април 2016 г. в Институт по биоразнообразие и екосистемни изследвания – БАН, гр. София, България. Част от докладите са публикувани в пълен текст, а други като кратки съобщения. Семинарът е организиран от секция „Биология“ към СУБ и Институт по биоразнообразие и екосистемни изследвания – БАН, гр. София, България и с любезната финансова подкрепа на фирмите БУЛГАП ЕООД и Л.К.Б - България ЕООД. Публикуваните в „Сборник по Екология - 2016” материали са рецензирани и редактирани.

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All manuscripts have been reviewed and edited.

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The editors

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До

Организационния комитет

на "Семинар по екология - 2016"

Уважаема Проф. СТЕФКА ЧАНКОВА

Уважаема Доц. АННА ГАНЕВА

Уважаеми участници в Научния "Семинар по екология - 2016",

Скъпи гости, дами и господа,

Семинарът по екология се утвърди през годините като привлекателен научен форум, предоставящ възможности за изява и представяне на научни резултати в областта на управлението и устойчивото ползване на природните ресурси, на екологията и опазване на природната среда, на биоразнообразието и консервационната биология .

В резултат на редица обстоятелства, като напр. изменението на климата, социални и икономически фактори, демографските промени, екологията се превръща в много важен сектор на научното познание за изследване на околната среда и човешкото влияние. С натрупването на все повече научна информация за глобалните процеси в земната биосфера и изменението на климата, те се очертават като може би най-голямото предизвикателство на 21 век. Нещо повече, замърсяването на въздуха, деградацията на почвата, опустиняването и обезлесяването са неразривно преплетени. Те очертават необходимостта от намиране на решение за цялостен подход за преодоляване на глобалните климатични промени и редуцирането на вредните последствия от човешката дейност.

Семинарът по екология беше анонсиран за първи път през 2007 год. От самото начало интересът към научната проява бе много голям, като

броят на участниците с всяка изминала година нараства. В заседанията участват вече и чуждестранни учени от Македония, Германия, Русия, Франция. Семинарът даде възможност за изява на видни учени, но също така позволи на младите участници – докторанти и студенти да обменят опит и знания с утвърдените научни капацитети в различни области на екологията и биологията.

В заключение искам да посоча, че Институтът по биоразнообразие и екосистемни изследвания (ИБЕИ) при БАН и Секцията "Биология" към Съюза на учените в България (СУБ), в качеството си на организатори на този форум, се явяват същевременно и гаранти за неговото високо научно ниво.

Пожелавам на участниците и организаторите на Семинара успешна и ползотворна работа и много творчески успехи.

С уважение:

София /проф. дбн Димитър Иванов,

21.04.2016 г. Научен секретар, БАН/

СЪЮЗ НА УЧЕНИТЕ В БЪЛГАРИЯ
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ДО УЧАСТНИЦИТЕ В
НАУЧНИЯ „СЕМИНАР ПО
ЕКОЛОГИЯ-2016“ С
МЕЖДУНАРОДНО УЧАСТИЕ

Уважаеми колеги,

Бих искала да поднеса приветствие на участниците в станалия вече традиционен ежегоден Семинар по екология от името на Управителния съвет на Съюза на учените и Секция Биохимия, биофизика и молекулярна биология и лично от мое име.

Проблемите на околната среда са свързани не само с опазване на редките видове на флората и фауната на нашата страна, но и с осигуряване на здравословен и качествен начин на живот на човека. Научните изследвания свързани с причините за замърсяване на биосферата са от голямо значение за създаване на държавни регламенти за опазване на околната среда. Изясняването на механизмите на въздействие на редица вредни за околната среда химични, физични и антропологични фактори върху растителните и животински видове биха довели до създаване на нови подходи за съхранение на значими за човека видове с цел получаване на екологично чисти храни и нови фармацевтични продукти, тъй като екологичната наука отдавна е престанала да бъде самоцелна.

Този семинар е много полезен за младите учени, тъй като те придобиват умения в представяне и оформяне на получените от тях

резултати. Предоставя възможност за обмяна на резултати, както и ползотворна дискусия за изясняване на причините за наблюдаваните промени.

Пожелавам успешна и ползотворна работа и бъдещи успехи на всички участници на настоящия семинар.

София, 21 април 2016 г.

Проф. Диана Петкова
Член на Управителния съвет на СУБ и
Председател на Секция Биохимия, биофизика и молекулярна биология

БЪЛГАРСКА АКАДЕМИЯ НА НАУКИТЕ
ИНСТИТУТ ПО БИОРАЗНООБРАЗИЕ И ЕКОСИСТЕМНИ ИЗСЛЕДВАНИЯ
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21 април 2016 г, София

До Организационния комитет
на Семинар по Екология 2016
с международно участие

Уважаеми организатори, уважаеми колеги и гости!

От името на Ръководството на ИБЕИ Ви приветствам с добре дошли на поредното издание на Семинара по екология с международно участие, организиран от Секция "Биология" към Съюза на учените в България и Институт по биоразнообразие и екосистемни изследвания при БАН, утвърдил се вече като традиционно място за среща на специалисти, провеждащи екологични изследвания от различни научни институции в София, страната и чужбина.

През 2016 година в семинара отново включва голям брой участници. Разнообразните им по тематика доклади са организирани в 6 тематични сесии с устни доклади и две постерни сесии, посветени съответно на биоразнообразие и консервационна биология; ландшафтна екология; екосистемни изследвания, в това число екосистемни услуги; влияние на абиотичните и биотични фактори върху живата природа и механизми на адаптация на живите огранизми към това влияние; екология и образование. Няма съмнение, че и тази година на семинара отново ще бъдат представени нови научни постижения, ще възникнат нови творчески контакти и идеи за нови съвместни разработки.

На добър час с най-искрени пожелания за ползотворна работа и интересни дискусии в през двата работни дни на семинара!

проф. д-р Снежана Грозева,
Научен секретар на ИБЕИ

REPORTS

Topic:
BIODIVERSITY AND CONSERVATION BIOLOGY

CURRENT STATE OF MICROSPORIDIA RESEARCH IN BULGARIA

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Abstract: Microsporidia are primary pathogens related to the Fungi and infect animals from all major taxa. This group, a monophyletic phylum of single-cell organisms, is particularly well known from insects; some species play an important role in the natural regulation of insect populations. Investigations of insect microsporidia in Bulgaria began in the early 1960s; however, systematic research on microsporidian species began in the 1990s in close cooperation with scientists from Germany, Czech Republic, Austria, Slovakia and the USA. Approximately 25 microsporidian species in the genera *Nosema, Vairimorpha, Endoreticulatus, Amblyospora, Janacekia, Polydispyrenia, Thelohania, Bohuslavia* and *Chytridiopsis* have been recovered from the insect orders Diptera, Lepidoptera, Orthoptera and Coleoptera, and two new species were described. Life history, morphology, host tissue specificity, virulence and prevalence of microsporidia from Bulgarian insect populations are reviewed. Special attention is paid to pathogens of the gypsy moth *Lymantria dispar*, one of the most important pests in Bulgarian broadleaf forests.

Keywords: microsporidia, insect pathogens, Bulgaria

Introduction

Microsporidia are single-cell pathogens related to the Fungi. Together with the Aphelida and Cryptomycota, they form a sister group (Opisthosporida) of the true Fungi [1]. Microsporidia infect animals from all major taxa. Insects are the most commonly reported hosts and at least 80 microsporidian genera are known from insects [2]. As primary pathogens, microsporidia often play an important role in the regulation of insect populations. Effects of infection are generally rather chronic, leading to only low or moderate mortality. Microsporidian infection frequently decreases host reproduction and feeding, and epizootics in the host population can reduce populations and, thus, damage to host plants. Therefore, microsporidia may be candidates for use in biological control programs. While few species are sufficiently virulent for use as microbial pesticides, *Paranosema locustae* has long been commercially available for the control of grasshoppers and crickets. In general, though,

microsporidia are probably more suited for use as classical biological control agents that are introduced to augment epizootics or into naïve host populations to initiate establishment.

Review

The first microsporidium reported from insects in Bulgaria, *Nosema lymantriae*, was isolated from the gypsy moth, *Lymantria dispar* by Panayotov et al. [3]. Atanasov [4] reported *Nosema carpocapsae* from the codling moth, *Cydia pomonella*, and other Bulgarian researchers reported *N. lymantriae, Nosema serbica* and *Vairimorpha disparis* from *Lymantria dispar, Nosema carpocapsae* from *Cydia pomonella*, and *Grapholita mollesta* and *Endoreticulatus* sp. from *Lobesia botrana* [5, 6, 7]. Pilarska [8] also reported the occurrence of microsporidia in black flies in Bulgaria.

Systematic research on microsporidian species with emphasis on *Lymantria dispar*, one of the most economically important pests of broadleaf forests in Bulgaria, began in 1990s. An international team including scientists from Bulgaria, Germany, Czech Republic, Austria, Slovakia and the USA aimed to contribute to the knowledge of this relatively little studied taxon by studying life cycles, mechanisms of host infection and transmission, host-pathogen interactions and host specificity of microsporidia. The gypsy moth periodically causes severe defoliation of broadleaf forests, especially oak (*Quercus* spp.), in Bulgaria leading to decrease in diameter growth and physiological weakening of the trees. Gypsy moth populations from different central and eastern European countries were screened for microsporidia and 4 microsporidian species and more than 20 isolates were found in 8 countries. Three species were isolated from gypsy moth in Bulgaria: *Endoreticulatus schubergi, Nosema lymantriae* and *Vairimorpha disparis*.

Vairimorpha disparis [9] was first recovered in 1984 from Rupite in southern Bulgaria. It infects only the fat body tissues of the host. Initially, the isolations were considered to be a mixed infection of two microsporidian species, but later studies showed that it was a dimorphic species in the genus *Vairimorpha*. Detailed studies were conducted on morphology, ultrastructure, host specificity, and impacts on the host. *V. disparis* is virulent, transmitted horizontally and has a narrow host range [10, 11, 12]. No correlation between the prevalence of the pathogen and the density of the host, gypsy moth, was documented. *V. disparis* was persistent even when *L. dispar* densities were low during a 17–year period.

Nosema lymantriae was recovered in 1996 and 1997 in Levishte and Veslec in northern Bulgaria, and in 2009 in Tchekerica and Gorni Domlyan in central Bulgaria. *N. lymantriae* infects the fat body, silk glands, Malpighian tubules and gonads. Our studies showed that this species is transmitted horizontally and vertically [13] and that the pathogen is host density dependent. A narrow host range was also documented for this species [14].

Endoreticulatus schubergi was first detected in 1996 near Asenovgrad [9]. It infects the gut epithelium and is transmitted horizontally; however, laboratory studies indicated that it can also be transmitted to the offspring of infected females on the surface of the eggs [15]. Its prevalence in the host population increases with the age of the host larvae as the season progresses. A negligible impact on the growth, development and food consumption of infected gypsy moth larvae, pupae and adults was documented [16].

Because of their host specificity, *N. lymantriae* and *V. disparis* were chosen to be released in two naïve gypsy moth populations in Bulgaria in 2008. Larvae infected with *N. lymantriae* were released in Karlanovo in southern Bulgaria and larvae infected with *Vairimorpha disparis* were released in Opletnya (North Bulgaria). To confirm the presence of infected larvae in the experimental sites and evaluate other naturally occurring pathogens, treated sites and control sites were monitored in 2008, 2009, 2010 and 2011 by collecting gypsy moth larvae and non-target Lepidoptera.

In 2008, 54.8% of the gypsy moth larvae collected in Karlonovo were infected with *N. lymantriae*. Similarly, 57.1% of the gypsy moth larvae collected in Opletnya were infected with *V. disparis*. In 2009, 8.1% of the GM larvae collected in Karlanovo were infected with *N. lymantriae*. In 2010, *N. lymantriae* was recovered again in 10.3% of the collected larvae and in 2011 in 6.3%. No gypsy moth larvae recovered in 2009 from Opletnya site were infected with *V. disparis*. In 2010 an additional 3,000 larvae infected with *V. disparis* were released in the Opletnya site. In 2010 *V. disparis* was detected in 4.3% of the collected larvae but was not recovered in 2011 [17].

The brown tail moth, *Euproctis chrysorrhoea*, a serious pest of fruit and forest trees, was also surveyed for the presence of microsporidia. Three microsporidian species were recovered – the newly described *Nosema chrysorrhoeae* [18], *Nosema* sp., and *Cystosporegenes* sp. from the Karlovo region in 2011.

Nosema chrysorrhoeae was discovered in 1998–2000 near Karlovo in central Bulgaria. It infects only the silk glands of the host and is horizontally transmitted.

Nosema sp. was also recovered in 1998–2000 near Dolni Lozen, Sofia region, but always in mixed infections with *Cystosporegenes*. It infects the silk glands and fat body tissues of the host and prevalence ranged between 25-36% [19].

Investigations of microsporidia parasitizing bark beetles, serious pests of spruce and pine trees in Bulgaria, began in 2004. More than 30 bark beetle species were surveyed and three microsporidian species were isolated: *Chytridiopsis typographi*, *Chytridiopsis* spp. and *Nosema* spp. *Chytridiopsis typographi* was isolated from *Ips typographus* and *Chytridiopsis* spp. were also recovered from *Ips acuminatus, I. amitnus, Orthotomicus proximus, O.longicollis,* and *Cryphalus saltuatius*. *Nosema* sp. was isolated from *Hylurgus ligniperda* [20, 21].

Grasshoppers were surveyed for microsporidian pathogens and another new species of *Endoreticulatus, Endoreticulatus poecilimoni*, was described from the grasshopper *Poecilimon thoracicus* collected in northwestern Bulgaria [22]. It infects only the midgut tissues of its host, and despite high infection prevalence and intensity, high density populations of infected *P. thoracicus* persisted over a 3-year period.

Conclusion

Since research on microsporidia has begun in Bulgaria, 24 microsporidian species of the genera *Nosema, Vairimorpha, Endoreticulatus, Amblyospora, Janacekia, Polydispyrenia, Thelohania, Bohuslavia* and *Chytridiopsis* have been recovered. These pathogens parasitize all the insect orders studied, including Diptera, Lepidoptera, Orthoptera and Coleoptera. Life

history, morphology, host and tissue specificity, virulence and prevalence data of these pathogens in Bulgarian insect population were produced. Research continues and microsporidian species were recently isolated from additional species of Lepidoptera in the Geometridae, Tortricidae, Erebidae. Investigations of insect pathogens, including those producing chronic infections, are important for understanding the population dynamics of insects.

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POPULATION DYNAMICS OF GOLDEN JACKAL (*CANIS AUREUS* L., 1758) AND RED FOX (*VULPES VULPES* L., 1758) IN BULGARIA DURING THE PERIOD OF 2000-2015

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Abstract

The main aim of the present study is to investigate the impact of the expanding population of jackal (*Canis aureus*) on the population number of the red fox (*Vulpes vulpes*).

Materials and Methods: The population dynamics of both species was tracked using the available data from the official results of spring game counts, carried out by the Executive Agency for Forests (EAF) throughout the country, during the period of 2000-2015.

Results: Foxes and jackals as sympatric species in the lowlands often compete directly or indirectly. As a result of competition, the number of foxes in the recent years reduced unlike that of the jackal's number. Elsewhere, foxes retreated and occupied territories with greater altitude, obviously unsuitable for jackals, which prefer places with higher temperatures.

Conclusion: The present analysis illustrates the relationship and trends in the population dynamics of the two game species, and indicates a direction of their sustainable management.

Keywords: golden jackal, red fox, population dynamics, mapping trends

Introduction

Red fox (*Vulpes vulpes* L. 1758) and golden jackal (*Canis aureus* L. 1758) are the most numerous middle-sized game carnivores in Bulgaria. The red fox is widely distributed across the country, from the Black Sea coast to the subalpine zone; its highest population density is in typical lowland habitats, mainly in Northern Bulgaria. Forest habitats in both mountains and planes are also typical for the species [1]. The various foods conditions,

interaction with other predators, land cover and land use are the main reasons for fluctuating the dynamics of the fox population [2].

The jackal is an autochthonous species in Bulgarian mammalian fauna [3]. There is evidence that the species has been distributed in the open spaces over the country since the Middle Ages, especially in the Southeast. In the middle of the last century, it was distributed only in the Strandzha Mountain and the Black Sea coast. In 1962, it was declared as a protected species. In recent decades, the population numbers of the jackal sharply expanded and in 1984, when the conservation status of the species was cancelled, it almost entirely occupied the lowland habitats throughout the country. Main reasons for the increase of the population number were the conservation status of the species, the improving food base as a result of intensive hunting and the changed role of the jackal as a top predator by reducing the number of wolves [3].

The aim of this study is to trace the population dynamics of the golden jackal and red fox in recent decades in the context of the impact of the expansion of the jackal population on the red fox numbers. The analysis of relationships and trends in population dynamics of both species might be used in planning of activities concerning the sustainable use of this hunting species.

Materials and Methods

The estimation of the population size is done using the available data from the official results of spring game counts, carried out by the Executive Agency for Forests (EAF) throughout the country, during the period of 2000-2015. Population growth of the investigated species over two consecutive years was measured as the rate of increase, or $R=ln\ (Nt/No)/t$), where Nt is the population count at time t, and No is the initial population size [4]. In order to identify population trends, Mann-Kendall statistics was used because this statistical procedure being suitable for short time series having cases with monotonous trends and no seasonal or other cycles in the data [5]. To estimate the true slope of an existing trend (as change per year) the Sen's nonparametric method was used. The Sen's method can be used in cases where the trend can be assumed to be linear [6]. The close the value of the Sen's is to 0, the trend is lower. The sign of the slope tells if the trend is increasing or decreasing. The biogeographic regions (BGR) were determinate according to Gruev [7]. Statistical analyses were carried out using XLSTAT, version 2016.02.27313.

Results

According to the records of EAF for the last 16 years the population of the golden jackal has increased more rapidly than the population of the red fox in the whole country. The population number of both species reached parity in 2013, but the population of the jackal has increased in the following years, while the one of the fox has remained relatively constant (Fig.1).

The population growth showed positive values in 12 consecutive intervals for the population of jackal and in 11 for the population of red fox. The negative values were 3 for the jackal population and 4 for the red fox population, as in the last one they were well

expressed. The population growth of the jackal demonstrated a pronounced maximum in 2001 and minimum in 2015, while the population growth of the red fox was the highest in 2005 and lowest in 2014. Even in the intervals with negative values the population numbers of both species remain high during the investigated period (Fig.2).

The Mann-Kendall test at the national level demonstrated significant increasing trends for *C. aureus* and *V. vulpes* (Table 1).

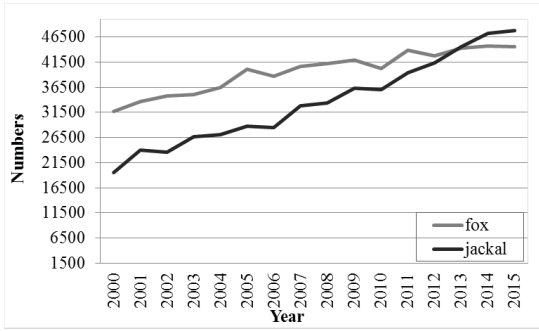

Fig. 1. Population numbers of red fox and golden jackal during the period 2000-2015

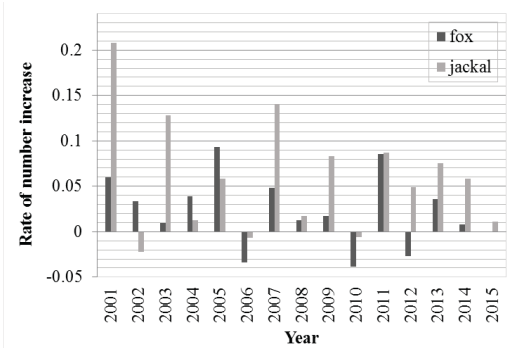

Fig. 2. Rate of population growth of red fox and golden jackal in two consecutive years in Bulgaria

Table1. Mann-Kendall trends test for 2 species at the national level

Species	Kendall's tau	S	Var(S)	p-value (Two-tailed)	alpha	interpretation
Vulpes vulpes	0.900	108.000	0.000	< 0.0001	0.05	significant increase
Canis aureus	0.950	114.000	0.000	< 0.0001	0.05	significant increase

Sen's trend lines showing the strength and direction of trend are presented in Fig. 3 A, B. The Sen's slope of the golden jackal population was 1831 and of the red fox population was 865.3, which indicates the lower increase.

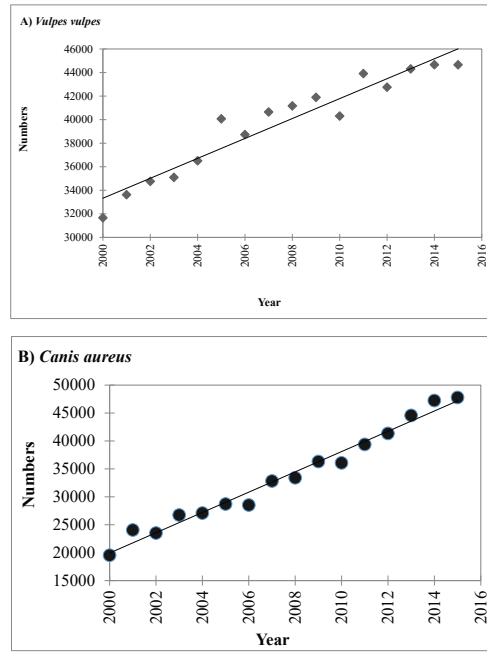

Fig. 3. National level Sen's estimates of trends for (A) red fox and (B) golden jackal

The result of the analysis at the biogeographical region (BGR) level is summarized in the Table 2 and Fig. 4 A, B.

Table 2. Mann-Kendall trends test for 2 species at the biogeographical level

Species	Statistics	*Vulpes vulpes*	*Canis aureus*
North BGR	Kendall's tau	0.822	0.733
	S	37.0	33.0
	p-value (2-tailed)	0	0.002
Middle BGR	Kendall's tau	0.911	0.911
	S	41.0	41.0
	p-value (2-tailed)	< 0.0001	< 0.0001
South BGR	Kendall's tau	0.333	0.911
	S	15.0	41.0
	p-value (2-tailed)	0.216	< 0.0001
Black Sea BGR	Kendall's tau	0.867	0.778
	S	39.0	35.0
	p-value (2-tailed)	0	0.001
Mountain BGR	Kendall's tau	0.333	not present
	S	15.0	not present
	p-value (2-tailed)	0.216	not present

The red fox population was mainly distributed in the North Bulgarian BGR (39%) and in the Middle Bulgarian BGR (about 26%), while in the Black Sea BGR only 7% of the population could be found. The same pattern was observed for the golden jackal population. It is manly distributed within the North Bulgarian BGR (about 43%) and only 8% in South Bulgarian BGR. Golden jackal was completely absent in the Mountain BGR, but within the Black Sea BGR its average population size (6637.5 individuals) during the investigated period considerably exceeded the red fox's average population number (4.240 individuals).

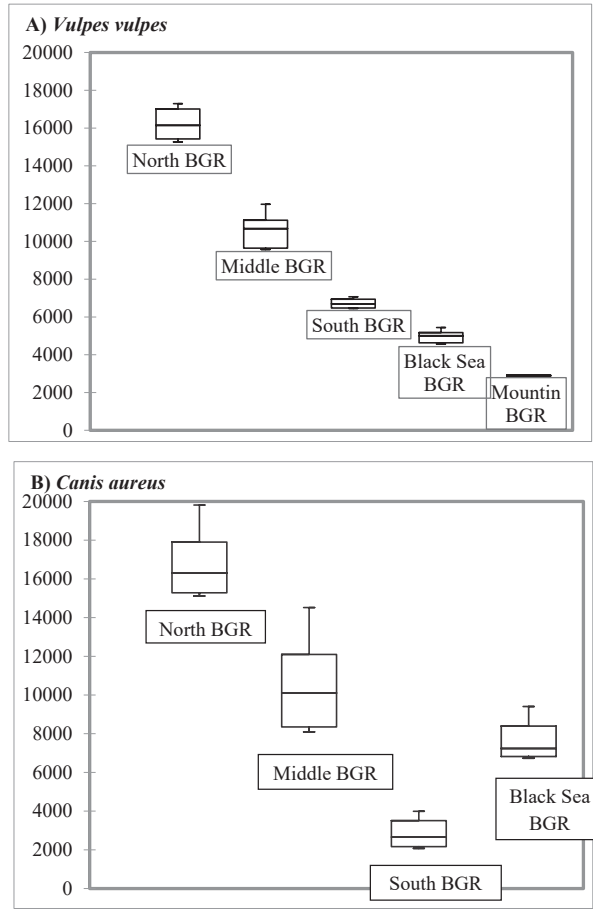

Fig. 4. National level Sen's estimates of the trends for *V. vulpes* (A) and *C. aureus* (B) populations

Discussion

Monitoring the abundances of carnivores is a basic management tool for the evaluation of the impact of biotic and anthropogenic factors such as the interspecies relationships, habitat fragmentation and hunting. The golden jackal has been intensively spreading in Bulgaria,

showing the characteristics of an invasive species. The results of the official hunting bag data showed a linear increase year by year and the density of the population could be similar or higher than the red fox (*V. vulpes*). After receiving protection status in 1962, the golden jackal has started gradually to expand its distribution in Bulgaria. The large amount of available food provided by intensive development of hunting in the country during this period has become main food base for the jackal. In 1984, it was excluded from the list of protected species. According to Spiridonov and Spassov [8] the population number of the species in the early 90's was about 5000 individuals, while in 2015 it reached 47,774 individuals (according to EAF). The growth has increased about 9-fold for 23 years. It appears that the largest jackal population in Europe is in Bulgaria [3]. One of the subjective reasons for the relatively high number of jackals is the fact that since 2009 the payment of bonuses for killed animal has stopped. The jackal has extended its distribution range in the country and has found suitable habitats in the mountainous and semi-mountainous parts [3]. The distribution of golden jackal at the European level is in continuous change due to the long-range dispersal rates [9, 10] as well as to the dominant increasing trend of this species.

The population number of red fox has increased constantly during the investigated period, as its growth rate remained lower after reaching the parity with the jackal population in 2013. The main stock of the species in 1983 was about 53,000 individuals [11], while in 2015 it dropped to 44,656 individuals (according to EFA, 2015). The downsizing was approximately 15% for 30 years.

The analysis of the population dynamics of both species in Bulgaria shows that their populations' numbers were increased for the reporting period as that of the jackal has a higher trend. In the North BGR, both species had the highest numbers, but the trends of increasing were less pronounced in the Middle BGR for the fox and in the South BGR for the jackal. In the lowlands of the North and South BGR, where the two species are sympatric, their competitive relationship is more pronounced and as a result the increase in the populations' number of the fox is smaller.

The diets of golden jackals and red fox overlap to a high extend [12], which increases the risk of interspecies competition. Studies revealed the golden jackal to be a "searcher hunter", feeding upon a broad range of small-sized prey such as rodents, hare, birds, reptiles and arthropods but also frequently consuming plants, scavenging on domestic animal remains [13] or carcasses left by large predators [14]. The jackal is a group-living canid, and not only a searcher but also a pursuer, successfully hunting medium- and larger-sized ungulates. The red fox is also a typical "searcher hunter" as well as a food generalist, utilizing a wide range of small-sized prey types or plants and carcasses in European habitats. Species with similar diets may diverge in resource use and hence overlap when food becomes scarce to reduce interspecific competition [15]. Additionally, foxes have adapted to the recent colonization of jackals by positioning territories in areas of higher elevation to avoid competition with jackals. Coexistence is probably possible because potential competition is reduced via niche partitioning.

Abundant food and flexibility of the omnivorous and opportunistic diet and feeding habits of golden jackal and red fox probably allows these two closely related, medium-sized carnivores to coexist sympatrically in the Bulgaria agricultural areas.

Acknowledgements: We thank Professor Peter Genov for his support.

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PINE MARTEN (*MARTES MARTES* L.): DISTRIBUTION, HABITAT PREFERENCE AND ACTIVITY IN VITOSHA NATURE PARK, BULGARIA

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Abstract

Aim: to study the distribution, habitat preference and activity of the pine marten in Nature Park (NP) Vitosha.

Materials and Methods: Camera traps were placed in NP Vitosha in 2013 and 2014 according to a predefined grid, set up to record the day and time for each registration. For each camera trap location, a standard form was filled, containing information about the habitat characteristics: altitude, forest type and forest visibility. Habitat preference analysis was performed using Ivlev's electivity index.

Results: 23 independent registrations of pine marten in 19 locations were obtained during the study. The pine marten predominates in mixed forests and also exhibits slight preferences towards coniferous forests. It prefers open forests and selects sites mainly above 1400 m a.s.l. The pine marten is predominantly active during the day, with little crepuscular activity.

Conclusions: There are publications on the pine marten presence in NP Vitosha but hard evidence was lacking till now. This study contributes to the better understanding of this species' ecology in Bulgaria.

Keywords: Pine marten, distribution, habitat preference, activity

Introduction

The distribution of the Pine Marten (*Martes martes* L., 1758) encompasses almost the whole continental part of Europe, except for most of the Iberian Peninsula and Greece, as well as parts of Belgium and the Netherlands [1]. The pine marten's populations decline and their ranges decrease in many parts of its distribution due to excessive hunting. The proportion and scale of these effects is difficult to determine since historical data are largely lacking. The pine marten is listed on Appendix III of the Bern Convention and Annex V of the Habitats Directive [1].

In Bulgaria, the pine marten's distribution is mosaic and the species is most likely present in the whole country, although systematic data have not been collected. The species inhabits mainly mountainous regions where old-growth forests remain [2, 3]. There has been a rapid decline in population numbers in the past 30 years due to the mass cutting of old-growth forests in the country. Subsequently, the pine marten was partially replaced by the more adaptive stone marten (*Martes foina* Erx.) [3, 4, 5]. The pine marten is currently listed

as Endangered (EN) in the Red Data Book of Bulgaria and is protected by the Biological Diversity Act (Appendix II and III) [3].

According to the existing data, the pine marten in Bulgaria inhabits predominantly coniferous (mainly spruce *Picea abies* L.) and common beech (*Fagus sylvatica* L.) forests above 1200 m.a.s.l., and less frequently oak (*Quercus robur* L.) and common hornbeam (*Carpinus betulus* L.) forests [3, 6, 7]. Its range is presumed to include the larger and higher Bulgarian mountains, such as Rila, Pirin, Stara Planina, Vitosha and Belasitsa [8], although hard evidence is lacking for some of these regions such as Vitosha Mountain where presence so far was based on a guesstimate. The pine marten possesses great ecological plasticity and is adapted to various forest, shrub and even grassland habitats in different parts of its range. There is no conclusive data based on field studies regarding the pine marten's habitat preferences in Bulgaria so far [4].

In this paper, we aim to fill this gap by using camera traps to study the distribution of the pine marten, its preferred habitats (regarding forest type, forest visibility, and site altitude) and activity patterns on the territory of NP Vitosha.

Materials and Methods

Study area: The study was conducted in Vitosha Mountain situated between the Balkan and Rila-Rhodope Massif is Bulgaria (Fig. 1). Vitosha's mean altitude is 1500 m a.s.l. reaching 2290 m a.s.l. at Cherni Vrah, the mountain's highest peak. The mountain can be divided into 4 zones based on the dominant plant species: mixed oak-hornbeam forests (at altitudes between 1100 and 1400 m a.s.l.), beech forests (between 1400 and 1840 m a.s.l.), coniferous forests (1700-2050 m a.s.l.) and subalpine zone (above forest level) [9].

Data collection and analyses: 30 camera traps (Ltl Acorn 5210) were set up in NP Vitosha between June 2013 and November 2014 according to a predefined grid with a cell width of 600 m (Fig. 1) [10]. Camera traps were set up on animal trails in suitable habitats (predominantly forests) for detecting middle and large sized mammals.

The locations were divided into 6 zones, and the camera traps were left in the field for 22-35 days in one zone before being moved to the next. Camera traps were set to record three photos and a video, printing the time and date on the photo. For each camera trap location habitat description was recorded, including information on the dominant tree species, forest type (deciduous, coniferous or mixed), forest visibility (open, with visibility > 10 m or closed, with visibility < 10 m), GPS coordinates and altitude.

Using Camera Base 1.6 [11] (adapted and translated into Bulgarian, Zlatanova 2014, unpublished) the resulting photos were imported and analyzed. To avoid overestimation of the species in a certain location or time period due to the activity of a single individual spending a relatively long period of time in front of the camera, we considered photos of prolonged stay of the same individual as 1 independent event (hereafter independent registration) unless it was clear that two or more different individuals were captured. The pine marten is often difficult to differentiate from the stone marten (*Martes foina* Erx.) in camera trap photos (especially nocturnal). Only registrations where one or more of the pine marten's differentiating characteristics (larger ears with white lining, black nose,

wider head and narrow snout – Fig. 1) are visible were considered for analysis [4]. The relative preference for different habitat types (forest type and visibility) and altitudinal classes (divided into 200 m intervals) was calculated using Ivlev's Electivity Index (D), partially modified by Jacobs [12]. The activity patterns were analysed as the percentage of registrations for each hour of the day. We made a cross-validation by creating a buffer of 800 m around each location (which corresponds to an area of approximately 2 km^2, the mean home range of pine marten estimated for Poland in similar habitats [13]) and extracted the landcover from Corine Landcover 2006. Pearson correlation was used to measure the link between the altitude and the number of buildings.

Fig. 1. Camera trap photo of pine marten in Nature Park Vitosha, with distinct differentiating characteristics

Results

The pine marten was registered in 23 occasions throughout the whole study period (Table 1).

Table 1. Pine marten registration in Vitosha NP during camera trapping in 2013-2014

Study Area	Independent registrations	Camera trap locations
2013 - Zone 1	1	1
2013 - Zone 3	1	1
2013 - Zone 4	1	1
2013 - Zone 5	4	2
Zone 6 -winter 2013-2014	2	2
2014 - Zone 1	3	3
2014 - Zone 2	4	4
2014 - Zone 3	1	1
2014 - Zone 4	1	1
2014 - Zone 5	5	3
Total	**23**	**19**

The distribution of the registrations is presented in Fig. 2.

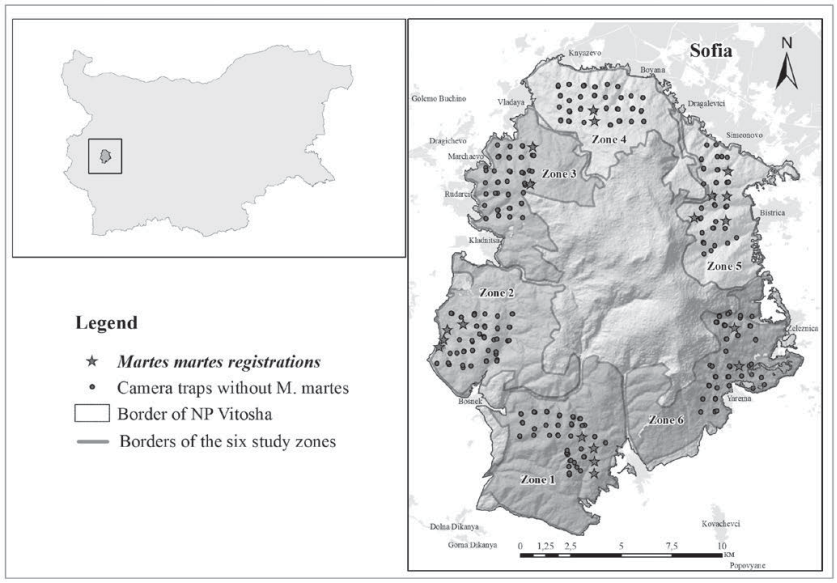

Fig. 2. Map of the camera trap locations and pine marten registrations in Nature Park Vitosha

Habitat preferences

The electivity index for the forest type (Fig. 3) indicates that the pine marten in Vitosha NP shows stronger preference to mixed forests (0.50) and to a lesser extent preference to coniferous forests (0.17) while it definitely avoids deciduous forests (-0.60) and shrubs and small trees (-1.00). The strong preference of the pine marten for mixed forests in Vitosha NP is not in coherence with the general belief that the pine marten in Bulgaria inhabits mainly coniferous forests [2–5] which is true for Pirin NP and other areas in Northern Europe. The cross-validation (Table 2) shows that although the presence of coniferous forests (22.08%) around each location is twice as big as that of the mixed forests (11.10%), mixed forests are definitely preferred.

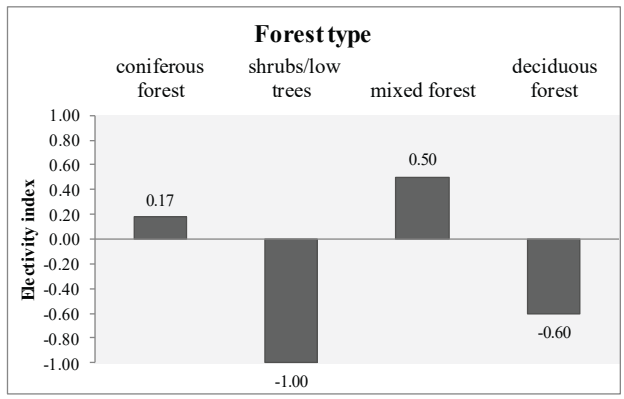

Fig. 3. Electivity index of the pine marten for the forest type

Table 2. Landcover in the 800 m buffer around each pine marten location

Landcover in the buffer	% of area covered
Broad-leaved forest	46.28%
Coniferous forest	22.08%
Land principally occupied by agriculture with significant presence of wild vegetation	4.53%
Mixed forest	11.10%
Natural grassland	5.55%
Sparsely vegetated areas	2.59%
Transitional woodland/ shrub	3.89%
Others	3.98%
Total	**100.00%**

The pine marten in Vitosha NP shows slight preference towards closed forests (0.25), slight avoidance of open forests (0.14) and total avoidance of the transitional areas between open and closed forests (Fig. 4). One reason for this might be the strong presence of many trophic competitors such as the stone marten, the red fox (*Vulpes vulpes*) and the wildcat (*Felis silvestris*), which creates the need for more covert places necessary for successful hunting.

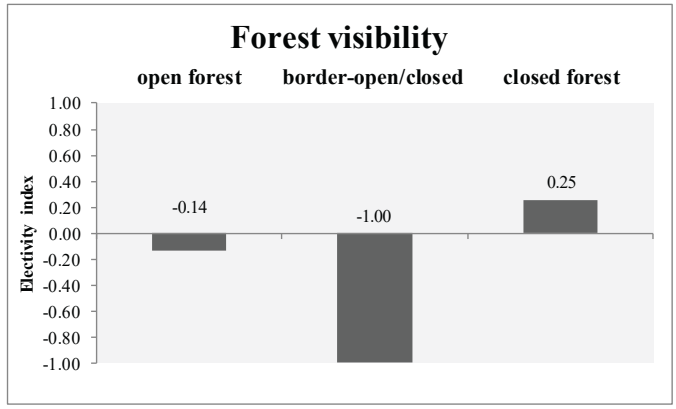

Fig. 4. Electivity index of the pine marten for the forest visibility

The most preferred range of altitude (Fig. 5) for the pine marten in NP Vitosha is that of over 1600 m. a. s. l. (0.67), followed surprisingly by the altitudinal range of 800-999 m a.s.l. (0.38) and 1400-1600 m a.s.l. (0.34). The analysis (Fig. 4) shows no correlation between the higher altitudes and the number of buildings (Pearson correlation, $p<0.05$).

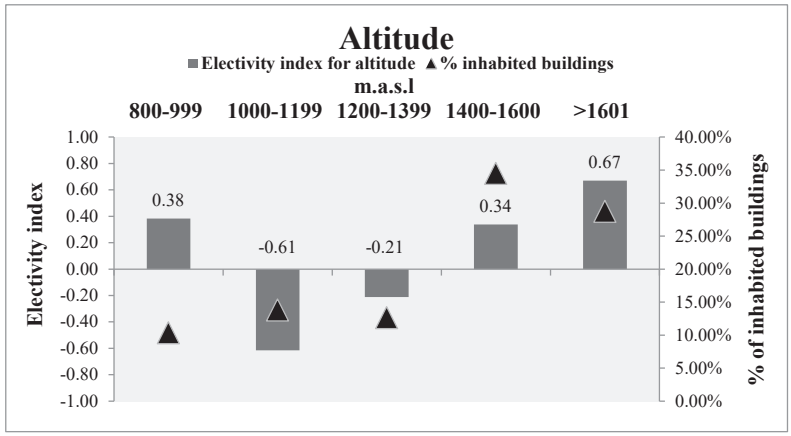

Fig. 5. Electivity index of the pine marten for different altitudinal classes

Additionally, the altitudinal distribution of forest types where the pine marten is recorded (Table 3) does not fully explain this inverted preference for altitude – the most preferred type of forest (mixed) is not well represented in the altitudinal ranges of 800-999 m a.s.l., and above 1400 m a.s.l. Most likely this skewed preference for altitude is formed by a combination of indirect factors affecting the pine marten's prey base.

Table 3. Altitudinal distribution of forest types in the locations where the pine marten was recorded

altitudinal range (m a.s.l.)	forest type	% cover	% total
800-999	coniferous forest mixed forest deciduous forest	50.00% 25.00% 25.00%	100.00%
1000-1199	mixed forest deciduous forest	50.00% 50.00%	100.00%
1200-1399	mixed forest deciduous forest	57.14% 42.86%	100.00%
1400-1599	mixed forest	100.00%	100.00%
>1600	coniferous forest	100.00%	100.00%

Activity patterns

The pine marten in Vitosha NP exhibits predominantly diurnal activity (n = 16, 69.6% of the all independent registrations), less nocturnal (n = 6, 26.1%) and crepuscular (n = 1, 4.3%). The hourly activity is presented in Fig. 6. There is an obvious peak in the morning between 7:00 and 10:00 h. (n = 9, 39.1% of all independent registrations), followed by smaller peaks around 14:00, 18:00 and 22:00 h. The pine marten has very limited activity between 23:00 and 7:00 h. (n = 2, 8.7 %).

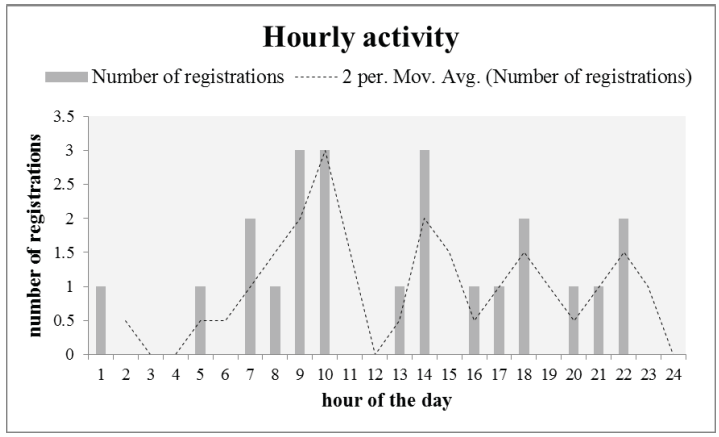

Fig. 6. Hourly activity of the pine marten in NP Vitosha

Discussion

There are publications on the pine marten's presence in NP Vitosha, but hard evidence was lacking till now. There are a small number of publications regarding the pine marten's biology and ecology in Bulgaria coming from a targeted field work in the country. For the first time in this paper, the habitat preferences and the activity patterns of the pine marten in NP Vitosha are analysed and presented. This study contributes to the better understanding of this species' ecology in Bulgaria. Comparing our results to the literature data, some dissimilarities emerge. In previous studies, the importance of deciduous forests for the pine marten has been overestimated, as well as the importance of the coniferous forests to a certain extent. Our results indicate that mixed forests are selected most often in NP Vitosha, which is probably explained by the more diverse food base there. The pine marten, somewhat surprisingly, shows a preference for sites with altitudes between 800–999 m a.s.l., while previous studies only indicate that the species inhabits areas above 1200 m a.s.l. [3, 6, 7].

Being one of the meso-carnivores in the mountain, the pine marten coexists in competition with more adaptable and ecologically plastic species as the stone marten, the red fox, the wildcat and the badger. It is fairly uniformly distributed in all parts of NP Vitosha, all forest types and altitudinal ranges. Comparing the rate of registrations, the stone marten is around 18 times more frequently registered in the mountain (n = 407) than the pine marten, while the red fox (n = 926) is around 40 times more frequently registered than the pine marten.

There is a need for further study on the species in the mountain to identify the intrinsic reasons for the skewed altitudinal distribution and the relationship with the other meso-carnivores.

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DIVERSITY, DISTRIBUTION AND CONSERVATION STATUS OF THE AMPHIBIANS (AMPHIBIA) IN PROTECTED AREA „ORANOVSKI PROLOM – LESHKO" (BG0001022), BULGARIA

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Abstract

Aim: To supplement and summarize the data about the diversity, distribution and conservation status of the amphibians in Protected Area BG0001022 „Oranovski Prolom – Leshko".

Material and Methods: The amphibian species and localities were registered during field trips in June 2008 – April 2016. The geographic coordinates have been determined with GPS.

Results: Totally 8 species (4 reported for the first time) have been found in the studied area, one caudate amphibian and seven anurans. All of them are protected according to the national and international legislation. All localities (published and new) have been summarized in tables.

Conclusion: The amphibian species that occur in the studied area represent 36.4% of the total number of amphibians (22) distributed in Bulgaria. The protected zone will be studied in details in the next two years. It is expected three other species: *Lissotriton vulgaris, Triturus ivanbureschi* and *Rana temporaria*, and many new localities to be registered.

Keywords: amphibians, diversity, new locality, conservation status, protected zone, Natura 2000

Introduction

The Protected Area „Oranovski Prolom – Leshko" (code BG0001022) is a part of the Natura 2000 network in Bulgaria. It has been designated under the EU Habitats Directive (Council Directive 92/43/EEC). The area was proposed as a Site of Community Importance (SCI) in March 2007 and approved by the European Commission on 12.12.2008. The Protected Area has been declared for conservation of 20 types of habitats and 28 species of wild fauna, including 7 invertebrate, 3 fish, 2 amphibian, 3 reptile and 13 mammalian species, including 9 bat species.

The batrachofauna in the area has been studied till now in connection with the realization of the project "Mapping and preservation of conservation status of natural habitats and species - Phase I" [1]. Only two species listed in the Annex II of the Habitats Directive have been searched for, the Yellow-bellied Toad *Bombina variegata* and the Balkan Crested Newt *Triturus karelinii* (currently known as *Triturus ivanbureschi*). Two specimens of Yellow-bellied Toad have been registered in only one locality. The Crested Newt has not been found but the suitable habitats have been mapped.

Data about the diversity and distribution of amphibians in the Protected Area and close to its borders are contained also in the publications of Pulev & Sakelarieva [2], and Pulev et al. [3]. Totally, 4 species were reported: one tailed amphibian *Salamandra salamandra* (in one locality) and 3 anuran species – *Bombina variegata* (in two localities), *Rana graeca* (in seven localities) and *Pelophylax ridibundus* (in one locality).

The aim of the present report is to supplement and summarize data about the diversity, distribution and conservation status of the amphibians in the Protected Area „Oranovski Prolom – Leshko".

Material and Methods

The Protected Area "Oranovski Prolom – Leshko" is situated in the northwestern part of the Blagoevgrad District, southwestern Bulgaria. With an area of 13245.47 ha [1], it falls within the territory of two municipalities – Blagoevgrad and Simitli.

The amphibian species and their localities were registered during field trips in June 2008– April 2016. The species were determined visually after Arnold & Ovenden [4] and Stojanov et al. [5]. In some cases, animals were captured for a while and released in the same place. All localities (published and new) have been summarized in tables. The geographic coordinates have been determined with GPS. The species conservation status has been determined according to the national and international legislation.

Results

Diversity: Totally, 8 amphibian species have been registered in the studied area so far, 1 caudate and 7 anuran species (Table 1). Four of them are reported for the first time for the area. All new species are anurans and are representatives of 3 families: Bufonidae – *Bufo bufo* and *Bufotes viridis*; Hylidae – *Hyla arborea,* and Ranidae – *Rana dalmatina*.

The largest number of specimens registered belongs to four species: the Eurasian Marsh Frog *Pelophylax ridibundus* (many specimens) (Table 3), Yellow-bellied Toad (many specimens, including several breeding pairs) (Table 9), Common Toad *Bufo bufo* (16 specimens) (Table 5) and the Balkan Stream Frog (12 specimens) (Table 8). Only one specimen of Agile Frog *Rana dalmatina* was recorded in the Protected Area (Table 7).

Table 1. Amphibian species and the number of localities registered in the Protected Area "Oranovski Prolom – Leshko"

Species	Number of localities	
	published	new
Salamandra salamandra (Linnaeus, 1758)	1	1
Bombina variegata (Linnaeus, 1758)	3	12
Hyla arborea (Linnaeus, 1758)	-	4
Bufo bufo (Linnaeus, 1758)	-	6
Bufotes viridis (Laurenti, 1768)	-	6
Rana dalmatina Fitzinger in Bonaparte, 1839	-	1
Rana graeca Boulenger, 1891	7	7
Pelophylax ridibundus (Pallas, 1771)	1	22
Total	**12**	**59**
	71	

Distribution: The total number of localities of the eight amphibian species registered within the protected area is 71; 12 of them have been published [1–3] and 59 are reported in the present article (Tables 1–9). The Eurasian Marsh Frog *Pelophylax ridibundus* has been recorded in 22 localities (Table 9), while the Agile Frog *Rana dalmatina* has been found only once (Table 7). The Fire Salamander *Salamandra salamandra* was registered twice, and the other species were recorded in 4 or more localities.

In some localities, more than one species have been recorded. The largest numbers of species have been found in:

- Emeshen (Tserovsko Dere) stream, in fountain tubs – 5 species (*Bombina variegata, Hyla arborea, Bufo bufo, Rana dalmatina,* and *Pelophylax ridibundus*).

- Stara Reka river, 2200 m from its mouth – 3 species (*Bombina variegata, Rana graeca,* and *Pelophylax ridibundus*).

Table 2. Localities of *Salamandra salamandra* in the Protected Area "Oranovski Prolom – Leshko".

№	Locality	Geographic coordinates	Altitude	Time of observation	Specimens observed	Data source
Published data						
1.	between the villages of Dolno Tserovo and Tserovo			13.05.1990	several larvae	[2]
New data						
1.	northeast of the village of Zheleznitsa, in a stream	N41°56′13″ E23°07′01″	436 m	28.04.2016 1:25 pm	several larvae	

Table 3. Localities of *Bombina variegata* in the Protected Area "Oranovski Prolom – Leshko".

№	Locality and species	Geographic coordinates	Altitude	Time of observation	Specimens observed	Data source
Published data						
1.	1400 m southwest of Tserovo, puddle in a gully	N41°56′50″ E23°07′01″	515 m		2 ad.	[2, 3]
2.	west of the village of Leshko				2 ad.	[1]
3.	Stara Reka river, 2200 m from its mouth	N41°55′31″ E23°05′13″	372 m	21.07.2013	many specimens	[3]
New data						
1.	Emeshen (Tserovsko Dere) stream, in fountain tubs	N41°58′30″ E23°06′34″	402 m	04.04.2015 6:00 pm	1 ad.	
				29.08.2015 7:30 pm	2 ad.	
2.	near Dzhaleva Mahala neighbourhood (the village of Zheleznitsa), in a stream	N41°56′00″ E23°05′14″	430 m	18.05.2009	several specimens	
3.	northeast of the village of Zheleznitsa, in a fountain tub	N41°55′56″ E23°06′32″	345 m	28.04.2016 3:00 pm	1 ad. female	
4.	near the village of Zheleznitsa, in Stara Reka river	N41°55′20″ E23°06′19″	313 m	23.07.2013 10:30 pm	several spec., 4 in copulation	
5.	near the village of Zheleznitsa, in Stara Reka river	N41°55′20″ E23°06′12″	321 m	25.04.2016 12:15 pm	1 juv.	
6.	near the village of Zheleznitsa, in Stara Reka river	N41°55′26″ E23°05′46″	348 m	23.07.2013 9:30 pm	1 juv.	
7.	near the village of Zheleznitsa, in Stara Reka river	N41°55′28″ E23°05′45″	353 m	25.04.2016 1:25 pm	several specimens	
8.	near the village of Zheleznitsa, in Stara Reka river	N41°55′33″ E23°05′34″	357 m	25.04.2016 2:00 pm	2 ad.	
9.	near the village of Zheleznitsa, in Stara Reka river	N41°55′32″ E23°05′26″	364 m	25.04.2016 2:10 pm	1 juv.	
10.	near the village of Zheleznitsa, in Stara Reka river	N41°55′32″ E23°05′23″	366 m	25.04.2016 2:30 pm	2 ad.	
11.	Korianska Mahala neighbourhood (the village of Leshko), in a wooden trough	N41°55′58″ E22°56′34″	780 m	15.04.2016 10:30 am	2 ad. male and female	
12.	Mariyanovska Mahala neighbourhood (the village of Gabrovo), in a fountain tub	N41°53′18″ E22°56′27″	869 m	27.04.2015 7:00 pm	1 ad.	

Table 4. Localities of *Hyla arborea* in the Protected Area "Oranovski Prolom – Leshko".

№	Locality	Geographic coordinates	Altitude	Time of observation	Specimens observed
New data					
1.	Emeshen (Tserovsko Dere) stream	N41°58′29″ E23°06′36″	401 m	19.04.20153:30 pm	1 ad. female killed on the dirt road
2.	Emeshen (Tserovsko Dere) stream, in fountain tubs	N41°58′30″ E23°06′34″	402 m	15.04.2015 11:30 am	3 egg clumps
3.	Dzhaleva Mahala neighbourhood (the village of Zheleznitsa)	N41°56′04″ E23°05′34″	320 m	15.06.2008 11:00 pm	1 ad.
4.	near Stamboliyska Mahala neighbourhood (the village of Gabrovo), in a small pool	N41°53′42″ E22°58′08″	815 m	01.04.2016 1:00 pm	2 ad. male, 4 egg clumps

Table 5. Localities of *Bufo bufo* in the Protected Area "Oranovski Prolom – Leshko".

№	Locality	Geographic coordinates	Altitude	Time of observation	Specimens observed
New data					
1.	Emeshen (Tserovsko Dere) stream, in fountain tubs	N41°58′30″ E23°06′34″	402 m	04.04.2015 6:00 pm	egg strings
				15.04.2015 11:30 am	egg strings
2.	Emeshen (Tserovsko Dere) stream	N41°58′25″ E23°06′15″	375 m	07.10.2015 9:00 pm	1 ad. male
3.	Emeshen (Tserovsko Dere) stream	N41°58′17″ E23°05′51″	335 m	27.03.2015 7:30 pm	1 ad. female
4.	Dzhaleva Mahala neighbourhood (the village of Zheleznitsa)	N41°56′04″ E23°05′34″	320 m	15.06.2008 4:30 pm	1 ad. male
				20.08.2010 2:30 pm	1 subad.
5.	near Kachulska Mahala neighbourhood (the village of Gabrovo), in a small reservoir	N41°53′11″ E22°55′57″	1018 m	17.03.2016	10 ad. dead, killed by an animals
6.	near Malinovska Mahala neighbourhood (the village of Gabrovo), in a small marsh	N41°53′12″ E22°57′56″	894 m	01.04.2016 2:00 pm	2 ad. male

Table 6. Localities of *Bufotes viridis* in the Protected Area "Oranovski Prolom – Leshko".

№	Locality	Geographic coordinates	Altitude	Time of observation	Specimens observed
New data					
1.	north of Dolno Tserovo neighbourhood (the village of Tserovo)	N41°57′12″ E23°06′18″	317 m	25.04.2016 11:20 am	1 ad. male killed on the road
2.	near the village of Zheleznitsa, in Stara Reka river	N41°55′20″ E23°06′15″	318 m	25.04.2016 12:05 pm	1 ad. male
3.	near the village of Zheleznitsa, in Stara Reka river	N41°55′20″ E23°06′09″	328 m	25.04.2016 12:25 pm	2 ad. male and female
4.	near the village of Zheleznitsa, in Stara Reka river	N41°55′19″ E23°06′08″	328 m	25.04.2016 12:30 pm	1 ad. female
5.	near the village of Zheleznitsa, in Stara Reka river	N41°55′19″ E23°06′05″	333 m	23.07.2013 10:15 pm	1 ad. male
6.	near the village of Zheleznitsa, in Stara Reka river	N41°55′33″ E23°05′26″	360 m	25.04.2016 1:45 pm	1 ad. male

Table 7. Localities of *Rana dalmatina* in the Protected Area "Oranovski Prolom – Leshko".

№	Locality	Geographic coordinates	Altitude	Time of observation	Specimens observed
New data					
1.	Emeshen (Tserovsko Dere) stream, in fountain tubs	N41°58′30″ E23°06′34″	402 m	19.03.2015	1 ad. male

Table 8. Localities of *Rana graeca* in the Protected Area "Oranovski Prolom – Leshko".

№	Locality	Geographic coordinates	Altitude	Time of observation	Specimens observed	Data source
Published data						
1.	1400 m southwest of Tserovo, puddle in a gully	N41°56′50″ E23°07′01″	515 m		1 ad., 2 subad.	[2, 3]
2.	Alina stream, left tributary of Leshnishka river, 550 m upstream of its mouth	N41°57′35″ E22°53′56″	847 m		several specimens	[2, 3]
3.	Leshnishka river, 1300 m east of Yovanovtsi neighbourhood (the village of Obel)	N41°57′23″ E22°54′36″	785 m	23.03.2014	1 ad.	[3]
4.	Stara Reka river, 2200 m from its mouth	N41°55′31″ E23°05′13″	372 m	21.07.2013	1 juv.	[3]
5.	Krastevski Dol stream, 1200 m from its mouth	N41°55′24″ E23°07′38″	418 m	05.05.2015	3 juv.	[3]
6.	Krastevski Dol stream, 350 m upstream of the previous location	N41°55′33″ E23°07′40″	461 m	05.05.2015	4 juv.	[3]
7.	about 600 m northwest of the village of Debochitsa	N41°52′24″ E22°56′57″	1007 m	20.08.2012	1 ad. male, 2 juv.	[3]
New data						
1.	northeast of the village of Zheleznitsa, in a stream	N41°56′11″ E23°06′58″	427 m	28.04.2016 1:15 pm	1 juv.	
2.	northeast of the village of Zheleznitsa, in a stream	N41°56′12″ E23°07′00″	433 m	28.04.2016 2:25 pm	1 juv.	
3.	northeast of the village of Zheleznitsa, in a stream	N41°56′13″ E23°07′01″	436 m	28.04.2016 1:25 pm	3 juv.	
4.	northeast of the village of Zheleznitsa, in a stream	N41°56′13″ E23°07′03″	435 m	28.04.2016 1:30 pm	1 juv.	
5.	northeast of the village of Zheleznitsa, in a stream	N41°56′15″ E23°07′05″	442 m	28.04.2016 1:40 pm	2 juv.	
6.	west of the village of Gabrovo, in Gabrovska Reka river	N41°53′45″ E22°55′53″	791 m	01.04.2016 10:00 am	2 juv.	
7.	near Stopanska Mahala neighbourhood (the village of Gabrovo), in a stream	N41°52′42″ E22°56′42″	892 m	01.04.2016 2:30 pm	2 juv.	

Table 9. Localities of *Pelophylax ridibundus* in the Protected Area "Oranovski Prolom – Leshko".

№	Locality	Geographic coordinates	Altitude	Time of observation	Specimens observed	Data source
Published data						
1.	Stara Reka river, 2200 m from its mouth	N41°55′31″ E23°05′13″	372 m	21.07.2013	many specimens	[3]
New data						
1.	Emeshen (Tserovsko Dere) stream, in fountain tubs	N41°58′30″ E23°06′34″	402 m	29.08.2015 7:30 pm	1 ad.	
				04.04.2015 6:00 pm	1 ad.	
				15.04.2015 11:30 am	2 ad.	
2.	northeast of the village of Zheleznitsa, in a fountain tub	N41°55′56″ E23°06′32″	345 m	28.04.2016 3:00 pm	2 juv.	
3.	northeast of the village of Zheleznitsa, in a stream	N41°56′12″ E23°07′00″	433 m	28.04.2016 1:20 pm	1 juv.	
4.	near the village of Zheleznitsa, in Stara Reka river	N41°55′20″ E23°06′13″	322 m	25.04.2016 12:10 pm	3 juv.	
5.	near the village of Zheleznitsa, in Stara Reka river	N41°55′20″ E23°06′11″	325 m	25.04.2016 12:15 pm	1 subad.	
6.	near the village of Zheleznitsa, in Stara Reka river	N41°55′20″ E23°06′09″	328 m	25.04.2016 12:25 pm	1 juv.	
7.	near the village of Zheleznitsa, in Stara Reka river	N41°55′18″ E23°06′04″	331 m	25.04.2016 3:15 pm	1 ad., 1 juv.	
8.	near the village of Zheleznitsa, in Stara Reka river	N41°55′28″ E23°05′44″	351 m	25.04.2016 1:25 pm	1 juv.	
9.	near the village of Zheleznitsa, in Stara Reka river	N41°55′32″ E23°05′27″	363 m	25.04.2016 2:10 pm	1 ad.	
10.	near the village of Zheleznitsa, in Stara Reka river	N41°55′32″ E23°05′23″	366 m	25.04.2016 2:30 pm	3 juv.	
11.	west of the village of Gabrovo, in Gabrovska Reka river	N41°53′45″ E22°55′53″	791 m	01.04.2016 10:00 am	1 juv.	
12.	west of the village of Gabrovo, in Gabrovska Reka river	N41°53′49″ E22°55′55″	781 m	01.04.2016 11:40 am	1 ad., 1 juv.	
13.	west of the village of Gabrovo, in a puddle on a dirt road	N41°53′58″ E22°55′50″	812 m	01.04.2016 10:30 am	1 juv.	
14.	west of the village of Gabrovo, in a puddle on a dirt road	N41°53′43″ E22°56′09″	812 m	01.04.2016 12:10 pm	1 subad.	
15.	west of the village of Gabrovo, in a puddle	N41°53′44″ E22°56′15″	825 m	01.04.2016 12:20 pm	2 juv.	
16.	near Milovska Mahala neighbourhood (the village of Gabrovo), in a puddle	N41°53′32″ E22°56′35″	883 m	17.03.2016	3 ad.	
17.	near Malinovska Mahala neighbourhood (the village of Gabrovo), in a small marsh	N41°53′12″ E22°57′56″	894 m	01.04.2016 2:00 pm	several specimens	
18.	near Stopanska Mahala neighbourhood (the village of Gabrovo)	N41°52′38″ E22°56′52″	930 m	01.04.2016 2:10 pm	1 juv.	
19.	near Stopanska Mahala neighbourhood (the village of Gabrovo), in a stream	N41°52′42″ E22°56′45″	879 m	01.04.2016 2:30 pm	2 juv.	

20.	near Stopanska Mahala neighbourhood (the village of Gabrovo), in a stream	N41°52′43″ E22°56′46″	876 m	01.04.2016 2:35pm	2 juv.	
21.	Korianska Mahala neighbourhood (the village of Leshko), in a wooden trough	N41°55′58″ E22°56′34″	780 m	15.04.2016 10:30 am	2 subad.	
22.	near Gorno Leshko neighbourhood (the village of Leshko), in Gravalia reservoir	N41°55′24″ E22°56′41″	777 m	15.04.2016 11:20 am	many specimens	

Conservation status

All amphibian species found in the Protected Area "Oranovski prolom – Leshko" are protected according to the national and/or international legislation (Table 10). Three species are included in the Appendix II (species whose conservation requires the designation of special areas for habitat protection), six species are included in the Appendix III (species protected in the territory of the whole country), and only one species is included in the Appendix IV (species under protection and regulated exploitation) of the Biodiversity Protection Act of Bulgaria. Six of the amphibian species are protected according to the Habitats Directive. Only one species (*Bombina variegata*) is listed in the Annex II (animal and plant species of community interest whose conservation requires the designation of special areas of conservation), 5 species are listed in the Annex IV (animal and plant species of community interest in need of strict protection) and only one species is listed in the Annex V (animal and plant species of community interest whose taking in the wild and exploitation may be subject to management measures) of the Directive. All species are protected according to the Bern Convention, four species are listed in the Appendix II (strictly protected fauna species) and the other four - in the Appendix III (protected fauna species). All 8 species are included in the IUCN Red List of Threatened Species, as Least Concern. None of the amphibians are objects of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), and are included in the Red Data Book of the Republic of Bulgaria.

Table 10. Species conservation status

Abbreviations: BPA – Biodiversity Protection act of Bulgaria, 2015; **92/43** – Habitats Directive 92/43/EEC, 1992; **BERN** – Convention on the Conservation European Wildlife and Naturel Habitats, 1982; IUCN – **IUCN** Red List of the Threatened Species, 2009 (**LC** – Least Concern)

Species	BPA	92/43	BERN	IUCN
Salamandra salamandra	III	-	III	LC
Bombina variegata	II, III	II, IV	II	LC
Hyla arborea	II, III	IV	II	LC
Bufo bufo	III	-	III	LC
Bufotes viridis	III	IV	II	LC
Rana dalmatina	II	IV	II	LC
Rana graeca	III	IV	III	LC
Pelophylax ridibundus	IV	V	III	LC

Discussion

The diversity and distribution of the amphibians in the Protected Area BG0001022 „Oranovski Prolom – Leshko" has not been studied thoroughly till now. Totally, 8 species have been found in the studied area, one caudate amphibian and seven anurans. The occurrence of 4 species has been confirmed, and 4 other are reported for the first time for the area. All these species represent 36.4% of the total number of amphibians (22) distributed in Bulgaria. The most widely distributed species are *Pelophylax ridibundus* and *Bombina variegata*.

All of the amphibians are protected according to the national and international legislation.

The diversity and distribution of the amphibian species in the Protected Area will be studied in details in the next two years. It is expected the number of localities to increase considerably. Probably three other species will be found as there are suitable habitats for their existence. These are *Lissotriton vulgaris, Triturus ivanbureschi*, and *Rana temporaria*.

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DISTRIBUTION OF PEREGRINE EARTHWORMS (LUMBRICIDAE) IN RELATION TO AFFORESTATION AND LAND USE IN SOFIISKA MOUNTAIN, WESTERN BALKAN MOUNTAINS, BULGARIA

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Abstract

Aim: The aim of the paper is to describe the distribution patterns of peregrine earthworm species in different biotopes in relation to afforestration and land use of Sofiiska Mt. (part of Western Balkan Mts.).

Materials and Methods: Earthworms were collected by the formaldehyde method complemented with digging and hand sorting.

Results: This paper presents the first results on earthworm (Lumbricidae) fauna of Sofiiska Mountain. During the investigation six earthworm species were identified. Earthworm communities were dominated by peregrine species: *Aporrectodea rosea* (Savigny, 1826), *Lumbricus terrestris* Linnaeus, 1758 and *Octolasion lacteum* (Örley, 1881). *Autochthonous lumbricids* were found only in the old-growth forests.

Conclusion: The exploration revealed that afforestation and agriculture falicitate peregrine earthworms invasion. The secondary deciduous and coniferous forests have dense populations of peregrine species. Endemic and native earthworms were almost entirely replaced by peregrine species.

Keywords: earthworms, Lumbricidae, afforestation, Balkan Mts., Bulgaria.

Introduction

The Balkan Mountains (Stara Planina) are a mountain arc of the alpides, located in the northern part of the Balkan Peninsula. The Western Balkan ridge is over 190 km long with an average height of about 849 m a.s.l., runs in an east-west direction and forms a barrier separating temperate and subtropical climatic zones [1].

Before the incorporation of the Balkan Peninsula in the Turkish Empire in the 14/15th centuries there is little evidence of man related environmental changes in the Balkan Mts. Incorporation of the Balkans into the Turkish Empire and opening of the Muslim markets of the Near East for mutton, fur, leather and wool, increased grazing pressure. The upper timberline has been lowered by several hundred metres over the last 4-5 centuries. This phenomenon has been well documented by the paleobotanical studies [2], with secondary grass communities with developing on deforested areas [3].

Particularly intensive was the deforestation in 18th and 19th century, when the areas for pasture and agriculture increased drastically and large autochthonous forests were destroyed, and virgin forests were significantly fragmentased. Cattle-breeding, farming, forest fires, ore melting and shipbuilding, on the other, contributed to the significant decrease of forest land [4]. As a consequence in a very short period of less than 200 years

the slopes of the mountain were almost deforested and subjected to serious soil erosion [5]. Afforestation activities occurred in the early 1930's and were intensive from 1950 to 1975 year [6].

Rosa [7], Černosvitov [8] and Plisko [9] were among the early scientists who studied the earthworm fauna of the Balkan Mountains. Their work was continued by Šapkarev [10] and Szederjesi [11]. The earthworm fauna has recently been summarized by Stojanović et al. [12], who listed 28 species from the Bulgarian part of the Balkan Mts.

Six lumbricid species were recorded from Sofiiska Mountain, which constitutes 40.0% of the earthworm diversity in the Western Balkan Mountains. Previous investigations of the earthworm fauna from the entire territory of the Western Balkan Mountains in Bulgaria comprised 15 species (Table 1) registered by Plisko [9] and Šapkarev [10].

Table 1. Earthworm species registered from Western Balkan Mts.

Species	Habitat	Zoogeographical distribution type	Ecological group
Allolobophora chlorotica (Savigny, 1826)	on stream banks and wet soils	Peregrine	Endogeic
Aporectodea caliginosa (Savigny, 1826)	cultivated soils, meadows, secondary forests	Peregrine	Endogeic
Aporrectodea handlirschi (Rosa, 1897)	deciduous and mixed forests	Trans-Aegean	Endogeic
Aporrectodea jassyensis (Michaelsen, 1891)	wet soils, meadows	Trans-Aegean	Endogeic
Aporrectodea trapezoides (Duges, 1828)	cultivated soils, meadows, secondary forests	Peregrine	Endogeic
Aporrectodea rosea (Savigny, 1826)	cultivated soils, meadows, forests	Peregrine	Endogeic
Cernosvitovia biserialis (Černosvitov, 1937)	in caves and under stones near streams in forests and meadows	Endemic	Endogeic
Dendrobaena octaedra (Savigny, 1826)	under stones and moss in forests	Peregrine	Epigeic
Dendrodrilus rubidus subrubicundus (Eisen, 1874)	wet soils near brooks and under bark of rotten trees	Peregrine	Epigeic
Eisenia fetida (Savigny, 1826)	areas with very high organic matter content	Peregrine	Epigeic
Eisenia lucens (Waga, 1857)	under bark of decaying logs or under leaves in beech forests	Central-European	Epigeic
Eiseniella tetraedra tetraedra (Savigny, 1826)	on river banks	Peregrine	amphibiotic endogeic
Lumbricus rubellus Hoffmeister, 1843	cultivated land and forests. Also near river banks	Peregrine	Epigeic
Lumbricus terrestris Linnaeus, 1758	cultivated soils, forests, meadows	Peregrine	Anecic
O*ctolasion lacteum* (Orley, 1891)	cultivated soils, forests and meadows	Peregrine	Endogeic
Proctodrilus tuberculatus (Černosvitov, 1935)	forest soils	Trans-Aegean	Endogeic

Pop and Pop [13] reported that ubiquitous *Lumbricus terrestris* Linnaeus, 1758 and *Octolasion lacteum* (Orley, 1891) have invaded new habitats in Carpathian Mts., replacing mostly endemic or rare species of the former earthworm communities. The invasive species with wide ecological tolerance, replace totally or partially autochthonous species with narrow ecological tolerance.

Materials and Methods

The studied region covered Sofiiska Mountain, which is part of the Western Balkan Mountains with the highest peak Goten (1294 m a.s.l.). Sofiiska Mountain has length of 35 km and about 10-12 km width [1]. The oak (*Quercus petraea* Liebl.) and hornbeam (*Carpinus betulus* L.) forests, predominate up to the altitude of 600 m. Above, there is a zone of beech (*Fagus sylvatica* L.) and coniferous (*Pinus nigra* Arn., *P. sylvestris* L. and *Picea abies* (L.) Karst.) forests up to 900 m a.s.l. S subalpine plant communities consist of grasses dominated by *Festuca, Agrostis, Carex, Vaccinium*. The secondary forests following afforestation and succession predominate on the slopes of Sofiiska Mt. The old-growth forest have been almost destroyed and vegetation consists now of several tree species (*F. sylvatica* and *Salix alba* L.). All main habitat types, such as secondary deciduous and coniferous forests, old-growth forests, cultivated lands, meadows and stream valleys were explored in Sofiiska Mt.

The extensive field investigation was carried out during the period 2011-2014. Earthworms were collected by the diluted formaldehyde method [14] complemented with digging and hand sorting. The combination of both methods provides a more complete sampling of species, because the formalin method alone is not efficient for collecting species living in a horizontal burrows. The specimens were killed in 70% ethanol, fixed in 4% formalin solution and in 96% ethanol, then transferred into 75% ethanol and deposited in the private collection of Hristo Valchovski (PCHV). Specimens were dissected under low power microscope and described. In each sampling point, five squares (50 x 50 cm and 20 cm depth) were dug out and the earthworm specimens were hand sorted. Data from the each habitat were pooled and density adjusted to one square meter. Statistical analyses of soil characteristics were conducted using SPSS software. Data present mean ± standard deviation.

Results

Earthworm communities comprised of the peregrine species: *Aporectodea caliginosa* (Savigny, 1826), *A. trapezoides* (Duges, 1828), *A. rosea* (Savigny, 1826), *L. terrestris*, *O. lacteum* (Örley, 1881), and the native *Eisenia lucens* (Waga, 1857). *Lumbricus terrestris* and *O. lacteum* appear to be the most common species in the studied area. *Aporrectodea rosea* and *E. lucens* were less numerous. Ubiquitous *A. caliginosa* and were found only in the lowest parts of the mountain (Table 2).

In secondary deciduous and mixed forests *L. terrestris* and *O. lacteum* have dense populations. *Octolasion lacteum* was found in typically diploid and large parthenogenetic polyploid form. In cultivated lands beside *L. terrestris* and *O. lacteum* the earthworm species

from genus *Aporrectodea – A. rosea, A. caliginosa* and *A. trapezoides* were also registered. In secondary coniferous forests surprisingly no any earthworm species were found. Even the commonly presented lubricid species from genus *Dendrobaena* in pine and spruce forests were not registered in the observation. In meadows and succession lands with shrubs *A. rosea, L. terrestris* and *O. lacteum* were abundant. In the subalpine grasslands located on the highest parts of the mountain – *A. rosea* was found. *Eisenia lucens* was registered only from old-growth beech forests and stream valleys with willows. Specimens from this species were found under bark of old rotten trees or under rocks beneath forest torrents.

Afforestation and agriculture in Sofiiska Mt. cause spreading of peregrine earthworm species. *Aporrectodea rosea, L. terrestris* and *O. lacteum* have invaded new habitats in secondary forests and arable lands from Western Balkan Mts. Peregrine lumbricids replaced endemic and rare species of the autochthonous earthworm communities. Nowadays the old-growth forests remain the "hot-spots" of native earthworm biodiversity.

Table 2. Earthworm species collected from Sofiiska Mt. (part of the Western Balkan Mts.)

Habitat	Altitude (m a.s.l.)	Coordinates	Date of sampling	Species
arable land	550	42° 52' 52N 23° 23' 35E	12 06 2013	*A. caliginosa; A. trapezoides; A. rosea; O. lacteum*
mixed forest	685	42° 52' 29N 23° 24' 29E	02 11 2013	*L. terrestris*
secondary beech and oak forest	760	42° 52' 40N 23° 24' 43E	23 06 2012 09 04 2013 25 06 2013	*O. lacteum; L. terrestris*
meadow	905	42° 52' 21N 23° 25' 05E	14 05 2011 16 03 2014	*O. lacteum; L. terrestris; A. rosea*
stream valleys with old-growth rotten willows	920	42° 52' 24N 23° 25' 06E	12 06 2013	*E. lucens*
under rocks beside torrent in an old-growth beech forest	950	42° 52' 11N 23° 25' 04E	16 03 2014	*E. lucens*
subalpine grasslands	970	42° 52' 04N 23° 25' 03E	16 03 2014	*A. rosea*

Total lumbricid density from all seven explored habitats ranged from 4 to 40 ind./m^2 with general average of 15.14 ± 2.1 ind./m^2 (Table 3). Earthworm populations from arable lands and meadows were with higher density than secondary deciduous and mixed forests. Native earthworm fauna from old-growth forests were with lowest abundance. Populations of *O.lacteum* were with highest density (24 ind./m^2) than other peregrine species. Five of the explored sampling points have numerous individuals (16-8 ind./m^2) in different combinations of peregrine lumbricids (*A. rosea, A. caliginosa, A. trapezoids, O. lacteum* and *L. terrestris*). In general density of peregrine species was higher in habitats, which were more affected of anthropogenic activities – agriculture and afforestation. Rare

and endemic earthworm species have lower density with populations concentrated in old-growth forests.

Table 3. Average abundance of earthworm species and total density of lumbricid populations

Habitat	Species abundance (ind./m^2)	Total density (ind./m^2)
arable land	*A. caliginosa* – 16±1.4 *A. trapezoides* – 8±1.2 *A. rosea* – 8±1.1 *O. lacteum* – 4±0.8	32±2.1
mixed forest	*L. terrestris* – 8±0.9	8±0.9
secondary beech and oak forest	*O. lacteum* – 4±1.1 *L. terrestris* – 4±0.9	8±1.1
meadow	*O. lacteum* - 24±4.3 *L. terrestris* - 8±1.6 *A. rosea* – 8±1.3	40±3.8
stream valleys with old growth rotten willows	*E. lucens* – 4±2.1	4±2.1
under rocks beside torrent in an old-growth beech forest	*E. lucens* – 4±1.5	4±1.5
subalpine grasslands	*A. rosea* - 10±3.1	10±3.1

Discussion

The current research from Sofiiska Mt. revealed that the invasive species with wide ecological tolerance replace the native earthworm species with narrow ecological tolerance. Similar changes in earthworm communities were registered in Carpathian Mountains by Pop and Pop [13].

The lumbricid fauna of the Bulgarian part of the Western Balkan Mts. the peregrine earthworms make more than 11 taxa = 73.3% of the total lumbricid species. Trans-Aegean species take part with 3 taxa = 20.0 %. The Balkan Mountains belong to the north-Aegean subdomain [15], which is the reason for presence of Trans-Aegean lumbricids. Endemic (1 taxon = 6.66%) and Central European (1 taxon = 6.66%) are less numerous. The low presence of endemic species is due to deforestation, land use and finally soil erosion of mountain slopes.

This field study showed shifts of earthworm communities in response to land use and afforestation activities. The secondary deciduous and coniferous forests have dense populations of peregrine species. Endemic and native earthworms were almost entirely replaced by peregrine species. Populations of autochthonous earthworm fauna were concentrated in old-growth forests and in the context of maintenance the biodiversity these ecosystems should be of a top conservation priority. Urgent investigations on belowground soil diversity need to be undertaken in forests from territory of the Western Balkan Mts. With focus on evaluation the distribution of native lumbricids and to prevent their extinction.

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SPECIES RICHNESS OF *DENDROBAENA* EISEN, 1873 (CLITELLATA: LUMBRICIDAE) GENUS IN TURKEY AND BULGARIA

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Abstract

Aim: The aim of the paper is to present a review on species richness of genus *Dendrobaena* (Clitellata: Lumbricidae) in Turkey and Bulgaria.

Results: The present study provided the first list of *Dendrobaena* earthworm taxa of Turkey and Bulgaria. In the explored regions 34 species are recorded, 30 of which are registered from Turkey, while in Bulgaria only 9 species were recovered. Five taxa were registered both for the territory of Turkey and Bulgaria. The genus is represented by 15 endemic species (44.1%) while peregrine species take only small part - 3 taxa (9%).

Conclusion: Among the lumbricid genera on the territory of Turkey and Bulgaria *Dendrobaena* is the species richest genus. The study revealed that conservation strategy is needed for the numerous group of rare and endemic *Dendrobaena* species to prevent them from extinction.

Keywords: Biodiversity, Bulgaria, Earthworms, *Dendrobaena*, Lumbricidae, Turkey.

Introduction

The genus *Dendrobaena* Eisen, 1873 was founded by Eisen [1]. Before that, species from the genus were considered as members of many different genera – *Enterion, Allolobophora, Helodrilus* and *Eophila*. The phylogenetic relationships among the species of *Dendrobaena* genus are yet unclear. Work in this particular domain is difficult due to polymorphism in certain species such as *Dendrobaena alpina* and *Dendrobaena veneta*.

Numerous scientists published data about *Dendrobaena* fauna in Turkey [2, 3, 4, 5, 6] and Bulgaria [7, 8, 9]. Their work were continued by [10, 11, 12, 13].

The genus *Dendrobaena* have three evolution and distribution centres: Carpatho-Balkan area, Trans-Caucasus and Franco-Iberian region [14]. It is not surprising that *Dendrobaena* species are a dominant taxon in the earthworm fauna of Bulgaria and Turkey.

Results

The literature review revealed 33 *Dendrobaena* species. In Turkey the genus is presented by a great number of taxa – 30 species. Their zoogeographical distribution type also is given. In Bulgaria 9 *Dendrobaena* species are distributed (Table 1). Five taxa are recorded both for the territory of Turkey and Bulgaria – *Dendrobaena alpina, D. attemsi, D. byblica, D. hortensis* and *D. veneta*.

Table 1. Distribution and origin of *Dendrobaena* species from Turkey and Bulgaria.

Species	Origin	Distribution
Dendrobaena alpina (Rosa, 1884)	Balkanic-Alpine	Bulgaria, Turkey and widely distributed in Europe
Dendrobaena attemsi (Michaelsen, 1902)	Balkanic-Alpine	Bulgaria, Turkey and widely distributed in Europe
Dendrobaena balcanica (Černosvitov, 1937)	Balkan endemic	Bulgaria and Greece
Dendrobaena bruna Omodeo & Rota, 1989	Endemic	Turkey
Dendrobaena byblica (Rosa, 1893)	Circum-Mediterranean	Cosmopolitan.
Dendrobaena cevdeti Szederjesi, Pavlicek, Coşkun & Csuzdi, 2014	Endemic	Turkey

Dendrobaena cognettii (Michaelsen, 1903)	Balkanic-Anatolian	Austria, Croatia, France, Greece, Hungary, Italy, Portugal, Spain, Switzerland and Turkey
Dendrobaena decipiens (Michaelsen, 1910)	Caucasus-Anatolian	Turkey
Dendrobaena fridericae Omodeo & Rota, 1989	Endemic	Turkey
Dendrobaena fridericae uludagi Omodeo & Rota, 1991	Endemic	Turkey
Dendrobaena hauseri Zicsi, 1973	Levantine-Anatolian	Israel and Turkey
Dendrobaena hortensis (Michaelsen, 1890)	Peregrine	Cosmopolitan
Dendrobaena hrabei (Černosvitov, 1934)	Balkan endemic	Austria, Bulgaria, Czech Republic, Greece, Hungary
Dendrobaena mahunkai Csuzdi, Pavlíček & Mısırlıoğlu 2007	Endemic	Turkey
Dendrobaena montana (Michaelsen, 1910)	Caucasus-Anatolian	Turkey
Dendrobaena nivalis Omodeo & Rota 1989	Endemic	Turkey
Dendrobaena octaedra (Savigny, 1826)	Peregrine	Cosmopolitan
Dendrobaena omodeoi Csuzdi, Pavlíček & Mısırlıoğlu, 2007	Endemic	Turkey
Dendrobaena orientalis Cernosvitov, 1940	Levantine	Armenia, Iran, Israel, Jordan, Turkey
Dendrobaena orientaloides Zicsi, 1985	Endemic	Turkey
Dendrobaena pantaleonis eutypica Omodeo & Rota, 1989	Endemic	Turkey
Dendrobaena pentheri (Rosa, 1905)	Caucasus-Anatolian	Cyprus, Georgia, Greece, Turkey
Dendrobaena persimilis Omodeo & Rota, 1989	Endemic	Turkey
Dendrobaena perula Omodeo & Rota, 1989	Endemic	Turkey
Dendrobaena proandra Omodeo & Rota, 1989	Endemic	Turkey
Dendrobaena ressli Zicsi, 1973	Caucasus-Anatolian	Turkey
Dendrobaena rhodopensis (Černosvitov, 1937)	Balkan endemic	Bulgaria, Montenegro, Serbia
Dendrobaena samarigera (Rosa, 1893)	Levantine-Anatolian	Israel, Lebanon, Syria and Turkey
Dendrobaena schmidti marinae Kvavadze, 1985	Caucasus-Anatolian	Italy, Russia and Turkey
Dendrobaena schmidti tellermanica Perel, 1966	Caucasus-Anatolian	Georgia, Russia and Turkey
Dendrobaena semitica (Rosa, 1893)	Levantine	Cisjordan and Gaza, Cyprus, Israel, Jordan, Lebanon, Syria, Turkey
Dendrobaena surbiensis (Michaelsen, 1910)	Caucasus-Anatolian	Georgia and Turkey
Dendrobaena szalokii Szederjesi, Pavlicek, Coşkun & Csuzdi, 2014	Levantine-Anatolian	Turkey
Dendrobaena veneta (Rosa, 1884)	Peregrine	Cosmopolitan

Discussion

In Turkey, among the recorded 80 earthworm species, 30 (37.5%) belong to genus *Dendrobaena* which is the species richest genus in the country [3]. Besides, 12 of them are endemic (40% of *Dendrobaena* species). Six taxa are with Caucasus-Anatolian range – 20%. Less numerous are the Levantine-Anatolian species (3 taxa – 10%), the Balkanic-Alpine (2 taxa – 6.66%), the Levantine (2 taxa – 6.66%) and the Peregrine species (2 taxa – 6.66%). The Balkan-Anatolian, Circum-Mediterranean and Trans-Aegean (3.33%) types are represented by one *Dendrobaena* species each. In Turkey the most common *Dendrobaena* species are *D. hortensis* and *D. veneta* [3].

In Bulgaria from 50 registered earthworm species [15], 9 (18%) belong to the *Dendrobaena* genus which is the species richest genus in the country. Among the *Dendrobaena* domain 3 taxa are endemic (33.3%). They are Balkan endemic species. 3 species are peregrine (33.3%). 2 taxa belong to the Balkanic-Alpine type (22.2%) and one taxon is Circum-Mediterranean (11.1%). The most common *Dendrobaena* species in Bulgaria are *D. alpina, D. attemsi* and *D. octaedra*.

Genus *Dendrobaena* contains many endemic species in the studied countries, among the 34 earthworm species registered in Turkey and Bulgaria 15 are endemic - 44.1%.

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FIRST DATA ON THE OLIGOCHAETA (ANNELIDA: CLITELLATA) COMPLEX IN SOME BULGARIAN EPHEMERAL WATER BODIES

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Abstract

Aim: The aim of the study is to determine which Oligochaeta species occur in temporal water bodies and what environmental factors determine the content and density of the oligochete complex.

Materials and Methods: The study represents data from 35 samples, taken in 2013-2014 from two temporary rivers, two temporary lakes and one artificial pond in Eastern and Western Bulgaria. The samples were taken in 15 days intervals during the period May-August. The species content, abundance and seasonal distribution pattern of the oligochets were analysed.

Results: A total of 18 taxa were identified (8 species of Naididae, 7 of Tubificidae and 1 of Lumbricidae, Lumbriculidae and Enchytraeidae). The species composition shifted along the hydroperiod gradients. During the early stage of the newly formed water bodies naidids species were the only representatives amongst the oligochets. As a result of a quick parthenogenesis naidids appeared in high density. In temporary aspect the density of naidids decreased and they were replaced by tubificid species.

Conclusion: During the hydroperiod the dynamics of the depth and wetland size have a significant influence on the environmental factors, which may contribute to the positive relationship with the number of species. Macroinvertebrate richness and abundance increased linearly along the hydroperiod gradient in response to changes of temperature and dissolved oxygen concentrations.

Keywords: temporary lakes, temporary rivers, Oligochaeta, abundance, life strategy

Introduction

Temporary fresh waters are bodies of water that experience a re-current dry phase of varying length that is sometimes predictable in both its time of onset and duration. This definition sets them apart from those permanent waters which may occasionally experience a drought but which contain faunas that are generally intolerant of water loss [1].

Wetland hydroperiod is known to influence invertebrate richness and community composition [2], abundance [3], and reproductive success [3]. The response of organisms

to wetland hydroperiod may largely depend on changes in the degree and frequency of disturbance events along the hydroperiod gradient [4]. As a habitat, temporary waters vary more in their physical and chemical environment than permanent fresh waters. Such extremes in the conditions demand that the fauna of temporary waters develop one or more strategies in order to exploit successfully the rich resources and the freedom from competition and predation press that often occurs in these habitats.

The Bulgarian Oligochaeta fauna from drying streams and ponds has never been studied in details. The aim of the study was to determine which Oligochaeta species occur in these types of water bodies and what environmental factors determine the composition and density of the oligochaete complex.

Materials and Methods

Site selection

The investigations were carried out on 35 benthic samples of two temporary rivers, two temporary lakes and one artificial pond (Fig. 1) in the east and west part of Bulgaria. The model objects were selected to present temporal water bodies in karst areas. The chosen rivers have a hydroperiod very common with those in the Mediterranean zone. Most of these rivers are drying up tributaries of bigger ones, but the model objects in this study are independent lotic water bodies with such a hydrological regime.

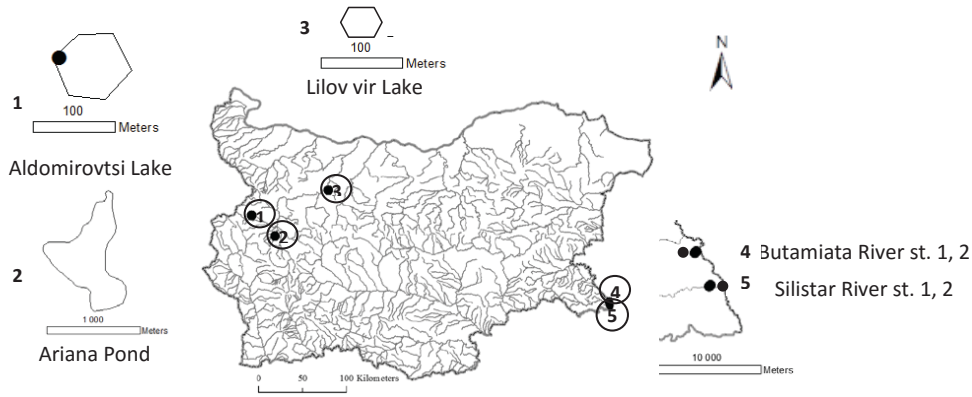

Fig. 1. Studied water bodies (author: Galia Georgieva)

Study Area

Aldomirovtsi Lake/marsh: the season climate changes determine a cyclic recurrence in the periods of drought, which assign the lake to the category of drying up lakes. The lake/marsh is situated in a karst region and the water can easily soak up through the karst. Most of the protected area (130 ha) is covered with reed. The real lake cup is 10 times smaller and free of fringing vegetation but the bottom is fully covered with Charophyta and *Myriophyllum* spp. (Table 1). The marsh is a protected landscape (IUCN V), defined for protection of the habitats of protected and rare species of waterfowl and plant associations of 40 species of higher plants.

Lilov vir Lake/marsh is a typical ephemeral marsh that in dry periods might not be flooded for years and in rainy periods might be full all year-round. Usually the hydroperiod is 1-3 months. The marsh is situated in one of the biggest karst regions in the country.

Ariana Pond is an artificial lake in the capital city of Sofia. The pond is flooded for 5 months and every year the bottom is scrubbed out.

Butamiata River: the river mouths into the Black Sea and during the summer the outflow is filled with sand and becomes a stagnant water body. The river flows through the Nature Park Strandzha Mountain - part of the National Ecological Network NATURA 2000 but its main part runs through oak forest.

Silistar River: the river flows south of the Butamiata River in the same protected area. In summer the mouth in the sea is filled with sand and fringed with reed.

Table 1. Morphometric characteristics of the studied water bodies

Water body	Altitude (m)	Area size (ha)	Depth variability (m)	Bottom character	Hydroperiod (days)
Aldomirovtsi L.	650.5	130	0.3-1.3	silt, macrophytes	107*
Ariana L.	542.9	3	0.8-0.9	concrete	120
Lilov vir L.	536.6	4	0.2-1.1	silt, macrophytes	120*
Butamiata st.1	4.6		0.25-0.45	gravel	110**
Butamiata st.2	1.7		1.2-1.6	sand	110**
Silistar st.1	28.3		0.4-0.95	gravel	110**
Silistar st.2	12.1		0.7-1.75	sand	110**

*In 2014 the Aldomirovtsi L. and the Lilov vir L. had no dry phase and their hydroperiod was relative.

**The studied rivers are lotic water bodies with relatively dry (stagnant) period of about 90-100 day per year.

Methods

The samples were taken at one sampling site in each lake in 15 days intervals during the period May-August, according to the hydroperiod. Two sampling sites from the rivers were chosen – one in the woodland and one in the outflow.

Macrozoobenthos was collected according to the adapted version of the multi-habitat sampling methodology (as described in details by [5]). Benthic samples were taken in correspondence to the European standards EN ISO 10870:2012 using a hand-net. The main physical and chemical parameters of the water body (pH, conductivity, temperature, dissolved oxygen and oxygen saturation, depth and transparency) were measured *in situ* along with the nutrients concentrations (NH_4-N, NO_2-N, PO_4-P, total phosphorus, total N) *in lab*. The measurements and analysis were accomplished according to the actual Bulgarian and European standards. The species composition, seasonal distribution pattern and abundance of the oligochets were measured. Oligochaete taxa were identified to the

lowest taxonomic level possible. The statistical analysis of the data was performed in SPSS v 20.0.0.

Results and Discussion

As a result a total of 18 taxa were identified (see Tables 4a and 4b), comprising of 8 species from Naididae, 7 from Tubificidae and 1 from Lumbriculidae. Preferably terrestrial species from Lumbricidae and Enchytraeidae were also recorded (Table 2a, b). The species composition shifted along the hydroperiod gradients. Boix *et a*l. [6] pointed out that the length of the hydroperiod and the seasonality are the main factors in determining the faunal composition and the structure of temporary aquatic communities.

Some differences in the Oligochaeta complex in ephemeral lentic and lotic water bodies were observed but the first inhabitants always were naidids. During the early stage of the newly formed water bodies the Naididae species were the only inhabitants. In order to conquest the new ecological niche they reproduced quickly with parthenogenesis. In result of this life-strategy naidids appeared in high density. As the hydroperiod increases, vertebrate and invertebrate predators become more diverse and abundant, and exert a more profound influence on the benthic community and oligochets in particular [7] and [8]. In temporary aspect, with the increase of the predator press and changes of the environmental conditions, the density of naidids decreased and they were replaced by tubificid species. The studied rivers incorporate unique and local hydrological conditions as the Mediterranean temporary water bodies [9]. In the stagnant zone outflows no species from genera *Limnodrilus* and *Tubifex* were registered. Upstream the rivers the number of naidid species was higher and the number of species as total was higher in the lotic than in the lentic permanent waters. Permanent rivers in Strandzha Mountain were studied [10]. In order to compare the oligochet composition from permanent and temporal rivers, we used data for Veleka River at Brodilovo village and in the mouth [11] and the corresponding sampling sites in the studied rivers. The site at Veleka R. was chosen because of its location, as it is far from the mouth as like as the sites from Silistar and Butamiata. A low level of species similarity (after Sorensen's coefficient) between the oligochaete complex of the permanent and temporal rivers was calculated (Table 2).

Table 2. Species similarity (after Sorensen's coefficient) between the oligochete complex of permanent and temporal rivers

	Silistar st.1	Butamiata st. 1	Silistar st.2	Butamiata st. 2
Veleka st 1.	14.28%	0%	-	-
Veleka st. 2	-	-	0%	25%

*Veleka st. 1-Brodilovo, st. 2 - outflow

In the natural temporary wetlands due to the extreme conditions only a few colonizing species occur. On the contrary is the situation in the artificial Ariana L. where only 2 species inhabited and were with high density. The annual drain out of the lake and scrub

the concrete bottom makes the inhabiting easier for some of the common oligochets. Some negative statistical significant correlation (SPSS Pearson's correlation) between PO_4 and the number of some naidids (*D. digitata, N. pardalis*) and positive with *Rh*. *coccineus* was calculated. Total nitrogen (TN) showed negative correlation with *L. claparedeanus*. The species *Rh*. *coccineus* showed a positive correlation with PO_4 and TN. Enchytraeidae sp. correlated positively with dissolved oxygen, TP (Total phosphorous) and nitrite forms. *D. digitata* had negative correlation with the depth and the transparency (after Secchi) respectively.

Table 3. Pearson's correlation between some environmental factors and oligochete taxa (N=21)

	L. variegatus	*D. digitata*	*Limnodrilus* ssp. juv.	*L. claparedeanus*	*N. elinguis*	*N. communis*	*N. pardalis*	*Lumbriculidae* spp. juv.	*Enchytraeidae* spp.	*Rh. coccineus*	*E. velutinus*
O_2 mg	-,015	-,222	,163	,127	,009	,237	-,261	,287	**,498***	-,349	-,077
O_2 perc	,004	-,196	,175	,138	,020	,288	-,237	,268	**,488***	-,338	-,133
Cond	,424	,175	,291	,165	-,292	,190	-,368	-,059	-,008	-,281	-,106
Temp	-,165	-,230	-,066	,115	-,246	-,165	-,145	-,326	,033	-,200	-,217
PO_4	-,012	**-,083****	-,050	-,050	-,113	,039	**-,068****	-,071	-,066	**,991****	-,079
TP	,159	,020	,107	,165	-,181	-,199	-,225	,288	**,700****	-,188	,055
NH_4	-,177	-,173	-,105	-,105	-,076	,378	-,136	-,048	-,045	-,038	-,081
NO_2	-,064	-,132	-,069	-,052	-,232	-,123	-,139	,142	**,485***	-,116	-,162
NO_3	,298	,251	,220	,220	**-,446***	-,254	-,020	-,088	,135	-,049	-,034
TN	-,016	-,090	-,055	-,055**	-,123	,034	-,074	-,044	-,066	**,994****	-,055
Debt	-,318	**-,530***	-,308	,272	**,535***	,006	-,295	,148	,192	-,240	-,017
Secchi	-,277	**-,471***	-,269	,401	,335	,148	-,208	,093	-,092	-,165	,177

* Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed).

O_2mg – Dissolved oxygen ($mg.l^{-1}$); O_2perc - Oxygen saturation (%); Cond – conductivity ($\mu S.cm^{-1}$); Temp Water Temperature (ºC); PO_4–P ($mg.l^{-1}$); TP- total phosphorus ($mg.l^{-1}$); NH_4–N ($mg.l^{-1}$); NO_2–N ($mg.l^{-1}$); NO_3–N ($mg.l^{-1}$); TN – total nitrogen ($mg.l^{-1}$); Debt – depth (m); Secchi – transparency (m).

The three types of temporary water bodies are separated depending on the impact of environmental factors on the oligochete complex (Fig. 2a and Fig. 2b).

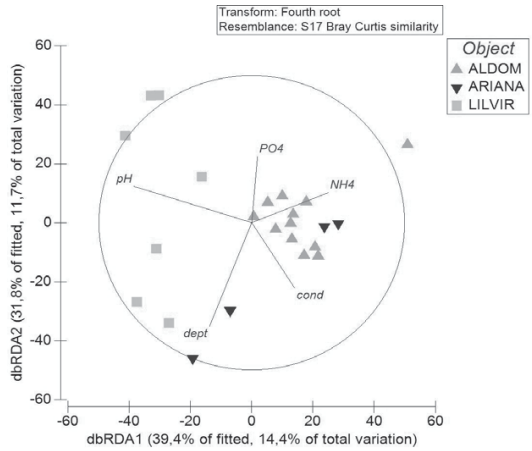

Fig. 2a. CCA of the environmental parameters and the abundance of the oligochete species in the lakes

Aldomirovtsi Lake is the closest in nature to the permanent swamps and nutrients are a major factor for the oligochets diversity.

Lilov Vir Lake is the kind of seasonal drying up water bodies. The depth here is quite variable, influenced by the amount of precipitation. The changes in the depth and respond to other environmental factors‘ fluctuations.

Ariana Lake is an artificial water body and is not affected by rainfall and droughts. Here the first succession stars every year from the very beginning. Species diversity is influenced by the depth and amount of nutrients.

There is some separation in different points in temporarily dried up rivers (Fig. 2b):

At sites 1 of the two rivers (located in the forest - green and light blue markings) the oligochete complex depends on the nitrite and phosphate.

At sites 2 (estuaries - dark blue and red) a connection can be seen with the concentration of oxygen, nitrates and temperature, which vary during the spring and the summer.

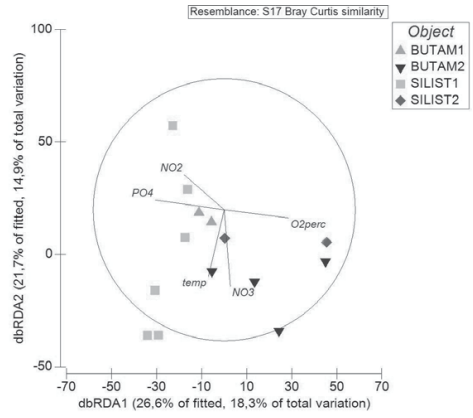

Fig. 2b. CCA of the environmental parameters and the abundance of the oligochete species in the rivers

During the hydroperiod the oligochete complex changes. The first settlers are the naidids that with paratomiya split their bodies and rapidly conquer the new ecological niche. After the accumulation of the organic deposits follows the development of the tubificid forms. In Lilov Vir L. this trend continues, but clearly it is expressed in pond Ariana. Although the concrete bed is cleaned before every filling, apparently the naidids survive the period of drought in the form of eggs and cysts and begin quickly parthenogenesis. While lovers of soft substrate tubificids are represented in much smaller numbers and species diversity.

Table 4a. Taxa list of the oligochets found in the studied temporary lakes

	Aldomirovtsi Lake									Lilov vir Lake							Ariana Lake			
	01/07/13	12/05/14	27/05/14	13/05/15	27/05/15	09/06/15	23/06/15	07/07/15	04/08/15	11/05/14	26/05/14	12/08/14	26/05/15	10/06/15	08/07/15	19/07/15	30/05/13	04/07/13	27/05/15	09/06/15
Nais elinguis Muller, 1774		*	*																	
Nais communis Piguet, 1906																	*	*		
Nais pardalis Piguet, 1906																		*		
Dero digitata (Műller, 1773)	*				*															
Dero obtusa Udekem, 1855				*																
Pristina bilobata Bretscher, 1903									*											
Aulophorus furcatus (Oken, 1815)				*																
Lumbriculus variegatus (Műller, 1774)	*																			
Potamothrix hammoniensis (Michaelsen, 1901)				*										*	*	*				
Tubifex tubifex (Műller, 1774)	*					*														
Tubificidae spp. juv.	*				*	*			*			*								
Limnodrilus claparedeanus (Ratzel, 1868)	*																			
Limnodrilus sp. juv.	*				*	*	*	*					*						*	*
Psammoryctides albicola (Michaelsen, 1901)								*												
Lumbricidae g. sp.										*										
Enchytraeidae g. sp.										*	*									

Table 4b. Taxa list of the oligochets found in the studied temporary rivers

	Butamiata River st. 1			Butamiata River st. 2				Silistar st. 1						Silistar st. 2	
	23/05/14	23/05/15	30/06/15	23/05/14	01/07/14	23/05/15	30/06/15	23/05/14	01/07/14	01/08/14	23/05/15	30/06/15	30/07/15	23/05/15	23/06/15
Nais elinguis Muller, 1774	*		*	*			*				*			*	
Nais communis Piguet, 1906					*		*	*							
Nais variabilis Piguet, 1906		*	*								*	*			
Limnodrilus sp. juv.											*				*
Lumbriculus variegatus (Műller, 1774)														*	
Tubificidae g. sp. juv.		*				*		*							
Potamothrix spp. juv.									*	*					
Potamothrix hammoniensis (Michaelsen, 1901)											*	*	*		
Rhyacodrilus coccineus (Vejdovsky, 1875)								*							
Embolocephalus velutinus (Grube, 1879)								*	*	*	*				
Lumbriculidae g. sp. juv.								*				*			
Enchytraeidae g. sp.								*							

Conclusion

During the hydroperiod the dynamics of the depth and wetland size have a significant influence on the environmental factors. Macroinvertebrate richness and abundance increased linearly along the hydroperiod gradient in response to changes of the temperature and the dissolved oxygen concentrations. During the dry period in the intermittent rivers the current is interrupted and the lotic water body turns into lentic. In the upstream of the studied rivers, flowing through oak woodlands, this phenomena and the rich ecotone zone makes the invasion of new species possible. In the outflow no differences in the species community were observed, because there is no change in the water body characteristics due to the nearly permanent lake of current. The species composition in oligochete complex undergoes significant changes during the hydroperiod. Naidids survive the dry period in eggs and multiply rapidly after the development of the peryphyton, which they use as a trophic base. Thus they are the first inhabitants of artificial temporarily dried up water bodies. However, data are insufficient and for more correct conclusions long term observations are required.

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NEW DATA ON THE DISTRIBUTION OF DOTHIDEOMYCETES AND SORDARIOMYCETES (ASCOMYCOTA) IN BULGARIA

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Abstract

Aim: This work presents recently discovered country records of stromatic and non-stromatic pyrenomycetes.

Materials and Methods: The size of the microscopic characters is given in the form of minimum and maximum values. Measurements under LM were usually taken in water. The microscopic features were examined in water and in solution of Cotton Blue in lactophenol. Photographs were taken with the help of Canon PS A460 under Boeco microscopes.

Results: The distribution of *Diatrypella favacea, D. quercina* (Xylariales), *Gnomonia geranii-macrorrhizi, Gnomoniopsis comari* s. lat., *Mamiania fimbriata*, O*phiognomonia rosae, Plagiostoma apiculatum* (Diaporthales) and *Pleospora herbarum* (Pleosporales) is given according to the data published. *Pleospora herbarum* (Pleosporales) is found on dead twigs of *Amorpha fruticosa* and *Buddleja davidii*. All of the species except *P. herbarum* are reported from new localities.

Conclusion: Seven pyrenomycetous fungi are reported from new localities (Forebalkan, Stara Planina Mts, Sofia region, Vitosha region, Rila and Rhodopi Mts), while *D. quercina, P. apiculatum* and *P. herbarum* are collected on new host-plants.

Keywords: *Diatrypaceae*, fungal diversity, *Gnomoniaceae, Pleosporaceae*

Introduction

During a period of over 12 years, studies on fungal diversity of pyrenomycetes in different parts of Bulgaria were carried out. With the help of standard light microscopic methods 8 pyrenomycetous fungi, with so far unknown localities or new substrata in the country, from Sordariomycetes (*Xylariales, Diatrypaceae; Diaporthales, Gnomoniaceae*) and Dothideomycetes (*Pleosporales, Pleosporaceae*) were recorded. Available information up to date, concerning the distribution of the examined species, follows the publications used.

Materials and Methods

The majority of the collected specimens studied herein are conserved at the Mycological Collection, Institute of Biodiversity and Ecosystem Research (SOMF). The size of the ascomata, asci and ascospores is presented in the form of mininum-maximum values. Measurements under LM were made from digital images taken in water with the help of software Carnoy 2.0 (© Peter Schols, 2001). The microscopic features were examined in water and in water solution of Cotton Blue in lactophenol. All photographs were taken with the help of Canon PS A460 under Boeco BM-180/T/SP LM and Boeco BOE3500 dissecting microscope. The identification is justified after [1, 2, 3, 4 and 5]. All of the fungal specimens were collected by the author, unless other stated.

Results and Discussion

Sordariomycetes
Xylariales
Diatrypaceae
Diatrypella (Ces. & De Not.) Nitschke, Pyr. Germ., p. 69, 1867.

Stromata conical-truncate, cushion-like or discoid, usually delimited by a black zone in host tissues, with umbilicate or sulcate ostioles. **Asci** cylindrical, polysporous, long stipitate. **Ascospores** allantoid, hyaline or yellowish.

Diatrypella favacea (Fr. : Fr.) Ces. & De Not., Comment. Soc. Crittog. Ital., **1**(4): 205, 1863 (Figs 1-2).

Stromata with ellipsoid ectostromatic disc and large, black, 6-sulcate ostioles at whitish or pallid-brownish surface, arranged in 2 rows. **Asci** (65–)70–85(–90) × 9–11 µm, clavate. **Ascospores** 7–8 × 2–2.3 µm, oblong ellipsoid, hyaline.

Specimen examined: Forebalkan (eastern), Lovech distr., Golyama Zhelyazna village, 07.09.2007, D. Stoykov, on dead twig of *Betula pendula* Roth (*Betulaceae*), sub *Valsa ceratosperma* (Tode : Fr.) Maire [5], SOMF 27597.

Known distribution: Vitosha region, Pirin and Rila Mts. On twigs of *B. pendula, Carpinus betulus* L., *C. orientalis* Mill. and *Corylus avellana* L. (*Betulaceae*) [6, 7, 8].

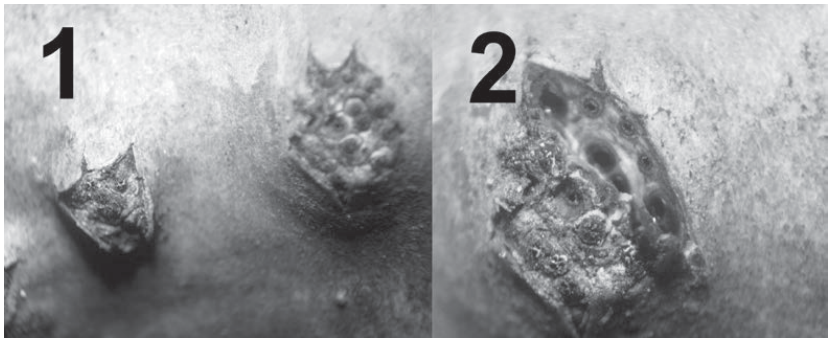

Fig. 1. Stromata of *Diatrypella favacea* on birch; Fig. 2. Cross section of stroma

D. quercina (Pers. : Fr.) Cooke, J. Bot. British and Foreign, 4: 99, 1866 (Figs 3-4).

Stromata 1.3–2.6(–3) mm in diam, dark brown to black, with ellipsoid ectostromatic disc and 6-sulcate ostioles at whitish or pallid-brownish surface, arranged in 2 rows. **Asci** 90–135(–150) × 12–14 µm, clavate to fusiform. **Ascospores** (7–)8–9(–10.5) × (2–)2.5–3.5(–4) µm, oblong ellipsoid, hyaline, ± curved.

Specimens examined: Stara Planina Mts: Sofia distr., between the villages of Chibaovtsi and Tsaritchina, 16.05.2011, leg. I. Assyova, det. D. Stoykov, SOMF 26643, on bark of old oak tree; Sofia region: Sofia city, Darvenitsa, in the yard of the Institute of Forest, Bulgarian Academy of Sciences, 15.06.2006, D. Stoykov, on dead twig of *Quercus thracica* Stef. & Nedjalkov (*Fagaceae*); Sofia city, Northern Park, 19.04.2015, D. Stoykov, SOMF 26624, on dead twigs of *Quercus rubra* L.; ibid., 05.03.2016, D. Stoykov, SOMF 26650.

Note. These are the second reports of *D. quercina*. Previously it was known from Vitosha region on twigs of oak [9]. *Quercus thracica* and *Q. rubra* are new host-plants of *D. quercina* for Bulgaria.

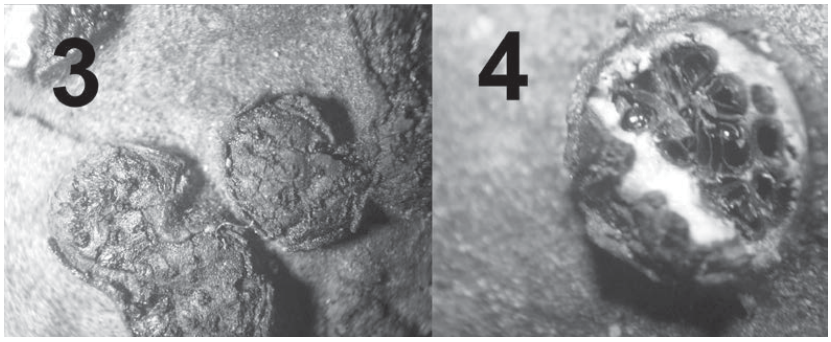

Fig. 3. Stromata of *D. quercina* on *Q. rubra*; Fig. 4. Cross section of stroma

Diaporthales

Gnomoniaceae

Gnomonia geranii-macrorrhizi Fakirova, Mycotaxon, 54: 329, 1995.

Specimens examined: Rila Mts: Rilomanastirska Gora Reserve, along the track from Rilski monastery to Brichebor peak, 25.05.2015, D. Stoykov, SOMF 26647; Rila National Park, Ibar Reserve, 08.06.2015, SOMF 26648; Centralen Rilski Reserve, along the track to Sarugyol circus, 02.07.2015, D. Stoykov, SOMF 26649, on dead petioles of *Geranium macrorrhizum* L. (*Geraniaceae*); The Rhodopes (central): Plovdiv distr., the town of Asenovgrad, along the road to Koru Dere locality, 04.07.2004, D. Stoykov, SOMF 28634, on dry petioles of *G. macrorrhizum*.

Known distribution: Sofia region, Forebalkan (eastern, western), Vitosha region, Belasitsa Mt, Slavyanka Mt, Pirin Mts, Sredna Gora Mts (western) and Western Rhodopes [5].

Note. Recently the fungus has been recorded in Western Europe (France) on dead petioles of *G. macrorrhizum* by Alain Gardiennet (pers. comm.)

Gnomoniopsis comari (P. Karst.) Sogonov, in Sogonov, Castlebury, Rossman, Mejía & White, Stud. Mycol., **62**: 47, 2008, **s. lat.**

Specimen examined: The Rhodopes (central): Plovdiv distr., Asenovrgad, Koru Dere locality, 04.07.2004, D. Stoykov, SOMF 28644. On dead stems of *Agrimonia eupatoria* L. (*Rosaceae*).

Known distribution: Black Sea coast (southern), Northeastern Bulgaria, Forebalkan (eastern), Stara Planina Mts (central – Gyosheva et al., in press; western), Sofia region, Vitosha region, Slavyanka Mt, Mesta River valley, Pirin Mts, Sredna Gora Mts (western), Western Rhodopes, Thracian Lowland [5, 10].

Note. For a detailed description see [5].

Mamiania fimbriata (Pers. : Fr.) Ces. & De Not., Comment. Soc. Crittog. Ital., **1**(4): 211, 1863 (Figs 5-6).

Stromatic capsules 1.3–2 mm in diam, always immersed in the leaf blades, with up to 10 ascomata. **Perithecia** black, globose to depressed globose. **Beaks** 300–470(–550) µm long, black, central, short, projecting the stoma from the underside of the leaves. **Asci** 40–47 × 7–8(–8.5) µm, ellipsoid, with irregularly biseriate ascospores and apical annulus ca 3 µm. **Ascospores** 8–9(–11.5) × 3–3.5(–4) µm, septum at 1/5 of the spore length; without appendages; smaller cell tapering; bigger cell pointed towards the apical annulus, biguttulate.

Specimen examined: Forebalkan (eastern): Lovech distr., Golyama Zhelyazna village, towards 'Peshtera Toplya' natural landmark, 42°58'15.5''N, 024°29'27.6''E, 23.08.2015, D. Stoykov, SOMF 26645. On living leaves of *Carpinus betulus* L. (*Betulaceae*).

Known distribution: Stara Planina Mts (Gyosheva et al., in press), Sofia region and Rila Mts [5, 6].

Note. *M. fimbriata* was found on living leaves of *Carpinus caroliniana* Walt. (*Betulaceae*) in Mexico [11]. The specimen's description given by Carrión and Chacón is quite similar to our collection, regarding its common morphology, but the asci contain slightly smaller ascospores: 6–8 × 2–3 µm.

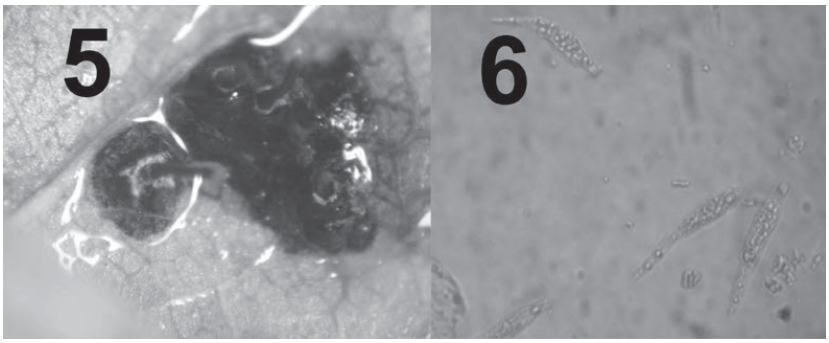

Fig. 5. Stroma of *Mamiania fimbriata* in wet condition; Fig. 6. Asci and ascospores.

Ophiognomonia rosae (Fuckel) Kirschst., Ann. Mycol., **37:** 129, 1939.

Perithecia (165–)215–260(–350) μm in diam, black or brownish, depressed globose, scattered, solitaire, immersed in the leaf blade, veins and petioles. **Beaks** (500–)590–650 × (30–)35–45 μm, filiform, black, narrowed and brightened towards the apex, straight or curved, widened at the base up to 65-80 μm, central. **Asci** 30–37 × 6–7 μm, long ellipsoid, 8-spored, with ascospores in fascicle; apical annulus 1–1.5 μm. **Ascospores** about 22–24 × 1–1.3 μm, long filiform, straight, septum median, appendages not seen.

Specimen examined: Vitosha region: Mt Vitosha, near the holiday home of the Bulgarian Academy of Sciences, 29.06.2001, leg. E. Dimitrova, det. D. Stoykov. On dead leaf and petioles of rosaceaeous plant.

Known distribution: Forebalkan (eastern), Sredna Gora Mts (westrern) and Mt Strandzha [5].

Note. This collection shows shorter beaks and slightly broader asci than the known published data [5].

Plagiostoma apiculatum (Wallr.) L.C. Mejía, in Mejía, Rossman, Sogonov & White, Stud. Mycol., **68**: 219, 2011 (Figs 7-10).

Perithecia immersed in bark, under the periderm, solitary, scattered, appearing initially as slight elevations, later with medium-sized beaks, black, globose. **Asci** (45–)51–80(–86) × 10–16(–18) μm, cylindrical, 8-spored, with biseriate to multiseriate arrangement, apical ring conspicuous. **Ascospores** (14.5)17–18 × (4.5–) 5–6 μm, oblong-ellipsoid, hyaline, with slightly tapering to rounded ends, straight or slightly curved, with median to submedian septum.

Specimen examined: Forebalkan (eastern): Lovech distr., Staro Selo village, 21.03.2015, D. Stoykov, SOMF 26646. On dead twigs and branches of *Salix alba* L. (*Salicaceae*).

Known distribution: Stara Planina Mts and Vitosha region. On *Salix* spp. [5].

Note. Description given by [5], based on studying of old specimens, shows thinner asci (6.5–12 μm wide) and slightly larger ascospores (size up to 24 × 7 μm) when compared to our recent record.

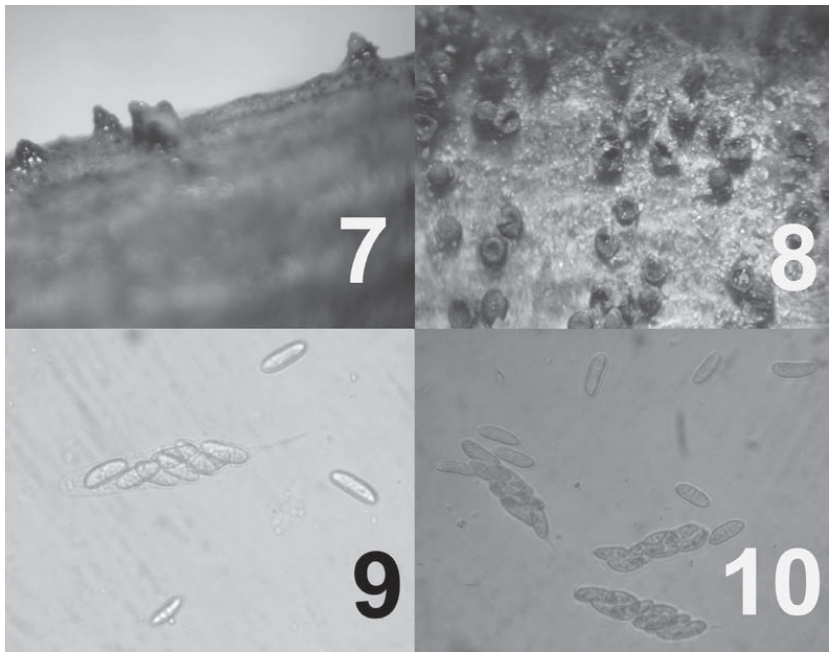

Fig. 7. *Plagiostoma apiculatum* – perithecia immersed in bark of *Salix alba*;
Fig. 8. Perithecia – view from the underside of the periderm;
Fig. 9 & Fig. 10. Asci and ascospores of *P. apiculatum*

Dothideomycetes
Pleosporales
Pleosporaceae
Pleospora herbarum (Pers. : Fr.) Rabenh., Comment. Soc. Crittog. Ital., 1: 217, 1863.

Ascocarps (200–)250–350 µm, black, dark-brownish in lactophenol, globose to subglobose. **Asci** 70–110(–115) × 25–30 µm, bitunicate, usually 8-spored, occasionally 2-spored, cylindrical to cylindrical-clavate. **Ascospores** 25–32 × 10.5–16 µm, pale to dark yellow-brown, oblong to almost fusoid, apex usually broadly rounded, sometimes acute, base rounded to almost truncated, predominantly with 7 transverse septa.

Specimens examined: On dry twigs of: *Amorpha fruticosa* L. (*Fabaceae*), Sofia region: the city of Sofia, Iztok quarter, 11.05.2015, D. Stoykov; *Buddleja davidii* Franch. (*Scrophulariaceae*), Sofia city, Geo Milev quarter, 19.08.2011, D. Stoykov.

Known distribution: Black Sea coast, Sofia region, Mt Belasitsa, Sredna Gora Mts (Lozenska Planina) [12, 13, 14].

Conclusion

Seven pyrenomycetous fungi of the Sordariomycetes were studied with standard methods of light microscopy based on the materials collected from new localities: *Diatrypella favacea* – Forebalkan (eastern); *D. quercina* – Stara Planina Mts and Sofia region; *Gnomonia*

geranii-macrorrhizi – Rila Mts and Central Rhodopes; *Gnomoniopsis comari* s. lat. – Central Rhodopes; *Mamiania fimbriata* – Forebalkan (eastern); *Ophiognomonia rosae* – Vitosha region; *Plagiostoma apiculatum* – Forebalkan (eastern). The following plants were found as new hosts to Bulgaria – *Amorpha fruticosa* and *Buddleja davidii* (*Pleospora herbarum*), *Quercus thracica* and *Q. rubra* (*Diatrypella quercina*); *Salix alba* (*Plagiostoma apiculatum*).

Acknowledgements: The author is grateful to the anonymous reviewer for the proposed recommendations. The part of this study held during 2011 year was supported by Grant DO 02-194/2008 of the National Scientific Fund (Ministry of Education and Science, Sofia).

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SHORT COMMUNICATIONS

Topic:
BIODIVERSITY AND CONSERVATION BIOLOGY

IN VITRO CLONAL PROPAGATION OF *VALERIANA OFFICINALIS* L. (VALERIANACEAE)

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Valerian is one of the most popular medicinal herbs used since ancient times, first in folk and then in the official medicine. The used plant parts are its roots and rhizomes (Radix Valerianae; Rhizoma cum radicibus Valerianae). Different pharmaceuticals are prepared from them: tinctures, tablets, thick extract, etc. Wild resources of valerian greatly decreased in recent decades as a result of improper plant collection. However, market demand is strong and steadily increasing. In Bulgaria, valerian is under a special regime of protection and use. Its collection from the natural habitats is prohibited for commercial purposes by annual order of the Ministry of Environment and Water in accordance with Art. 10 of the Medicinal Plants Act (2000).

The aim of the study was to establish a reliable protocol for *in vitro* clonal propagation of the medicinal plant *Valeriana officinalis* and to produce plants identical to the parent individual in order to initiate a pilot plantation, starting from a single valuable plant.

Material and Methods. Segments of leaves, stems, raceme stalks and leaf-buds of one selected valerian plant in blossom were used as primary explants. Plant surface disinfection was done soaking plant material consecutively in 70% ethanol for 1 min, undiluted or 50% solution of commercial bleach (chlorine < 5%) for 7 min, and distilled sterile water for 5, 10, and 15 min. *In vitro* cultures were initiated on eight agar-solidified medium variants: control basal MS medium [1], six media supplemented with 0.5 mg/l cytokinin alone: 6-benzylaminopurine (BAP) or kinetin (Kin), or in combination with the auxin α-naphthalene acetic acid (NAA), in proportion 2:1 and 1:2, and a half-strength MS medium free of plant growth regulators (PGRs). All media contained 30 g/l sucrose, and 6.5 g/l Plant agar (Duchefa, NL). *In vitro* cultures were initiated in plastic Vitro vent containers with grids (Duchefa, NL). Leaf and root explants excised from the *in vitro* obtained plantlets were sub-cultured on the same media, in plastic containers without grids. The diurnal temperature in the culture room was 23±2ºC, while the light regime was 16/8 h light/dark. Plantlets with well-shaped leaves and roots were potted in soil substrate (soil mixture, sand and coconut fiber, in proportion 2:1:1) and *ex vitro* adapted first in growth chamber (POL-EKO Aparatura, PL) under strict control of the ambient conditions (light, temperature and humidity) simulating as much as possible the natural diurnal dynamics of these parameters. Air humidity was gradually decreased from 90% to 60% for a period of four weeks, then

plants were placed on the shelves of a room phytotron with wider amplitude of the light, temperature and humidity. Finally, strengthened plants were acclimated to open field.

Results and Discussion. Surface sterilization was difficult as undiluted bleach caused necrosis of the initial plant material while its 50% solution was not enough strong to kill fungi. *In vitro* cultures of *V. officinalis* were initiated from leaf segments and raceme stalks without microbial contaminations. Leaf explants formed *in vitro* roots which were further sub-cultured on media supplemented with different plant growth regulators. Raceme stalks raised whole *in vitro* plantlets or shoots which were *in vitro* rooted on basal MS medium. Plant regeneration from these explants took about 9 weeks on basal MS free of plant growth regulators.

The regeneration rate depended on both: type of the explant and medium composition. Raceme stalks were the best primary explants. They formed an average of 2.7 shoots per explant on medium containing 0.5 mg/l Kin. This medium was also the most appropriate one regarding the percentage of the explants with regeneration answer: 67% of the explants formed one or several shoots. Contrary, the frequency of morphogenesis of the primary leaf explants was very low. The potential of the *in vitro* leaves to form roots when transferred to fresh medium was highly enhanced, even if it was found to depend on the explant orientation with abaxial or adaxial side toward the medium surface. High regeneration potential of *V. officinalis in vitro* leaves was reported by other authors as well [2]. They observed root formation on media with equal concentration of the cytokinins and auxins, and callus formation in the case of auxin predominance. In our study, root segments were found to be the most productive among all types of secondary explants, giving rise to an average of four plantlets for 4 to 6 weeks in the best variant, on medium supplemented with 1 mg/l NAA and 0.5 mg/l Kin.

Callogenesis was also observed on some of the media, especially when the auxin predominated. Calli were able to produce roots and plantlets. However, direct organogenesis was preferred in the present study, attempting to avoid the potential somaclonal variation which occurs much more frequently in callus-derived plantlets.

Plantlets with well-shaped leaves and roots were *ex vitro* adapted. The *ex vitro* survival rate was high: 91.3% in the growth chamber and 100% in the phytotron. Plants were fast-growing in the phytotron and reached up to 45 cm height for two months. Valerian is very vulnerable to greenhouse whitefly (*Trialeurodes vaporariorum*). Some of the plants were partially damaged by the attacks of these insects. Most plants survived after their repeatedly treatment with the preparations Aktara and Admiral which proved to be high efficient against *T. vaporariorum*. All 150 plants transferred to the open field developed normally and spent successfully the winter. They are currently in flowers.

Conclusion. *In vitro* clonal propagation was proved to be an effective method for rapid multiplication of high productive valerian individuals and for establishment of a nursery of a commercial plantation. Best primary explants were raceme stalks while *in vitro* roots distinguished as most productive secondary explants. *Ex vitro* plant survival was high under phytotron conditions as well as on the open field.

Acknowledgements: Authors are grateful to Bioprograma EAD for the financial support of the study.

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SEED GERMINATION AND *EX SITU* CONSERVATION OF *VERBASCUM ANISOPHYLLUM* MURB. (SCROPHULARIACEAE)

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Verbascum anisophyllum Murb. is a Balkan endemic plant with a total of 3 populations covering very restricted area of less than 50 km^2. Two of the populations are in Bulgaria: one located near Tsarvenyano village, in the Viden divide of Konyavska Planina Mt. (Znepole floristic region), the other one near Vukovo village (floristic region Valley of River Struma), while the third population is in the vicinities of Bosilegrad, Serbia. The species is critically endangered according to the IUCN criteria. It is included in the Red Data Book of Bulgaria, and protected by the Biodiversity Act (2002). In 2013, protected areas were declared for both Bulgarian populations. In addition, an Action Plan was elaborated in 2014 concerning the conservation measures intended for the period 2014-2023 in order to safeguard the species. Some *ex situ* activities were planned, among them seed conservation in the National Seed Gene bank in Sadovo, investigation of the plant biology and establishment of a living collection.

The present study aimed to evaluate the germination potential of the seeds both *in vivo* and *in vitro*, and to obtain plants for *ex situ* conservation of the species.

Materials and Methods: A monitoring of the two Bulgarian populations was done in 2015. Seeds were gathered in October 2015 from both populations, and their germination rate was assessed *in vivo* on wet filter paper in petri dishes, and *in vitro* on agar-solidified basal medium MS [1], in parallel. The ambient conditions in the culture room were: temperature

of 23±2°C, and 16 h light daily. To stimulate seed germination, seeds were pretreated with 0.35% gibberecllic acid (GA_3), 0.35% kinetin (Kin) or 0.2% KNO_3 solutions for 22 hours; control seeds were soaked in distilled water. Hundred seeds were used per variant, distributed in 5 petri dishes 20 seeds each, or in 4 plastic containers with grids (Duchefa, NL), 25 seeds each. Besides, seeds soaked in GA_3 or in water were germinated on MS medium as control and on medium K01, supplemented with 0.1 mg/l Kin. To study the germination potential in the time, 200 seeds gathered in 2012 from the population near Vukovo village, were treated similarly with GA_3 and tested *in vitro* on MS medium. Seedlings were potted in soil substrate (soil mixture, sand, and coconut fiber in proportion 2:1:1) and *ex vitro* adapted first in a growth chamber POL-EKO Aparatura with strict control of the ambient light, temperature and humidity, and then in a room phytotron.

Results and Discussion: Assessment of the conservation status of the two visited populations in 2015 was favorable. Both young rosettes and individuals with blossoms and fruits were observed. Seeds were easily disinfected with 70% ethanol and commercial bleach. Seed germination began at the end of the first week and occurred quickly, until the end of the fourth week. The seed germination rate in the control variant was low both *in vivo* and *in vitro*: 16 % and 6 % respectively.

The stimulating effect of the gibberellic acid was significant causing an average of 84% *in vitro* and 95% *in vivo* germinated seeds. The other two tested compounds (kinetin and KNO_3) did not influence the germination of the seeds. In all variants the percentage of *in vitro* germinated seeds was slightly lower, probably due to the inhibitory effect of the bleach. No significant differences were noticed between the germination potential of the two populations. The addition of 0.1 mg/l Kin in the medium did not enhance the germination rate. Furthermore, its presence in the medium led to abnormal development of many seeds, especially in the case they had been pretreated with GA_3. It is worth to say that GA_3 applied alone caused only elongation of the seedlings but they had well shaped leaves and roots. It impeded the development of the *in vivo* seedlings whose stems became too thin and seedlings layered. The combined application of the two plant growth regulators *in vitro* was found to be unsuitable, causing grave etiolation of some seedlings. High germination rate up to 90% and 89% was reported for two other endemic *Verbascum* species in Turkey: *V. bithynicum* Boiss., and *V. wiedemannianum* Fisch & Mey, when non-stimulated seeds were germinated in dark, at 20°C [2]. Authors noticed that the effect of GA_3 was not universal and it totally inhibited the germination of these *Verbascum* species seeds even if it was used in much lower concentration of 0.02%.

From all *V. anisophyllum* seeds gathered in 2012 from one of the Bulgarian populations, only one germinated which was probably due to the fast loss of seed viability. Interestingly, seeds of *Verbascum blattaria* L. germinated and produced normal plants after 120 years of storage in a bottle with moderately moist sand, buried on a sandy knoll [3]. Obviously, seed germination is specific and needs to be tested for every species.

In our study, the best plantlets were obtained in the control *in vitro* variants, on MS medium free of plant growth regulators. They were easily *ex vitro* adapted in the growth chamber for a month under conditions simulating the diurnal dynamics of the temperature, light, and air humidity. Plants continued their growth for another three months in the room

phytotron, with wider amplitudes of the ambient parameters. Plants were transferred to the greenhouse are now are ready to be acclimated outside.

Conclusion: Seed germination of *Verbascum anisophyllum* was successfully enhanced under laboratory conditions. The obtained plants are suitable for *ex situ* conservation of the species and for *in situ* reinforcement of its natural populations. The germination potential of the seeds decreases very quickly with the time. This is the reason to recommend the maintenance of *ex situ* collections of the species as more suitable than the seed preservation in seed gene bank.

Acknowledgements: This research was supported by the Bulgarian National Science Fund, Bulgarian Ministry of Education and Science (project DFNI-BO2/18).

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CONCEPT FOR ORGANIZING THE BLACK SEA TURKISH AQUATIC SITES FROM NATURA 2000 ECOLOGICAL NETWORK

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Introduction: Whether Turkey will become an EU member or not is a political question but nature conservation and biodiversity in particular require that the border aquatic areas of conservation have to be expanded and connected.

The study is a continuation of the research of one of the authors [1], who defends the understanding that NATURA 2000 ecological network can't be limited by country borders and the spatial connection of the sites is required for functioning as a network.

Materials and Methods: Taking the proposed MPAs by the Turkish researchers [2] who determined 5 sites for protection of the sea aquatories, the authors add several new zones to them and suggest the expanding of the already outlined ones, thus proposing a concept for the NATURA 2000 sites in the Turkish aquatory of the Black Sea.

When outlining the sites, the specific features of the Turkish shelf are considered – in one of the proposed sites, a significant factor is the vast area of depth less than 25 m, which determines the existence of greater richness of species. Other determining factor is the priority for preserving those species – existence of fields of Mediterranean monk seal and existence of wet zones – territories rich in species, having relation with the sea and absolutely dependent of the ecological status.

The comparative method and GIS based rework of maps and designation of the borders of the sites are also applied.

Results: The presented concept for MPAs includes sites, which are barely 2% from the aquatory of Turkey in the Black Sea, while the European legislation says that not less than 10% from the aquatory of the EU members has to fall in NATURA 2000 ecological network.

Site 1* - The changes are aimed at widening the site so it can be connected directly with the "Strandzha" NATURA 2000 site. Part of Strandzha Mountain both on Bulgarian and Turkish territory is distinguished with low population density, which leads to sustaining the natural features of the environment and determines the good ecological status – one of the criteria, which is taken under consideration when determining the NATURA 2000 sites.

Underwater habitats are distinguished with high species diversity. Most spread are the real sand dunes and rock dunes, which are home of a range of rare fish, the annular seabream (*Diplodus annularis*), the Sphinx blenny (*Aidablennius sphinx*), the wedge clam (*Donax trunculus*) and *Chamelea gallina, Cystoseira barbata,* the big and small mussels (*Mytilus galloprovincialis, Mytilaster lineatus*), etc.

Site 2* – This new site extends from Kiyiköy almost to Kumköy. It spreads alongside the land national park Çılıngöz and includes the aquatory to the lake Durugöl, which has a natural water connection with the Black Sea, and continues on the stretch of the coastal salt lakes near to Kumköy

Site 3* - According to some researchers the Mediterranean monk seal (*Monachus monachus*) is completely extinct from the Turkish shore of the Black Sea, but other sources indicate, that maybe in one certain aquatory of the Black Sea between Zonguldak and Sinop the species still can be seen. Due to that, fact site 3 is determined in the concept for MPAs. The proposed site 3* covers greater aquatory, not only with the aim to preserve the above mentioned species, but also to guarantee connection with the proposed site 4. The reason for this big increase of the scope and the connecting of the two proposed sites (3 and 4) is that in this part of the Turkish aquatory the biggest space of water depth less than 25 m is located. Everywhere else, the 25 m area is really shrunk and the depth is abruptly increasing on a small distance from the land. The shallow zone and the geomorphologically separated closed bays allow assuming that the biodiversity is rich and has to be protected. Site 4 is proposed also because of the availability of two rivers and deltas and adjacent to them wetlands, being connected with the Black Sea.

The last site 5 is proposed because of the availability of a reef formation, very rich in species diversity.

Conclusions: The review and analysis carried out by the researchers and the actions of the Republic of Turkey for organizing and localizing the future NATURA 2000 ecological network and the endangered marine zones, determine the following conclusions:

1. Whether the Republic of Turkey will join the EU or not, the designation of the areas of conservation in the country has to consider the natural continuation of the sites towards those in the Republic of Bulgaria, both on land and in the aquatory.

2. It is required the connections between the endangered zones on the border with the Republic of Turkey to be formalized in order to set up an actual network of NATURA 2000 sites in compliance with the requirements for continuity and enough space of preserved areas, able to guarantee the quality and quantity of the ecosystem services.

3. More thorough research has to be made for the actual areas of prioritized for protection species as the Mediterranean monk seal.

4. The aquatory sites determined so far, enlarged with those proposed in the current concept for MPAs still do not reach the required share and outlining additional sites or expanding the existing and proposed ones need to be discussed after more thorough scientific research.

5. The participation of Bulgarian experts in the designation of the Turkish NATURA 2000 network is recommended, which to a great extent depends on the activity of the Bulgarian country.

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REPORTS

Topic:
BIOTIC AND ABIOTIC IMPACT ON THE LIVING NATURE AND MECHANISMS OF ADAPTATION

ENVIRONMENTAL POLLUTION WITH TOXIC CHEMICALS – INFLUENCE ON THE BIOTA AND HUMAN HEALTH

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Abstract

The growing number of many different diseases during the last decades is a subject of great concern for the medicine and scientists also. A lot of ecological problems are obvious, like contamination of water and soil and destruction of their inhabitants. Many factors may be suspected as a cause of these disturbing phenomena, but one of the most serious is the contamination of environment with toxic chemicals. The main issue is that the basic vulnerable groups are babies, children and pregnant women.

Pesticides are chemical substances, designed to protect crops and destroy pests; about 1200 – 1400 active substances are now available. Heavy metals are naturally occurring or segregated in various industrial processes. They are elements, noted for their potential toxicity. Mycotoxins are toxic chemicals, produced by fungi in the crops. Most of them are associated with human and veterinary diseases. Industrial/other contaminants: dioxins, benzopyrene, formaldehide, acrylamide, DMN (methylnitrosamine), perchlorate, melamine etc., appear in various industrial or natural processes. Veterinary Medicinal Products (VMP) are substances, intended for treating, mitigating, preventing illnesses or influence on specific body functions in animals.

Considerable amounts of all these chemical products are released into the environment, where several substances seem to be persistent and pose a risks for the biota and human health. Implementation of measures and alternative approaches for mitigation of environmental impact and to ensure safety for consumers are necessary, like: Good Agricultural Practices, Good Veterinary Practices, Integrated Pest Management (IPM), organic agriculture, genetic engineering, phytoremediation.

Keywords: environment, contamination, toxic chemicals, ecological problems, mitigation measures

Introduction

Environmental pollution in recent decades is an undeniable fact. The boom of cancer deseases in humans and global reduction of pollinators – in particular, honeybees, are just a few of the consequences caused by improper treatment of natural resources through various anthropogenic activities, which led to strengthening the presence of toxic substnaces in the environment. Modernization and growing use of chemicals in the modern agriculture and powerful industrial development also, are among the main causes of pollution; some

pollutants have natural origin or are due to improper storage of products and improper food preparation.

I. Rapid Alert System for Food and Feed (RASFF). The most distributed pollutants in the environment are pesticides, heavy metals, mycotoxins, etc. Analysis of the RASFF for the period 2011 – 2015, has determined that the most numerous notifications are those, regarding mycotoxins – 2435; followed by those concerning pesticide residues – 2099; after them are notifications of heavy metals – 1334; annoucements for veterinary medicinal products and industrial contaminants are respectively 408 and 332 (see Fig. 1), (RASFF Portal, https://webgate.ec.europa.eu/rasff-window/portal/?event=SearchForm&cleanSearch=1).

II. Pesticides. They are chemical substances, designed to protect crops and destroy pests; about 1200 – 1400 active substances are now available. They are a class of biocides. Pesticides are one of the most widespread and common contaminants in food. Pesticides are chemical substances included in the composition of plant protection products (PPP), designed to protect crops from various diseases, pests and weeds; they are used in the household also (mainly insecticides) in the routine spraying rooms to combat insects or rodents. Pesticides enter the human body mainly in two ways: through direct contact with substances (inhalation or through the skin) or by food consumption, when residue in it are high.

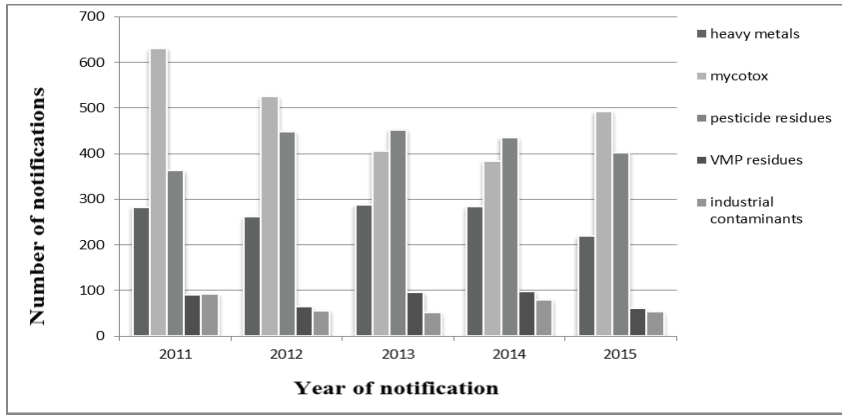

Fig. 1. Distribution of notifications for different contaminants for the period 2011 – 2015

According to their active substances, pesticides are chemicals and biopesticides. Biopesticides include microbial pesticides and biochemical pesticides.

According to their purpose and function, the main groups of pesticides are herbicides, insecticides, fungicides, etc. They also belong to different chemical groups. Prominent insecticide families include organochlorines, organophosphates, and carbamates. Prominent families of herbicides include phenoxy and benzoic acid herbicides (e.g. 2,4-D), triazines (e.g., atrazine), ureas (e.g., diuron), and chloroacetanilides (e.g., alachlor). Fungicides are from the classes of dithiocarbamates, dicarboximides, benzimidazoles, etc.

Pesticides from the group of neonicotinoids: they are also synthetic analogues of the natural pesticide – nicotine but with a much more pronounced effect. Over the past two

years the use of three of them (clothianidin, imidacloprid and thiamethoxam) was banned precisely because of their high toxicity and environmental threat. After the expiry of the period of their ban, the European Authority for Food Safety appreciates the threat to bees from their use as a foliar spray and collect new evidence of a change in the status of bee colonies due to imposed restrictive measures.

Much of the pesticides fall into the group of so-called persistent organic pollutants (POPs), which are toxic organic substances and are particularly dangerous in terms of their accumulation in the environment, where they are retained for a prolonged period of time. POPs accumulate in living organisms through the process of bioaccumulation, which means that POPs gradually bioconcentrate through the food chain in fish, birds and mammals and eventually in humans. Because of their migration, they can be found in regions where there were never used.

Pesticides are used to control organisms that are considered to be harmful. The main source for their distribution in the environment is the improper use of plant protection products. Pesticides are one of the causes of water pollution, and some pesticides are persistent organic pollutants and contribute to soil contamination. Another problem, connected with chemical substances is the storage of unused pestidices, the so called obsolete pesticide. Due to breach the integrity of their packaging, they are exported by rains and groundwater into the environment and they easily contaminate in agricultural production and food for people and animals.

The most significant problem, araising from their application is the possibility to harm or destroy some nontarget species and, as a consequence – damaging the ecosystems and their inhabitants. In addition, pesticide use reduces biodiversity, contributes to pollinator decline, destroys habitat (especially for birds), and threatens endangered species.

The emergence of resistance of pests to a chemical substance is another negative result of frequent or unauthorized use of pestides. This demands the increase of the used dose or application of new, more powerfull substances, to kill pests.

Influence on human health: The presence of pesticides in the body may cause a number of acute and delayed health effects in people who are exposed to them. A variety of adverse health effects, ranging from simple irritation of the skin and eyes to more severe effects, such as violations in functioning of the organism and lethal effect at higher doses can be provoked. The presence of pesticides in the body causes the emergence of a number of health problems: destroying the nervous system and the occurrence of problems with mental development, memory loss, loss of coordination, reduced speed of response to various stimuli, reduced visual abilities, uncontrollable behavior, reduced motor skills. Exposure to pesticides can cause a disorder of the endocrine glands, a large number of active substances are proven endocrine offenders. Some pesticides are carcinogenic and can cause malignant diseases such as leukemia and cancers; in the last decades a strong spread of breast cancer is explained by the increased use of pesticides and other chemicals. Harmful effects can cause further manifestation of diabetes, infertility, miscarriage, damage to the thyroid gland, liver, damage to the genetic material in the cells and the immune system disorder. Exposure to pesticides is associated with a number of other possible health effects including asthma, allergies and hypersensitivity, problems with reproduction and the developement of the fetus. Medics direct their attention to the possible link between the widespread use of pesticides and Parkinson's disease – a slowly progressive neurodegenerative disease in which brain cells die. [1, 2, 3, 4].

III. Heavy metals. They are relatively dense metals that are noted for their potential toxicity, especially in environmental context; cadmium, mercury, lead, arsenic, chromium, copper, iron, etc.

They are also widespread contaminants in food. As the term in modern ecology, they are classified as particularly toxic elements which in high quantities harm plants and plant production and hence fall into the food chain, which constitute a danger to human health.

Heavy metals are the main pollutants which were mainly due to industrialization through industrial waste. The presence of these toxic elements has been increased around the world, such as areas with high soil contamination are located in countries with greater industrial activity, where the nearby farmlands are affected by atmospheric exposure to heavy metals.

Toxic heavy metals are found naturally in the earth, and become concentrated as a result of human caused activities. Common sources are from mining and industrial wastes; vehicle emissions; lead-acid batteries; fertilisers; paints; treated woods; and aging water supply infrastructure. They are also compounds in some plant protection products.

Heavy metals enter plant, animal and human tissues via air inhalation, diet and manual handling. Motor vehicle emissions are a major source of airborne contaminants including arsenic, cadmium, cobalt, nickel, lead, antimony, vanadium, zinc, platinum, palladium and rhodium. Water sources (groundwater, lakes, streams and rivers) can be polluted by heavy metals leaching from industrial and consumer waste; acid rain can exacerbate this process by releasing heavy metals trapped in soils. Plants are exposed to heavy metals through the uptake of water; animals eat these plants; ingestion of plant- and animal-based foods are the largest sources of heavy metals in humans. Absorption through skin contact, for example from contact with soil, is another potential source of heavy metal contamination. Toxic heavy metals can bioaccumulate in organisms as they are hard to metabolize.

Toxic heavy metals "can bind to vital cellular components, such as structural proteins, enzymes, and nucleic acids, and interfere with their functioning." Symptoms and effects can vary according to the metal or metal compound, and the dose involved. For humans, typical presentations associated with long-term exposure to any of the "classical" toxic heavy metals, or chromium (another toxic heavy metal) or arsenic (a metalloid) can have carcinogenic, central and peripheral nervous system and circulatory effects.

Adverse effect of heavy metals on human health: the most serious problem with heavy metals is the inability to be broken down and therefore remain in the environment, especially in soil for decades. Transmitted in the food chain or through contaminated surface and groundwater, they represent a huge risk to human health. Many heavy metals are known carcinogens. They enter the human body through food and water and even through contact with the skin, accumulate in the body and in certain quantities they damage some organs. Heavy metals can manifest a broad range of adverse health effects: destruction of bones – osteomalacia, neurological damage, cancer, blood poisoning, anemia, diabetes, reduced growth and development, nervous system damages, nephrotic syndrome, etc.

The adverse health effect from different heavy metals is:

Cadmium: lung inflammation, lung cancer, softening of bones, excess protein in urine; possible kidney damage;

Mercury: diarrhea, fever, vomiting, inflammation of gums and mouth, nausea, nonspecific kidney disorder, neurasthenia, pink disease, tremor;

Lead: encephalopathy, nausea, vomiting, anemia, foot and wrist palsy, kidney diseases

Chromium: gastrointestinal hemorrhage, hemolysis, lung scarring, lung cancer;

Arsenic: nausea, vomiting, diarrhea, encephalopathy, neuropathy, arrhythmia, diabetes, cancer;

Copper: it is an essential trace element that is vital to the health of all living things, but the excess copper intake causes stomach upset, nausea, and diarrhea and can lead to tissue injury and disease.

Soils contaminated by toxic heavy metals can be remediated by one or more of the following technologies: isolation; immobilization; toxicity reduction; physical separation or extraction. Isolation involves the use of caps, membranes or below-ground barriers in an attempt to quarantine the contaminated soil. Immobilization aims to alter the properties of the soil so as to hinder the mobility of the heavy contaminants. Toxicity reduction attempts to oxidise or reduce the toxic heavy metal ions, via chemical or biological means into less toxic or mobile forms. Physical separation involves the removal of the contaminated soil and the separation of the metal contaminants by mechanical means. Extraction is an on or off-site process that uses chemicals, high-temperature volatization, or electrolysis to extract contaminants from soils. The processes used will vary according to contaminant and the characteristics of the site [5, 6].

IV. Mycotoxins. They are toxic secondary metabolites produced by fungi, commonly known as molds. The term "mycotoxin" concerns the toxic chemical products produced by fungi that readily colonize crops. One mold species may produce many different mycotoxins, and the same mycotoxin may be produced by several species. The most important ones associated with human and veterinary diseases, include aflatoxin, citrinin, ergot alkaloids, fumonisins, ochratoxin, patulin, trichothecenes, DON, and zearalenone. One of the most important reasons for the production of mycotoxins is improper storage of agricultural products.

Aflatoxins are a type of mycotoxins produced by *Aspergillus* species of fungi. The term aflatoxin refers to four different types of mycotoxins produced, which are B_1, B_2, G_1, and G_2. Among them, aflatoxin B_1 is the most toxic, a potent carcinogen and has been directly correlated to adverse health effects, such as liver cancer, in many animal species. Aflatoxins are largely associated with commodities produced in the tropics and subtropics, such as cotton, peanuts, spices, pistachios and maize.

Ochratoxin is a mycotoxin that comes in three secondary metabolite forms, A, B, and C, produced by *Penicillium* and *Aspergillus* species. *Aspergillus ochraceus* is found as a contaminant of a wide range of commodities including beverages such as beer and wine. *Aspergillus* carbonarius is the main species found on vine fruit, which releases its toxin during the juice making process. Ochratoxin A has been labeled as a carcinogen and a nephrotoxin, and has been linked to tumors in the human urinary tract.

Citrinin is a toxin that has been identified in many species of *Penicillium* and several species of *Aspergillus*. Some of these species are used to produce human foodstuffs such as cheese (*Penicillium camemberti*), sake, miso, and soy sauce (*Aspergillus oryzae*). Citrinin is associated with yellowed rice disease in Japan and acts as a nephrotoxin . Although it is associated with many human foods (wheat, rice, corn, barley, oats, rye) its full significance for human health is unknown. Citrinin can also act synergistically with Ochratoxin A to depress RNA synthesis.

Ergot alkaloids are compounds produced as a toxic mixture of alkaloids in the sclerotia of species of *Claviceps*, which are common pathogens of various grass species. The ingestion of ergot sclerotia from infected cereals, commonly in the form of bread produced from contaminated flour, cause the human disease ergotism. There are two forms of ergotism: gangrenous, affecting blood supply to extremities, and convulsive, affecting the central nervous system. Modern methods of grain cleaning have significantly reduced ergotism as a human disease, however it is still an important veterinary problem.

Patulin is a toxin produced by the *P. expansum, Aspergillus, Penicillium,* and *Paecilomyces fungal* species. *P. expansum* is especially associated with a range of moldy fruits and vegetables, in particular rotting apples and figs. It is destroyed by the fermentation process and so is not found in apple beverages. Although patulin has not been shown to be carcinogenic, it has been reported to damage the immune system in animals. In 2004, the European Community set limits to the concentrations of patulin in food products.

Fusarium mycotoxins are the largest group of mycotoxins, which includes more than 140 known metabolites of fungi. They are synthesized by many species of fungi, mainly by *Fusarium* (*F. graminearum* and *F. culmorum*). *Fusarium* mycotoxins are produced by over 50 species of *Fusarium* and they infect the grain of cereals, like wheat and maize and their products. They include a range of mycotoxins, such as fumonisins, which affect the nervous systems of horses and may cause cancer in rodents; trichothecenes, which are most strongly associated with chronic and fatal toxic effects in animals and humans; zearalenone, which is not correlated to any fatal toxic effects in animals or humans; deoxynivalenol (DON), which affects animal and human health causing acute temporary nausea, vomiting, diarrhea, abdominal pain, headache, dizziness, and fever. Due to the high toxicity of *Fusarium* mycotoxins and high occurrence of the fungi species producing them, these mycotoxins belong to the most animal and human health endangering ones.

The various food processes that may have effects on mycotoxins include sorting, trimming, cleaning, milling, brewing, cooking, baking, frying, roasting, canning, flaking, alkaline cooking, nixtamalization, and extrusion. Most of the food processes have variable effects on mycotoxins, with those that utilize the highest temperatures having greatest effects. In general, the processes reduce mycotoxin concentrations significantly, but do not eliminate them completely. Mycotoxins greatly resist decomposition or being broken down in digestion, so they remain in the food chain in meat and dairy products. Even temperature treatments, such as cooking and freezing, do not destroy some mycotoxins.

Mycotoxicosis is the term used for poisoning associated with exposures to mycotoxins. The symptoms of mycotoxicosis depend on the type of mycotoxin; the concentration and length of exposure; as well as age, health, and sex of the exposed individual. The synergistic effects associated with several other factors such as genetics, diet, and interactions with other toxins have been poorly studied. Therefore, it is possible that vitamin deficiency, caloric deprivation, alcohol abuse, and infectious disease status can all have compounded effects with mycotoxins. In turn, mycotoxins have the potential for both acute and chronic health effects via ingestion, skin contact, and inhalation. These toxins can enter the blood stream and lymphatic system; they inhibit protein synthesis, damage macrophage systems, inhibit particle clearance of the lung, and increase sensitivity to bacterial endotoxin [7, 8, 9].

V. Industrial/other contaminants: dioxins, benzopyrene, formaldehide, acrylamide, DMN (dimethylnitrosamine), perchlorate, melamine, etc. The sources of their presense in the environment are from industrial waste, smoking, preparation of some food.

Affecting ecosystems in many ways, they have an influence on human health, which can be summarized as DNA damages, allergies, effect on estrogen receptors and enzymes; liver problems, damages of the immune, nervous and endocrine system, respiratory problems, reproductive/developmental toxicity, formation of tumors and cancer.

Dioxins are not produced in order and have some use. They are sub-product of various industrial processes (eg. bleaching of paper, production of chemicals and pesticides), other combustion (burning or incineration of waste, forest fires). Furthermore, they can also be the result of some natural processes such as volcanic eruptions or forest fires. Dioxins are persistent contaminants and therefore are found in low levels everywhere, in the air, soil, water, sediment, in some foods such as dairy products, meat, fish, shellfish; accumulate in the fatty tissue of animals. They are chemically stable and remain a long time in the body, exert their toxicity by acting on estrogen receptors and enzymes. Short-term exposure of humans to high levels of dioxins may result in skin and liver problems, damage the immune, nervous, endocrine and reproductive systems and tumor formation. Chronic exposure of animals to dioxins increases the risk of developing different types of cancer, including sarcoma, non-Hodgkins lymphoma, and Hodgkin's disease [WHO, 2014. http://www.who.int/mediacentre/factsheets/fs225/en/].

Initially, acrylamide was seen as a chemical, used in industry for the preparation of certain polymers. It is known that acrylamide is found in a wide variety of foods, such as semi-finished French fries, potato products for cooking at home, potato chips, when they are prepared at a temperature higher than 120°C, cereals, coffee, crispbread, biscuits and other bakery products since its production during the cooking process is dependent on the temperature. It is not found in foods that have been cooked, or in those which have not been heated. Acrylamide levels increase when the food is subjected to heating for a long period of time. Acrylamide has been found to cause the appearance of tumors of the nervous system, oral cavity, the peritoneum, thyroid gland, mammary gland and uterus [10].

Benzopyrenes are organic compounds from the chemical class of polycyclic aromatic hydrocarbons and widespread pollutants in the environment, formed by the incomplete combustion or pyrolysis of organic material. The most common is their isomer benzo(a) pyrene. It is naturally emitted by forest fires and volcanic eruptions and can also be found in coal tar, smoke from cigarettes and burnt wood from burning diesel fuel and burned foods such as coffee, etc. The population may be exposed to the emission of benzo(a)pyrene by cigarette smoke, air, water, food and pharmaceuticals. Benzo (a)pyrene interfere with the transcription of DNA and therefore is considered a pollutant and carcinogen in Group 2A (probable carcinogen for humans). Benzopyran is connected to the occurrence of lung cancer and colon cancer [11].

Dimethylnitrosamine (DMN) is an organic compound secreted as a byproduct of various industrial processes and delivered in very small amounts in cooked or smoked foods. DMN is secondary or industrial waste product of some industrial processes and it is necessary for the synthesis of rocket fuel. DMN is found at low levels in many unfit for human consumption

products, including canned meat, fish, beer and tobacco smoke. It is highly toxic, especially to the liver and it is probable human carcinogen, but not fully proven experimentally.

Formaldehyde is a naturally-occurring organic compound; it is an important precursor to many other materials and chemical compounds. It is mainly used in the production of industrial resins. In view of its widespread use, toxicity, and volatility, formaldehyde is a significant consideration for human health.

Melamine is a chemical compound, used in production of melamine-formaldehyde resins (plastics, adhesives, varnishes, superplasticizers for concrete), ion exchange resins, tannins, hexachloromelamine, used in the manufacture of dyes and herbicides and in cosmetics also. Melamine is used in the production of growth promoters of plants (plant growth regulators). It is also used as a non-protein nitrogen source in the diet of domestic livestock. The addition of melamine increases the nitrogen content of the milk and therefore its apparent protein content. Addition of melamine into food is not approved by the FAO/WHO Codex Alimentarius (food standard commission), or by any national authorities. Melamine alone causes bladder stones in animal tests. When combined with cyanuric acid, which may also be present in melamine powder, melamine can form crystals that can give rise to kidney stones. These small crystals can also block the small tubes in the kidney potentially stopping the production of urine, causing kidney failure and, in some cases, death. Melamine has also been shown to have carcinogenic effects in animals in certain circumstances, but there is insufficient evidence to make a judgment on carcinogenic risk in humans [12].

Perchlorate (ion: ClO_4^-) is a chemical pollutant that enters the environment from natural and anthropogenic sources. Biomonitoring studies show the presence of background levels of perchlorates in the general population, suggesting that perhaps they are ubiquitous pollutant in the environment. Identified other sources of pollution, which include: the use of fertilizers from natural origin, which is the possible presence of perchlorate; industrial emissions of perchlorate in the environment; natural formation of perchlorate in the atmosphere and surface water and the formation of perchlorate in the decomposition of chlorine-containing products, such as sodium or calcium hypochlorite. Perchlorate is a potent competitive inhibitor of the thyroid sodium-iodide symporter – in large amounts perchlorate interferes with iodine uptake into the thyroid gland. Some studies suggest that perchlorate has pulmonary toxic effects as well [EFSA Panel on Contaminants in the Food Chain (CONTAM), 2015. http://www.efsa.europa.eu/en/efsajournal/doc/3869.pdf].

VI. Veterinary Medicinal Products. Veterinary pharmaceuticals are widely used across Europe to treat farm animals. After application to an animal, a veterinary medicinal product may be absorbed and partially metabolised before being excreted with urine and faeces. The resulting manure or slurry can then be released directly to the environment or collected and stored before being applied to land. Once released to land, the medicines and its metabolites may run off into surface waters or leach to groundwater where they may impact environmental as well as human health. Contamination of the environment can be reduced by appropriate risk mitigation measures, e.g. limiting the application rate, the amount of contaminated manure being spread on agricultural lands or the access of treated pasture animals to surface waters [13].

The growing contamination of surface water, soil, and food with residues from veterinary medicinal products has increasingly attracted the attention of policymakers and

the general public. It has become apparent that measures are needed on various levels – from pharmaceutical approval, to regulations for use, to technical processes for wastewater treatment – in order to enhance protection of the environment and human health from adverse effects of hazardous pharmaceutical residues.

Adverse environmental effects of veterinary medicinal products. Veterinary medicinal products are intended for use in treating, mitigating, or preventing illnesses or to influence specific body functions in animals. They are used by veterinaries and pet owners as well as by professional livestock holders such as breeders and feedlot operators. Considerable amounts of these medicinal products are released into the environment by intensive fattening operations. These include drugs that target parasites, protozoa, worms and insects, antibiotics that combat pathogenic bacterial, substances for treating infections, and immunological veterinary pharmaceuticals. These substances enter the environment via animal excrements, livestock manure, waste water, and other farm waste. Medicinal products enter surface water directly as a result of their use in aquacultures. Several substances seem to be persistent in the environment [14].

The key steps (from an environmental perspective) in the life cycle of a medicinal product are manufacturing, consumption and waste management. The consumption phase is considered to be the biggest contributor to the emissions of medicinal products into the environment, notably through excretions and incorrect disposal of unused medicines through sinks and toilets. Between 30 and 90% of the orally administered dose is generally excreted as active substance in the urine. However, the nature and amount of medicinal residues mainly depend on the volumes and nature of the administered substances, their modes of administration, and metabolisation rates. Medicinal products can also directly enter the environment through feed surplus, notably in the case of aquaculture. The residues remaining after wastewater treatment depend on the composition of the medicinal product, wastewater treatment process and initial concentrations in the influent. Once in the environment, medicinal products are transformed and transferred among different compartments, depending on the nature of the compounds and the characteristics of the host compartment. Medicinal products can degrade biotically or abiotically in soils and water, a process that in general reduces their potency, even if some degradation products might be persistent and thus of concern. The mechanisms of transformations and transfer in the environment lead to the exposure of biota and constitute a potential risk for ecosystems. Although the scientific assessment of ecotoxicological effects of medicinal products on organisms is less developed compared to pesticides for example, it is becoming increasingly clear that some medicinal products, in particular antiparasiticides, antimycotics, antibiotics and (xeno)estrogens, pose environmental risks in specific exposure scenarios. For humans, the possible impacts are less clear than for the environment, but there are concerns notably regarding certain type of molecules, even if to date there is no clear evidence of short-term health effects on humans. Antibiotics, antiparasiticides, antimycotics and anticancer medicinal products are pharmaceutical groups that are especially intended to kill their target organism or target cells and might prove to be the most important pharmaceutical compound saffecting human healt via environmental exposure. Chronic low-level exposure to medicinal products can occur through drinking water and through residues in leaf crops, root crops, fishery products, dairy products, and meat [Executive Agency for Health and Consumers, 2013. http://ec.europa.eu/health/files/environment/study_environment.pdf].

Conclusion

The fast industry development and modernization of farms in recent decades led to increasing deposition and accumulation of chemicals in the environment and in the body of the occupants of ecosystems and in people also. The entering of these hazardous substances in the human body leads to induction of a number of negative effects. They are toxic for all inhabitants of the ecosystem, but the worst result is that main vulnerable groups are babies, children and pregnant women.

The presence of pesticides in the body causes irreversible acute or delayed health effects – violations in functioning of organism (cancer, endocrine disruption, affecting the nervous system, reproductive problems, etc.), birth defects, fetal death and lethal effect at higher doses. Heavy metals influence on the human health is expressed as effects on bone – osteomalacia, neurological damage, cancer, blood poisoning, anemia, diabetes, reduced growth and development, nervous system damages, nephrotic syndrome, etc. The negative effect of the presence of industrial contaminants (dioxins, benzopyrene, formaldehide, acrylamide, DMN, perchlorate, melamine) occurs in DNA damages, allergies, effect on estrogen receptors and enzymes; liver problems, damages of the immune, nervous and endocrine system, respiratory problems, reproductive/developmental toxicity, formation of tumors and cancer. Mycotoxins enter the blood stream and lymphatic system, where inhibited the protein synthesis and particle clearance of the lung, increase sensitivity to bacterial endotoxin as well.

Measures and alternatives, which are capable to mitigate the environmental impact and to alleviate the negative concequences for the living organizms and consumer health, must be applied.

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ANTIMICROBIAL EFFECT OF Al_2O_3/Ag, Al_2O_3 AND Ag THIN FILMS ON *ESCHERICHIA COLI* - INDUSTRIAL AND PATHOGENIC STRAINS

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Abstract

Aim: Study of the antimicrobial activity of nanostructured thin films deposited by radio-frequency magnetron sputtering on glass substrates for application in nanomedicine.

Materials and Methods: Thin films of Al_2O_3, Al_2O_3/Ag and Ag on two strains of *Escherichia coli* - industrial and clinical isolate were tested. Two methods for investigation of the antimicrobial activity of thin films were used: diffusion assay and determination of bacterial growth in dynamic regime.

Results: The effect of different thin films was compared. The industrial and pathogenic strains of *Escherichia coli* demonstrated partial retention in the growth in the presence of Al_2O_3/Ag. The Ag thin films demonstrated retention in the bacterial development during the first 4 hours. The Al_2O_3 thin film has no inhibition effect on both studied strains.

Conclusions: Synergistic effect of the both thin films in the Al_2O_3/Ag bilayer structure, due to the formation of oxidative species on the surface in contact with the bacterial suspension could be a reason for their antibacterial effect.

Keywords: radio-frequency magnetron sputtering, thin films, *Escherichia coli*, nanomedicine

Introduction

Antimicrobial surfaces of many materials are a challenge for the scientific community and of great interest for the human society to prevent spreading of epidemics and to improve the results from the treatment of different diseases. These materials have potential for application in water disinfection, manufacturing of food packaging materials, wound dressings, etc. The increasing interest in development of inorganic disinfectants, like dispersed nanoparticles or nanostructured thin films, is related with the toxicity of organic disinfectants to human existence, unfortunately still used in practice. One of the most innovative approaches to create new materials with antimicrobial effect is the development of the nanobiotechnologies, which provide nowadays materials for effective treatment of different diseases in the clinical practice. The joint development of microbiology and nanotechnology is mainly in two directions: one of them is production of bactericidal agents [1] and the other is creation and design of sensors for detection of the microorganisms [2].

The antimicrobial effect of the materials is due to different processes based on their surface biochemical functionality. The utilization of nanostructured materials as antimicrobial agents originates from their high surface to volume ratio. This characteristic reveals a great potential for application of nanoparticles, which can be synthesized with different dimensions, shapes and from variety of precursors [3, 4]. However, the utilization of metal oxide nanoparticles as antimicrobial agents encounter different challenges related to their properties, in particular, their segregation in the solutions, which requires additional care for homogenization of the solutions [5]. Static films of metal oxide are appropriate as surface coating on incubator walls, in sterile rooms, on wound dressings, etc., as an antimicrobial film. Barrier or antimicrobial properties of thin films of Al_2O_3 and ZnO deposited with Atomic Layer Deposition (ALD) method on commercial polymer films typically used as packaging materials are presented in [6]. The antibacterial effect against *Escherichia coli* of immobilized Cu and CuO nanoparticles on silica prepared by sol-gel technique is studied in [7]. The proposed mechanism for antibacterial effect describes the better antibacterial activity of the Cu than CuO nanoparticles in dark and under light irradiation. Nanostructured thin films with bactericidal or inhibition effects against microbiological organisms are also prepared by r.f. magnetron sputtering [8].

In this study, the research team was provoked from the potential antimicrobial effect of different thin films. The choice of test material was realized on the base of the following criteria:

- the form of the nanomaterial (powder, solution, or thin film). The static structure on the basis of nanograins, which build the film has an advantage as stability. The challenge for the nanoparticles in a solution is their leaching and segregation;
- the method of synthesis. We can use r.f. magnetron sputtering for good monotoring of the film structure;
- the effectivness against clinically significant microorganisms.

Taking into account the above criteria in the preliminary analysis of the problem, also the fact that relatively few studies have been conducted to date with Al_2O_3, our aim was to investigate the antimicrobial effect of r.f. magnetron sputtered thin films Ag, Al_2O_3 and Al_2O_3/Ag on two strains of *E. coli*.

Materials and Methods

The nanostructured thin films of Al_2O_3, Ag and Al_2O_3/Ag were deposited on glass substrates without intentional heating during the deposition by r. f. magnetron sputtering of Al_2O_3 and Ag targets and the two layered structure Al_2O_3/Ag by sequential sputtering of Al_2O_3 and Ag targets, respectively. The sputtering atmosphere and pressure in the case of Al_2O_3 was Ar (2 Pa) and in the case of Ag was Ar (0.2 Pa). The sputtering power of Al_2O_3 and Ag thin film was 50 W and 30 W, respectively. The thickness of Al_2O_3 films is 20 nm and this of Ag is 8 nm, measured with profilometer Taylor Hobson. The structure of the obtained thin films is columnar, typical for the films deposited by r. f. magnetron sputtering. The thickness of the films meets the requirements for multiple applications, as it is well known that Al_2O_3 is stable to different environmental conditions.

The microorganisms in this study were supplied by the National Bank for Industrial Micrororganisms and Cell Cultures and National Center of Infectious and Parasitic Deseases: *E. coli* 3548 (ATCC 10536) by NBIMCC and pathogenic *E. coli* ATCC 25922 by NCIPD.

Two methods for assessment of the antimicrobial effect of the thin films were used. The first method was diffusion assay in solid medium – an exponentially growing culture of *E. coli* (100 µl quantity) with optical density OD_{620} = 0.2 was evenly distributed in petri dishes with MPA (meat peptone agar) medium and one of the studied thin films, preliminary sterilized by UV light, was put on the surface of the spread suspension. The petri dishes were incubated initially for 20-24 hours in a refrigerator at 6±2^{O}C to ensure the diffusion of the active substance. The processed petri dishes were incubated for 24 hours at 37^{O}C for stimulation of the *E. coli* growth. On the next stage the availability of sterile zones was observed and measured in mm.

The second method was investigation of bacterial growth in dynamic regime. The determination of the toxic effect was studied through the classical Koch's method and measurement of the optical density. Two strains of test bacteria *E. coli* were used - industrial and clinical strains. The experiment in six-well plate was conducted. First, two controls' glasses, without thin films was flamed with ethanol and put in 2 wells of 6-well plate. In the four wells thin film coated glass substrates were placed sterilized by 30 minutes UV-irradiation. In the well with blank control sterile nutrient medium (meat peptone broth) was added over the thin sterile film without bacteria to measure the thin film light absorption. In the remaining 5 wells culture medium inoculated with a test microorganism with initial concentration (OD 0.01) of overnight bacterial culture, was added. The inoculum and medium quantity in all variants were the same for all samples and control at the beginning of the experiment (at the ratio 1:10). This optical density corresponds to 10^7 CFU/ml. The optical density was measured every hour and in every 2 hours consecutive decimal dilutions were prepared for determination of the bacterial amount of the samples and the controls. The measurement of optical density was carried out with spectrophotometer Specol 11 at λ=620 nm every hour up to 24th hour to check the bacterial and nanoparticles light absorption.

Results

For preliminary rapid screening of the thin films, diffusion assay was carried out in strict congruence with all test procedures described in section Materials and methods. The aim of this experiment was to trace the antibacterial effect of the tested thin films in solid medium. The results are shown in Table 1. As was expected, the pathogenic strain was a little bit more sensitive than the industrial one.

Table 1. Antibacterial effect of thin films recorded by diffusion method [9]

Structure and thickness of layers		Test bacteria	Zone of inhibition, /mm/
Ag	8 nm	*E. coli ATCC 10536* *E. coli ATCC 25922*	0 ± 0.5 (10536) 0 ± 0.5 (25922)
Al_2O_3	20 nm	*E. coli ATCC 10536* *E. coli ATCC 25922*	0 ± 0.5 (10536) 0 ± 0.5 (25922)
Al_2O_3/Ag	20 nm/8 nm	*E. coli ATCC 10536* *E. coli ATCC 25922*	1.0 ± 0.5 (10536) 2.0 ± 0.5 (25922)

Two strains of the Gram-negative bacteria *E. coli* were selected for the study of the bacterial growth in the presence of thin films of Ag, Al_2O_3 and Al_2O_3/Ag, in dynamic regime (Fig. 1 to 3).

Fig. 1 (A and B) displays *E.coli* growth in the presence of Ag thin film. Retention in the growth during the first 4 -5 hours in the presence of Ag thin film was observed by the method of Koch and optical density measurements (Fig. 1A and 1B, respectively). As the nutrient medium for the inoculum activation and for the experiment had the same content and the inoculum was in exponential phase, the delay of bacterial growth was due to the influence of the Ag atoms eluted from the thin Ag film and adaptation of bacteria. Same mode of the dissolution is also established during the first stage of interaction with bacteria in [5]. As the measurements by Koch method were conducted every two hours, we have no results on the 5^{th} h obtained by this indirect method. The measurements at the 24^{th} hour are not represented in Fig. 1 for clarity of the cells reaction in the early hours of the bacterial culture development. It is supposed, that metal ions were eluted from the thin film and they interacted with the cell wall, which decreased the bacteria multiplication rate. There is evidence that both silver state (atoms and ions) have a bactericidal effect [10, 11]. It could be suggested [5] that after the 5^{th} hour the dissolution of Ag ions decreases and after bacterial adaptation they continue to grow exponentially. The comparison of the results obtained by both methods demonstrates the same trends in the development of bacteria under the influence of the Ag film. The Koch's method gives clearer results for the interval till the 6^{th} hour than the optical density measurements.

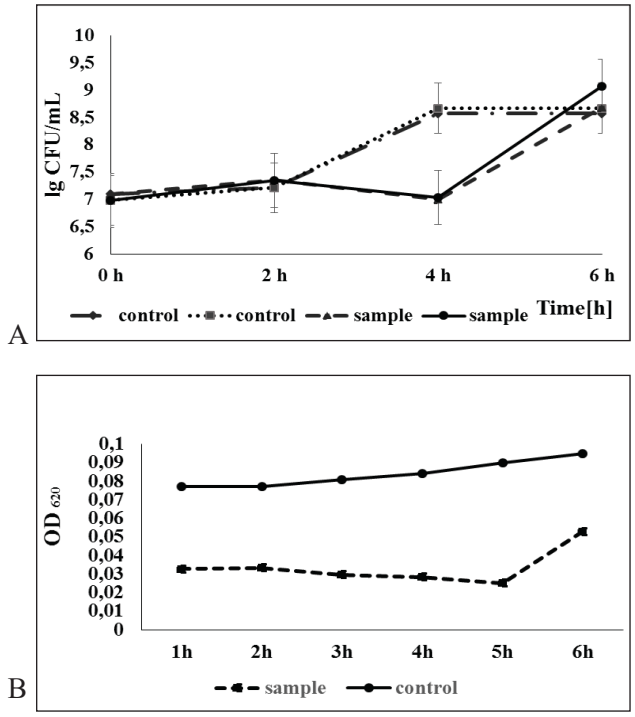

Fig. 1. Growth of *E. coli* in presence of Ag thin films. A- Survival of the cells of the two strains in the presence of Ag thin film determined by the Koch method. B- Growth of both strains *E. coli* in presence of Ag thin film measured by OD_{620}.

The results of the influence of a 20 nm thin film of Al_2O_3 on the growth of the same strains-industrial and clinical - of *E. coli* are presented in Fig. 2A (Koch's method) and 2B (optical density measurements). No obvious differences in the bacterial development between the both strains of *E. coli* (clinical and industrial), were observed. The lag-phase is prolonged till the 3rd hour according to the results obtained by the optical density measurements (Fig. 2B). The same trend is demonstrated by the results of the Koch's method, but not so clear, due to lack of results at the 3rd hour (Fig. 2A). The cultivation method revealed very similar results for the cell quantity in suspensions with and without thin film Al_2O_3 till the 6th hour and small differences at the 24th hour within the experimental error. At the end of the experiment (24 h) the optical density of the pathogenic strain was insignificantly lower than the control and the industrial strain density. The quantity of survived pathogenic *E.coli* cells measured as CFU/ml was 10 times less (in the Fig. 2B) than the control and industrial strain, so it can be concluded that the thin Al_2O_3 film does not influence the growth of *E. coli*. The lack of a growth till the second hour in the control is a normal adaptive phase of the culture in the new medium.

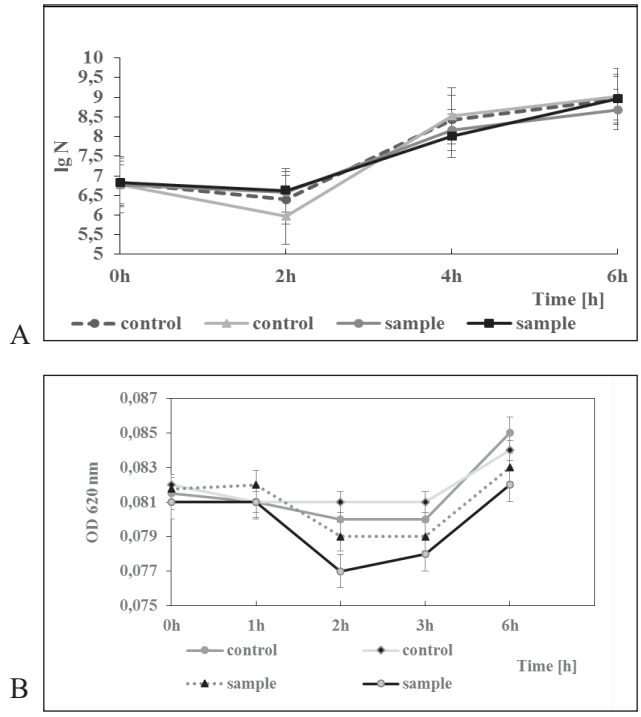

Fig. 2. Growth of *E. coli* in the presence of Al_2O_3 thin film. A- Survival of the cells in the presence of thin film of Al_2O_3 determined by the Koch method (dotted line – *E.coli* ATCC10536, solid line *E.coli* ATCC25922). B- Growth of the test culture as measured by OD_{620} (dotted line – *E.coli* ATCC10536, solid line *E.coli* ATCC25922)

Both strains of Gram-negative bacteria *E. coli* were investigated for the inhibition of bacterial growth in the presence of a thin film of Al_2O_3/Ag in dynamic regime (Fig. 3A- Koch's method and 3B – optical density measurements). There were almost no differences between the two strains. The results demonstrate a lag (adaptive) phase during the first two hours in the bacterial development. Between the second and the third hours they started to multiply, but the influence of thin films appeared. A strong decrease in bacterial quantity was observed between the third and fourth hour – the bacterial colonies were not detected on the nutrient media at the 4th hour in the quantity of 0.1 ml sieved in a petri dish, but obviously single cells survived in a bulk sample. These single cells, undetected on the medium, demonstrated an hour later some detectable growth and started to multiply rapidly after the 5th h. The bacterial density was very similar at the controls and the experimental replicates at the 6th hour. It could be suggested that the nutrient medium is not depleted till the 5th h in a sample as in the control variant and the bacteria quickly achieve the density of the control at the 6th hour. Other possible explanation of the observed results is that the organic substance from the lysed dead cells neutralized the active components eluted from the thin film and at the same time they are additional nutrient medium for the alive cells. The both explanations are equally possible.

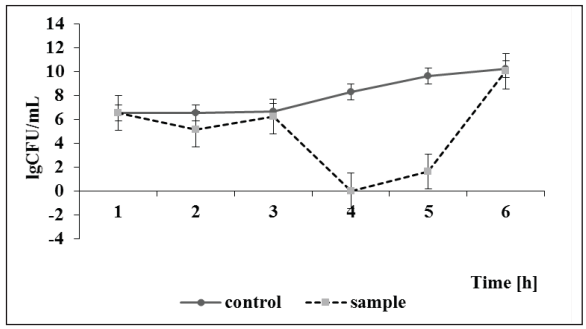

Fig. 3. Growth of industrial *E. coli* strain at presence of thin film Al_2O_3/Ag [9]. Average values of the 2 replicates of control (solid line) and 3 of the sample (dotted line) in lg CFU/ml

Discussion

It can be concluded, that the inhibition effect of the studied thin films - Al_2O_3/Ag, Al_2O_3 and Ag on the bacterial growth of *E. coli* depends on the composition of the thin films and the test method. The results from the diffusion method in the solid medium and the bacterial development in liquid medium are indicative that the studied thin films have no pronounced effect on the both strains of *E. coli*. In [12] the authors have studied the inhibition effect of Ag nanoparticles with size of about 400 nm, which cover the surface of polyurethane, on *E. coli*. They indicated with iso-sensitest agar supplemented with Ag nanoparticles small inhibition zone and established temporary antimicrobial effect on *E. coli*, similar to our results.

The two layer structure of Al_2O_3/Ag has an inhibition effect during the first 4-5 hours. Similar structure but prepared by an impregnation method has been studied in [13]. The authors used scavengers for reactive oxygen species (ROS) on the surface of Al_2O_3/Ag structure and they established no inhibition of the bacterial growth in comparison with the pure Al_2O_3/Ag structure without scavengers [13]. This indicates a formation of ROS when the Al_2O_3/Ag structure is in contact with the bacterial suspension and deactivation of the ROS if a scavenger is applied. These ROS can be a reason for the bacteriostatic effect. As can be seen from Fig. 3, the industrial strain of *E. coli* 3548 shows some retardation in its development during the first 4-5 hours, but after that it adapts to the environment and begins to grow exponentially till the 24th hour.

The Ag thin film had a weak inhibition effect on *E. coli* up to the 5th hour of the experiment, but on the 6th hour the bacterial densities in the control and in the sample were similar. This result probably shows that in low concentrations the silver nanoparticles or their ions have weak effect on the development of *E. coli* and after the 4th hour the culture adapts and shows clearly visible exponential growth of cells in the sample. The Al_2O_3 thin film has no pronounced inhibition effect on the two studied strains of *E. coli*. The future experiments with thicker Ag layer on Al_2O_3 are planned to study the antibacterial effect.

During the test of the thin films with different component composition, but with the same test organism (*E. coli*), an increase in the dehydrogenase activity of the treated cells compared to untreated ones was established [14]. This could be interpreted as a stress response of the

test micro-organisms as a result of their treatment with nanostructured thin films, which caused membrane disruption.

Future studies are planned with new thin films prepared through new technological characteristics for improvement of their antibacterial effect.

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EFFECT OF SALT STRESS ON THE GROWTH AND ANTIOXIDANT DEFENSE OF TWO *LYCIUM* SPECIES

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Abstract

Aim: The effect of salt stress on growth parameters and antioxidant defense system in *Lycium barbarum* and *Lycium chinense* species, grown *ex vitro* in hydroponic at three levels of salinity, 50 mM, 100 mM, 200 mM sodium chloride (NaCl) solution was evaluated. We are looking for the most sensitive physiological markers of the salt tolerance in order to develop a practical strategy for *in vitro* selecting tolerant species.

Materials and Methods: Seeds and *in vivo* explants from the species of *L. barbarum* and *L. chinense* were used for developing of *in vitro* multiplication protocol. *In vitro* propagated plants were transferred to nutrient solution for 48 days and were treated with NaCl solutions for 10 days.

The roots and shoots dry mass of plants was measured gravimetrically after heating at 60^{O}C for 48 h to a constant weight. The activities of antioxidant enzymes SOD, POX, CAT, APX, GR, low molecular antioxidants – GSH, Asc, H_2O_2, as well as MDA were determined spectrophotometrically.

Results: The roots and shoots dry mass of *Lycium chinense* were reduced more than that of *Lycium barbarum* during NaCl treatment.

Conclusions: Our results suggest that *Lycium barbarum* was more tolerant to salt stress than *Lycium chinense* at the salinity conditions tested. It responded to high salinity level with increasing SOD activity, MDA and H_2O_2 levels and improved dry mass accumulation.

Keywords: *Lycium*, salinity, growth, protective enzymes, antioxidants

Introduction

Natural or ”primary salinity” is more widespread in arid and semi-arid regions of the world and results from the accumulation of soluble salts in soils or groundwater over long geological periods. The occurrence of secondary salt – affected soils is due to application of different non-effective agricultural practices and continues to grow [1].

Deeper soil pollution and salinity require using as an alternative fast-growing woody species or shrubs with deep root system and the ability to grow on nutrient-poor soil. The investigators [1] proposed that increased salt tolerance of perennial species (such as woody species and shrubs) used for fodder or fuel production is a key component in reducing the spread of secondary salinity, while increased salt tolerance of crops will directly improve production in soils with primary salinity. The use of salt tolerant species might reduce areas of degraded soils and change their purpose for cultivation of high-yielding plants producing woody biomass, biofuel or economic important bioactive products.

Species from the genus *Lycium* (Solanaceae) are perennial shrubs which inhabit arid and semiarid regions of Asia, America and Africa [2], and its ripe fruits are unique from the

view of pharmacology and medicine. The plants are used in traditional Chinese medicine for the treatment of pneumonia, cough, hematemesis, inflammation, and diabetes mellitus. The great world economic interest about production of *Lycium* is showed. The billion investments for cultivation, conservation, processing, packing and distribution are made. There is business interest for any sort of production on the basis of *Lycium*: drying fruits, packed as a juice, extract, snacks and etc. Because of overexploitation and deterioration of *Lycium* natural habitats, the number and individuals of these species has dropped considerably in recent decades and *in situ* conservation strategies should be adopted to protect and restore all existing populations [3]. Micro-propagation of these endangered species is also recommended, but the breeding high quality of varieties is extremely urgent. In future, analysis of chemical constituents, important for medicine and genetic structure analysis will assist in breeding excellent germplasm of *Lycium* species [3]. *Lycium* species used in the current paper are propagated and rooted according to the technology registered by BioTree Ltd., Bulgaria.

In this research, the effect of NaCl on the growth and antioxidant defense in leaves of two *Lycium* species – *Lycium barbarum* and *Lycium chinense* grown *ex vitro* in hydroponic culture after transplantation the explants were compared so as to provide fundamental base for vegetation restoration in salinized soils.

Materials and Methods

Plant material. Seeds and *in vivo* explants from the species of *L. barbarum* and *L. chinense* were used for developing of *in vitro* multiplication protocol. For induction of shoots, explants of *L. barbarum* were cultured on 4 $g.cm^{-3}$ Murashige and Skoog nutrient medium included 30 $g.cm^{-3}$ sucrose and 8 $g.cm^{-3}$ agar. For shoots multiplication MS medium was supplemented with 1$ml.cm^{-3}$ indolilacetic acid (IAA). For induction of shoots, explants of *L. chinense* were cultured on 2.37 $g.cm^{-3}$ McCawn Woody plant medium included 24.3 $g.cm^{-3}$ sucrose, 8 $g.cm^{-3}$ agar and 0.3 $ml.cm^{-3}$ adenine. For shoots multiplication the medium were supplemented with 0.125 $ml.cm^{-3}$ giberelinic acid (GA_3), 1 $ml.cm^{-3}$ indolilacetic acid (IAA) and 0.5 $ml.cm^{-3}$ indol-3-butyric acid (IBA). After multiplication the shoots were transferred to rooting medium based on half strength basal salts MS medium or McCawn medium, supplemented with compounds described above. The pH of all media was adjusted to 6.0 using 0.1 N HCl and 0.1 N NaOH before autoclaving. All cultures were incubated under controlled conditions – 16 h photoperiod, light intensity of 100 $\mu mol.m^{-2}.s^{-1}$ and 24/18±1ºC day/night temperature. After three weeks of rooting, the shoots were rinsed with 1.5 $ml.l^{-1}$ Proplant solution.

Hydroponic experiment. The experiments were set as four treatments including control, where treatment was 7 replications. The uniform explants were selected and transplanted to polyethylene vessels containing 1.2 cm^3 of 1/4 Hoagland solution (pH 5.9) in growth chamber with a 16-h photoperiod (PAR 100 $\mu mol.m^{-2}.s^{-1}$ on the upper leaf surface, 25/17±1ºC day/night temperature, relative humidity 54/45%). Each vessel contained two plants which represented one replication. After 21 days of cultivation the plants were transferred to 1/2 Hoagland solution. The salt treatment was applied on the

48th day after transplanting of explants when the plants had adapted to the conditions of 1/2 Hoagland nutrient solution and 0 (control), 50, 100, and 200 mM NaCl were added. The solutions were aerated every day and were changed in every 3 d to prevent depletion of nutrients and NaCl. Plants were harvested after 10 d of treatment. Toxicity symptoms (e.g. discoloration, pigmentation, yellowing and stunting) were assessed by eye throughout the experiment.

Measurement of plant growth. At the end of the experiment the plant samples were collected, washed with tap water and rinsed with distilled water before being separated into shoots and roots and fresh mass of each plant sample was measured gravimetrically. Dry mass of shoots and roots was determined after oven-drying (60^{O}C) for 2 days until constant weight was obtained.

Determination of enzymatic antioxidants. In order to prepare crude extracts for the determination of the enzymes superoxide dismutase (SOD), glutathione reductase (GR), guaiacol peroxidase (POX), catalase (CAT) and glutathione reductase (GR) the plant material was grinded with 4 cm^3 of the extraction buffer (100 mM potassium phosphate buffer, pH 7.8; 5 mM ethylene diaminotetraacetic acid, EDTA); 2% polyvinyl pyrrolidone (PVP) that was added to 0.3 g of tissue powder. The extraction buffer for the determination of ascorbate peroxidase (APX) contained: 50 mM potassium phosphate buffer, pH 7.0; 1 mM ascorbate; 1 mM EDTA; 0.2% PVP and was added to 0.15 g of tissue powder. The suspensions were centrifuged (16 000 g, 15 min, 4^{O}C). All enzymes were assayed spectrophotometrically by tracing the changes in absorbance at 27^{O}C using Boeco S-22 UV/VIS spectrophotometer (Germany).

SOD (EC 1.15.1.1) was estimated in reaction mixture of 50 mM Tris-succinate buffer (pH 8.2), 8 mM pyrogallol, 100 µl extract. The decomposition of pyrogallol was determined by following the increase in absorbance at 412 nm for 3 min [4].

POX (EC 1.11.1.7) was estimated in a reaction mixture of 100 mM potassium phosphate buffer (pH 7.0), 20 mM quaiacol, 200 µl extract, 1 mM H_2O_2. The oxidation of quaiacol was measured by following the increase in absorbance at 470 nm for 2 min [5].

CAT (EC 1.11.1.6) was estimated in a reaction mixture of 100 mM potassium phosphate buffer (pH 7.0), 50 µ1 extract, 15 mM H_2O_2. The decomposition of H_2O_2 was determined by following the decline in absorbance at 240 nm for 3 min [6].

GR (EC 1.6.4.2) was estimated in a reaction mixture of 300 mM potassium phosphate buffer (pH 7.5), 3 mM $MgCl_2$, 0.1 mM EDTA, 10 mM GSSG, 200 µl extract, 0.15 mM NADPH. The oxidation of NADPH was determined by following the decline in absorbance at 340 nm for 3 min [7].

APX (EC 1.11.1.11) was estimated in reaction mixture of 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2, 200 µl extract, 0.5 mM Na ascorbate. The rate of hydrogen peroxide–dependent oxidation of ascorbate was determined by monitoring the change in absorbance at 290 nm for 3 min [8].

The protein content was determined after a standard procedure [9].

Nonenzymatic antioxidant metabolites assays. For the low molecular antioxidant metabolites extraction, 0.2 g of fresh mass of the fully developed leaves were ground into fine powder with liquid nitrogen, then 5 ml1 M $HClO_4$ were added. After 25 min

centrifugation at 15 000 g at 4ºC, the supernatant was placed on ice and pH was adjusted to pH 7 (for glutathione) and pH 6 (for ascorbate) with 5 M K_2CO_3. The potassium perchlorate was removed by further centrifugation and the clear supernatants were used for the assays [10]. The concentration of total (GSH+GSSG) glutathione was determined with an enzyme recycling assay [11]. The assay was based on sequential oxidation of glutathione by 5, 5`-dithiobis (2-nitrobenzoic acid) (DTNB) and reduction by NADPH in the presence of GR. The mixture in 1 ml contained 125 mM potassium phosphate buffer and 6.3 mM EDTA pH 6.5, 0.3 mM NADPH, 3 mM DTNB and 50 µl of the supernatant. The reaction was initiated by adding of 10 µl of GR (5 IU/ml) and the change in absorbance at λ=412 nm was recorded. Standard curves were generated with reduced and oxidized glutathione. The results were expressed per 1 g FW.

Reduced form of ascorbic acid (Asc) was estimated as the decrease in absorbance for 1 min at λ=265 nm, in a reaction mixture, consisting of 100 mM potassium phosphate buffer, pH 5.6, 5 µl ascorbate oxidase and 100 µl supernatant. The reaction was initiated with the addition of 100 µl of the supernatant and the decrease of the absorption of samples was recorded at λ=265 nm. Standard curves were generated with Asc [12]. The results were expressed per 1 g FW.

For the determination of H_2O_2, 0.3 g fresh mass of the fully developed leaves were homogenized in a mortar at 4°C with 3 ml 0.1 % trichloroacetic acid (TCA) and centrifuged for 20 min at 15 000 g. Than 500 µl of the supernatant were mixed with 500 µl phosphate buffer, pH 7.4 and after the adding of 1 ml of 1 M KI, samples were incubated in dark for 60 min and absorption was measured at λ=390 nm. The content was calculated using a standard curve of H_2O_2 in the range of 1 – 100 $nmol.ml^{-1}$ of hydrogen peroxide [13].

For malondialdehyde (MDA) estimation, 500 µl of the supernatant were mixed with 500 µl phosphate buffer (pH 7.4) and after the addition of 1 ml 0.5 % (w/v) thiobarbituric acid dissolved in 20 % trichloroacetic acid, the samples were boiled for 30 min. After rapid cooling of the samples in an ice-bath, absorption was measured at 532 and 600 nm using the extinction coeficient 155 $mM^{-1}.cm^{-1}$ [14].

Statistical analysis. All data reported in this work are a mean value from of at least five to six independent experiments. The mean values ±SD and exact number of experiments are given in the tables. The significance of differences between the control and each treatment was analyzed by the Fisher`s LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis.

Results and Discussion

Seedlings growth is commonly limited by increasing the concentration of NaCl. Our results showed that with the increasing of salinity, the levels of dry mass of roots and shoots of *L. chinense* decreased. The differences between all variants of treatments are statistically significant. Roots dry mass of *L. barbarum* increased gradually, while shoots dry mass is highest at 50 mM NaCl in comparison with the control. The differences the between first three variants of treatments are statistically insignificant (Table 1).

Table 1. Mean values of roots and shoots dry mass of *Lycium barbarum* and *Lycium chinense*, grown in hydroponic in response to salt stress

Treatments	Root dry mass [g]	Shoot dry mass [g]
Lycium barbarum		
Control	0.275±0.057a	1.476±0.326a
50 mM NaCl	0.283±0.042a	1.554±0.187a
100 mM NaCl	0.296±0.038a	1.047±0.264b
200 mM NaCl	0.313±0.042b	0.914±0.267c
Lycium chinense		
Control	0.132±0.049a	0.489±0.159a
50 mM NaCl	0.081±0.016b	0.284±0.029b
100 mM NaCl	0.082±0.014b	0.252±0.027c
200 mM NaCl	0.066±0.017c	0.194±0.042d

Mean ± SD (n = 5-6).Values with the same letter are not significantly different when means are separated by Fisher`s LSD test ($P<0.1$).

The increased accumulation of lipid peroxides is indicative of the increased production of toxic oxygen species. MDA concentration is increased with increasing of salinity levels and the maximum values are observed at 200 mM/l NaCl in the leaves of *L. barbarum*, while in the leaves of *L. chinense* highest values are established at 100 mM NaCl (Table 2). The results indicated that salt stress produced more reactive oxygen species, resulting in more increased lipid peroxidative products and oxidative stress in *L. barbarum*. The concentrations of H_2O_2 and total glutathione were elevated with increasing salinity levels, but the concentration of reduced ascorbate declined gradually in the leaves of *L. barbarum*. Hydrogen peroxide, glutathione and ascorbate levels changed in a different manner in the leaves of *L. chinense*. Highest concentration of H_2O_2 was established at 100 mM NaCl. The levels of glutathione and ascorbate were lower in comparison with the control at all variants of treatments (Table 2).

Table 2. Mean values of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), total glutathione (GSH+GSSG) and reduced ascorbate (Asc) contents in the leaves of *Lycium barbarum* and *Lycium chinense*, grown in hydroponic in response to salt stress

Treatments	MDA	H_2O_2	GSH+GSSG	Asc
	[nM g^{-1} FW]	[nM g^{-1} FW]	[nM g^{-1} FW]	[nM g^{-1} FW]
Lycium barbarum				
Control	1.33±0.08a	3.04±0.21a	12.93±1.18a	10.46±1.48a
50 mM NaCl	0.91±0.02b	3.08±0.26a	12.83±1.16a	11.32±1.64a
100 mM NaCl	6.63±0.42c	3.01±0.31a	19.05±1.77b	7.62±0.37b
200 mM NaCl	16.04±1.01d	4.72±0.28b	19.72±1.78b	4.35±0.35c
Lycium chinense				
Control	7.39±0.64a	3.14±0.29a	16.73±4.85a	2.61±0.65a
50 mM NaCl	8.08±0.89a	3.28±0.43a	9.33±1.16b	2.19±0.99c
100 mM NaCl	12.78±1.23b	4.14±0.56b	8.77±1.75c	2.15±0.32c
200 mM NaCl	6.78±0.89a	3.48±0.14a	14.06±2.33a	2.49±0.48bc

Mean ± SD (n = 5-6). Values with the same letter are not significantly different when means are compared by the Fisher`s LSD test (P<0.05).

Table 3. Mean values of superoxide dismutase (SOD) – [$\Delta E.mg^{-1}.min^{-1}$], catalase (CAT) -[$\mu M.mg^{-1}.min^{-1}$], quiacol peroxidase (POX) - [$\mu M.mg^{-1}.min^{-1}$], glutathione reductase (GR) - [$\mu M.mg^{-1}.min^{-1}$] and ascorbate peroxidase (APX) - [$\mu M.mg^{-1}.min^{-1}$] activities in the leaves of *Lycium barbarum* and *Lycium chinense*, grown in hydroponic in response to salt stress

Treatments	SOD	CAT	POX	GR	APX
Lycium barbarum					
Control	0.34±0.11a	36.9±2.9a	45.8±3.4a	29.2±7.0a	314.7±35.5a
50 mM NaCl	0.47±0.05b	34.2±3.2a	28.9±2.9b	30.5±5.3a	90.2±4.2b
100 mM NaCl	0.56±0.04c	30.9±9.7b	24.9±5.1c	36.6±1.8b	74.1±2.2c
200 mM NaCl	0.63±0.16d	28.8±4.5c	20.3±1.4cd	31.9±6.9a	62.2± 1.6d
Lycium chinense					
Control	0.22±0.03a	29.6±5.6a	17.4±2.2a	50.5±8.6a	164.8± 3.2a
50 mM NaCl	0.53±0.02b	23.7±5.2b	13.9±2.8b	20.7±2.0b	160.4± 5.6a
100 mM NaCl	0.56±0.04bc	21.4±1.9c	19.1±3.1a	14.3±3.1c	158.9± 6.6b
200 mM NaCl	0.59±0.07d	10.2±3.8d	14.1±2.8b	23.8±5.9b	126.7±29.9d

Mean ± SD (n = 5-6).Values with the same letter are not significantly different when means are compared by the Fisher`s LSD test (P<0.05).

The level of the antioxidant enzymes, such as superoxide dismutase (SOD), quiacol peroxidase (POX) and catalase (CAT) may determine the sensitivity of plants to lipid peroxidation [15]. In our study, with increasing salinity levels, the activity of the antioxidant

enzyme - SOD is enhanced in the leaves of *L. barbarum* and *L. chinense* and the differences between all variants of treatments are statistically significant. The activities of CAT, POX and APX decreased in the leaves of *L. barbarum* plants, but GR activity is highest at 100 mM (the difference is statistically significant as compared to the control). CAT activity decreased at 200 mM NaCl after treatment of *Periploca sepium* Bunge seedlings in hydroponic [16], indicating that the ability of this antioxidant enzyme to eliminate oxygen species is limited because it possesses relative substrate specificity. Highest POX activity is observed in *L. chinense* plants treated with 100 mM NaCl, but the difference is not statistically significant as compared to the control. The measured activities of GR and APX after the treatment with increasing the concentrations of NaCl are lowest than that of control plants of *L. chinense* (the differences are statistically significant). The results showed that SOD provides a better defense mechanism against salt-stress-induced oxidative damage in *L. barbarum* and *L. chinense* seedlings. The results obtained for *Periploca sepium* Bunge seedlings grown in hydroponic at the same levels of salinity showed that POX and CAT activities rose with increasing of salinity levels [16]. Our results showed that different enzymes and low molecular quenchers (H_2O_2, glutathione, ascorbate) participat in the antioxidant defense of *Periploca sepium* Bunge and Lycium species during salt stress.

In conclusion, *Lycium barbarum* possesses better salt tolerance capacity than *Lycium chinense*, which can help to adapt to increasing salinity levels. It responded to high salinity level with increasing SOD activity, MDA and H_2O_2 concentrations. The results suggest a possibility to improve saline soil by utilizing *Lycium barbarum* because it possesses better antioxidant defense and enhances dry mass accumulation in the roots and shoots.

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MULTIPLICATION AND POLYPHENOLICS PRODUCTION OF *SIDERITIS SCARDICA* THROUGH DIFFERENT TISSUE CULTURE APPROACHES

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Abstract

The aim of the present work was to study the effect of different approaches of tissue culture development on the multiplication as well as polyphenolic production capacity of *Sideritis scardica in vitro*.

Material and Methods: Organic (plant growth regulators benzyl adenine, BA and naphtalene acetic acid, NAA), as well as inorganic (activated charcoal, AC) treatments were applied to shoot cultures of the species.

Results: Higher multiplication rates were obtained by means of plant growth regulators treatments. However, plants tended to have a shorter sub-culture period and form lower and more compact shoot clumps, as compared with the charcoal-treated *S. scardica* plantlets.

Conclusion: As a cost-effective and inorganic agent, activated charcoal seems to be a prospective tool in development of *in vitro* culture system for both prolonging the sub-culture period and inducing *in vitro* multiplication in this plant species.

Keywords: *Sideritis scardica in vitro culture,* plant growth regulators, activated charcoal, multiplication, phenolics.

Introduction

The *Sideritis genus* (Lamiaceae) comprises of over 150 species, distributed mainly in the Mediterranean region, the Balkans, the Iberian Peninsula, Central Europe and West Asia. The genus name comes from the Greek ”*sideros*”, meaning ”*iron*” and is related to the use of these plants to treat wounds caused by metal weapons [1]. The species have also

been applied as anti-inflammatory, anti-ulcerogenic, digestive and antimicrobial remedies in the indigenous ethnobotanical practices of the people in the region of their distribution [2]. In addition studies have shown the antiproliferative, anti-HIV and antifeedant activities of compounds isolated of representatives of the genus. Among the biologically active constituents eluicidated for the representatives of the genus are diterpenes, flavonoids, as well as essential oils [1 and references cited within]. *Sideritis scardica* is a Balkan endemic species, traditionally utilized as a pulmonary treatment, as well as anti-flu and wound healing remedy [3 and references cited within]. It has been known under the common names "*Pirin tea*", "*Mursalski tea*" or "*Alibotush tea*" in Bulgaria, "*Sharplaninski tea*" in Macedonia and "*Greek Mountain tea*" and "*Olympus tea*" in Greece [3]. Interesting neurological activity of *Sideritis* species has been established. Thus, improvement of swimming performance has been established in mice for preparations of *S. libanotica* ssp. *kurdica*, *S. lanata*, *S. perfoliata* and *S. athoa* [4]. Recently the activity of the extract of the Balkan endemic *S. scardica* has been proven to be effective in the Morris water maze test on the cognition and spatial memory performance of Alzheimer model mice [5].

The species is enlisted under the category "*Endangered*" in the Red List of Bulgarian vascular flora [6]. The low germination rate and collection pressure imposes significant risk on its natural populations [7]. Therefore *ex situ* conservation approaches for its conservation have been utilized in Bulgaria, including field cultivation for the needs of the market. In addition plant cell tissue and organ culture techniques provide a controlled and independend of the environmental factors source for the purposes of raw material supply and fundamental studies.

Different approaches have been utilized for the multiplication and biomass formmation of *in vitro* cultured plants. Most often plant growth regulators (PGR) such as cytokinins and auxins have been applied in order to obtain a higher multiplication rate *in vitro*. AC is an inorganic component comonly used in tissue culture medium for various purposes. It might act as either an inhibitor or a stimulant of the plant growth depending on the species or type of tissue [8]. While much information is present on its effect in micropropagation of *in vitro* cultured plants [9], as far as the authors knowledge is concerned, little is still known on its effect on secondary metabolite production. Here are presented the results of the first experiments on tissue culture initiation and *in vitro* multiplication of *Sideritis scardica*, collected from its wild habitat in Bulgaria, by means of organic (PGR) and inorganic (AC) treatments applied. The effect of these medium supplementations on multiplication and total phenolics and flavonoids *in vitro* have been discussed.

Material and Methods

Plant material

Plant material was collected at the Schabran peak of the Slavianka Mountain. Seeds were washed in 70% Ethanol for 30 seconds then sterilized in 0.1% $HgCl_2$ for 8 minutes. After five times washing in sterile distilled water, seeds were inoculated into seed germination medium consisiting of the half strength Murashige and Skoog salts [10], Gamborg [11] vitamins, 20 g/l sucrose and 6 g/l agar.

In vitro culture

For the purpose of *in vitro* multiplication experiment modifications of PGR and AC (Steam activated, 100% assay, Duchefa) were applied (Table 1).

Table 1. Formularion of the organic and inorganic treatments of *in vitro* cultures *S. scardica* shoots

Medium abbreviation	PGR supplementation	Medium abbreviation	AC supplementation
Control	PGR-free, Gamborg vitamins, 30 g/l sucrose, 6.5 g/l agar	**Control**	PGR-free, Gamborg vitamins, 30 g/l sucrose, 6.5 g/l agar
Sm	RM + 0.2 mg/l BA + 0.02 mg/l NAA	**C1**	C0 + 0.02 g/l
Sr_1	RM + 0.2 mg/l BA + 0.5 mg/l NAA	**C2**	C0 + 0.05 g/l
Sr_2	RM + 0.2 mg/l BA + 1.0 mg/l NAA	**C3**	C0 + 0.2 g/l
Sr_3	RM + 0.5 mg/l BA + 0.5 mg/l NAA	**C4**	C0 + 0.5 g/l
Sr_4	RM + 0.5 mg/l BA + 1.0 mg/l NAA		

Total phenolic and flavonoids quantification

100 mg of air dry weight of the plant material were extracted with hot ethanol. After 30 minutes maceration and centrifugation, aliquots of the obtained extract were used for the spectrophotometric assays. Total phenolics were determined by the Folin & Ciocalteu's colorimetric method of Singleton et al. [12] and total flavonoids content of leaf samples of the plant was measured using a colorimetric assay in accordance to a modification of the method of Zhishen [13]. Results were expressed as percentage of the content of polyphenolics in non-treated controls.

Results and Discussion

In vitro *culture development*

Within one month, 1% of germination was recorded. The axillary shoots formed were transferred to PGR-free medium for stock shoot storage with a period of sub-culture 1 month. Longer storage in the medium resulted in darkening of the plant clumps and necrosis.

Comparison between PGR and AC formed shoots showed that the first type of treatment led to the formation of elongated elyptical leaves with larger areas of the leaf blades, longer petiols and well expressed toothing of the leaf margins (Fig. 1). Leaves of AC treated plants were with ovate shape, smaller area of the leaf blade, shorter petiols and less expressed toothing of the leaf margins. The addition of activated charcoal in the medium prolonged the sub-culture period, as compared with the control plants to at least 2 months without significant browning and necrosis of the plantlets.

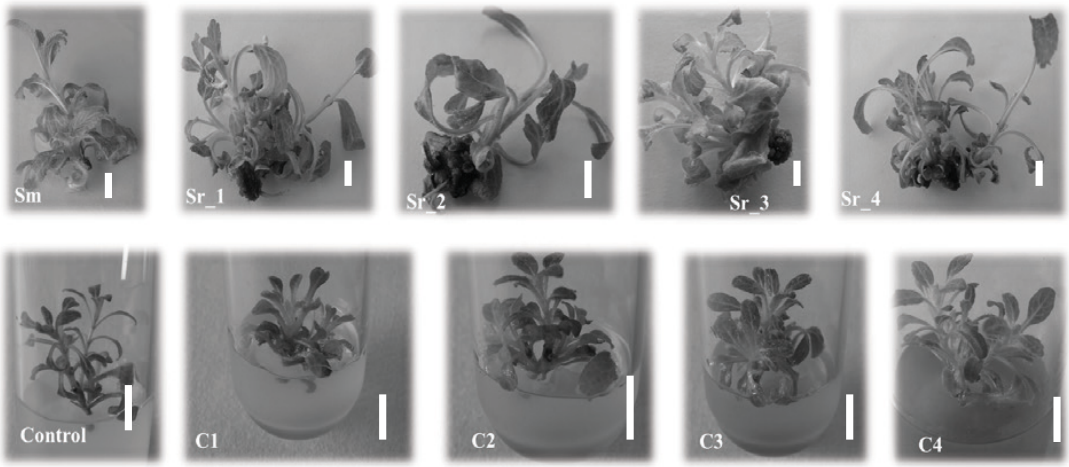

Fig. 1. Morphological development of *Sideritis scardica* in the different culture media modifications. Space bar = 1 cm

Polyphenolics levels in vitro

As a general observation, AC treatment affected the levels of flavonoids as compared with the non-treated control with 0.5 g/l AC supplementation (C4) leading to considerable elevation of polyphenolics levels. On the other hand, in PGR treated plants levels of both phenolics and flavonoids were similarly affected by the treatments as none of the media led to a profound stimulation of these secondary metabolites as compared with the non-treated control (Fig. 2).

The effects of AC may be attributed to establishing a darkened environment for the root; adsorption of undesirable/inhibitory substances; adsorption of growth regulators and other organic compounds, or the release of growth promoting substances present in or adsorbed by AC [8].

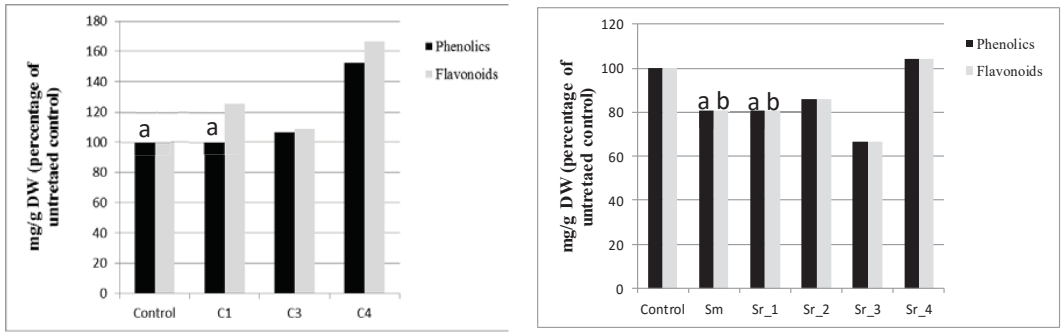

Fig. 2. Total phenolic and flavonoid content in the different treatments.
Arbitrary presentation of data where control values are regarded as 100%. Data for C2 not shown. Same letters denotes non-significant differences (< 0.5)

AC has a fine network of pores and a large inner surface, therefore it has a capacity to absorb many substances. Its stimulating effect on morphogenesis is attributed to the irreversible absorption of certain inhibitory compounds, excreted into the medium and lowering of the toxic metabolites, phenolic exudation and brown exudate accumulation [9].

Conclusion

Further research is needed in order to characteize the phytochemical sructure of compounds produced in the two types of treatments and assess the prospective of the two approaches for biotechnological delivery of plant material with phytotherapeutic potential.

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GROWTH-TEMPERATURE RATES OF *PHYTOPHTHORA* ISOLATES OBTAINED FROM VARIOUS ECOSYSTEMS IN BULGARIA

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Abstract

Aim: Our aim was to investigate the effect of the temperature on mycelial growth *in vitro* of sixteen *Phytophthora* isolates.

Materials and Methods: The isolates were exposed to a range of temperatures varying from 4°C to 40°C, their radial growth rate was recorded per day and the results were compared with referent data.

Results: Maximum growth rate of tested isolates was between 25 and 30°C, with several exceptions.

Conclusions: The revealed variable intraspesific adaptability of investigated *Phytophthora* isolates to the temperature as a major environmental factor could have an important role in the adaptation of the pathogens in the changing climate conditions.

Keywords: *Phytophthora*, mycelial growth, temperature

Introduction

The genus *Phytophthora* comprises of over 100 described species, most of them plant pathogens. Last decades research indicates that invasive *Phytophthora* species are regularly associated with decline and dieback of alder, beech, chestnut and oak stands across Europe. Taking into account climatic trends and widespread nursery infestations in Europe, a proliferation of *Phytophthora* damage may be expected, increasing the instability and vulnerability of forest ecosystems dominated by tree species susceptible to *Phytophthora* [1]. *Phytophthora* spp. are oomycetes, with unique biological and ecological traits as rapid creation of various reproduction structures, rapid evolution of new races and/or strains; and ability to produce hybrid species [2]. Various phenotype traits are used for characterization of *Phytophthora* isolates, including general temperatures for development and colony morphology. Temperature and precipitations are the most significant environmental factors for growth, reproduction and pathogenesis of plant pathogens.

In this study we use growth-temperature rates to characterize several *Phytophthora* isolates, obtained from different ecosystems in Bulgaria. We investigated the influence of temperature on the mycelium growth of sixteen isolates from six *Phytophthora* species.

Materials and Methods

The sixteen isolates used in this study belong to the following species: *Phytophthora plurivora*, *P. cryptogea, P. cambivora, P. citricola, P. rosacearum* and *P. megasperma* (Table 1). The species determination of isolates was proved by classical and molecular methods [3].

Two Petri dishes, each containing 15 ml vegetable agar media (VA) were inoculated with small (approximately 0.5 x 0.5 cm) pieces of 5-7 days old culture of each isolate, placed in the center. The VA was prepared with 100 ml vegetable juice "Gemuse saft Alnatura", cleared with 3 g Ca CO_3 in 900 ml distilled water.

For temperature-growth relationships, plates with VA medium of the tested *Phytophthora* isolates were incubated for 24 h at ambient temperature to initiate growth. Then two replicate plates per isolate were transferred to 4, 10, 15, 20, 25, 30, 35 °C and 40 °C (the last temperature for *P. rosacearum* isolate only). Radial growth rate was recorded after 4 – 11 days along two lines intersecting the centre of the mycelium colony at right angles [4] and was calculated as radial growth per day. Eight point measurements on four directions for each isolate were performed. The obtained results were compared with referent data.

Table 1. *Phytophthora* isolates, investigated in the study

Species	Isolate	Host species	Location
P. plurivora	Tran 2/1	Black alder (*Alnus glutinosa* (L.) Gaertn.)	Along the Erma river
	Tulovo 7/2	English oak (*Quercus robur* L.)	Protected area "Tulovska koria"
	Tulovo 4/1	English oak (*Quercus robur* L.)	Protected area "Tulovska koria"
	Chuypetlovo 3	White willow (*Salix alba* L.)	Along the Struma river
	Velingrad 1/1	White alder (*A. incana* Moench.)	Velingrad town
P. cryptogea	Belasitsa 10/2	Chestnut (*C. sativa* Mill.)	Belasitsa Mountain
	IGSF soil	Pepper (*Capsicum annum* L.)	City of Sofia
	Bankya 1/1	Spruce (*Picea abies* (L.) H.Karst)	Bankya town
	Starosel 2	Prune (*Prunus domestica* L.)	Starosel village
	Vitosha 16	Spruce (*Picea abies* (L.) H.Karst)	Nature Park Vitosha
P. cambivora	Vitosha 4/1	Spruce (*Picea abies* (L.) H.Karst)	Nature Park Vitosha
	Vitosha 11	Spruce (*Picea abies* (L.) H.Karst)	Nature Park Vitosha
	VB 6/2	Chestnut (*Castanea sativa* Mill.)	Berkovitsa town
P. citricola	GD1	Raspberry (*Rubus idaeus* L.)	Gotse Delchev town
P. rosacearum	Tulovo 8/2	Prune (*Prunus domestica* L.)	Tulovo town
P. megasperma	Vr. stena 3/2a	Black alder (*Alnus glutinosa* (L.) Gaertn.)	Treklyanska river

Results

General temperatures for minimum and maximum mycelium growth of all isolates were determined. All isolates were affected in the similar manner by increasing temperature, although to a different extent. All have shown scanty growth at 4°C. Only one isolate of *P. cambivora* VB 6/2, obtained from young chestnut plant in a nursery, did not grow at all at 4°C. Most of the isolates have shown little growth at 30°C (Fig. 3). The maximum growth rate of most isolates was between 25 and 30°C, with some exceptions. All tested isolates showed very little or no mycelium growth at 35°C with exclusion of *P. rosacearum* isolate.

Two isolates - *P. plurivora* Tran 2/1 and *P. cambivora* have shown lower growth rate at 20°C than at 15°C (Fig. 1 and Fig. 3).

Mycelium growth of most isolates was significantly greater in the diapason between 20 and 30°C, than at 10°C and 15°C. Differences among the species and individual isolates varied in the effects of temperature on colony size.

Among the five isolates of *P. plurivora*, four had a maximum growth rate at 25°C, but one (Tulovo 7/2) – at 20°C (Fig. 1).

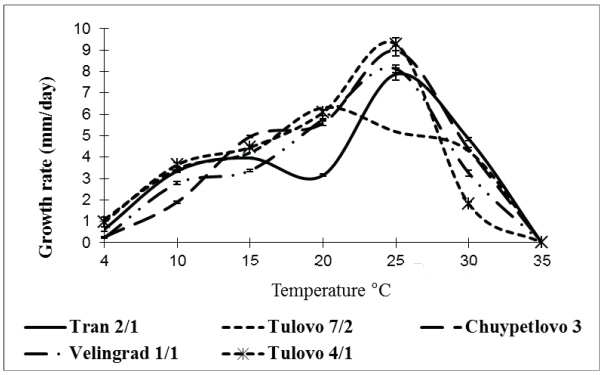

Fig. 1. Temperature-growth relationships of *P. plurivora* isolates

All five *P. cryptogea* isolates have shown maximum mycelium growth also at 25°C, but one of them, IGSF soil, demonstrates much higher mycelium growth comparing to the other isolates at higher temperature at 30°C) (Fig. 2).

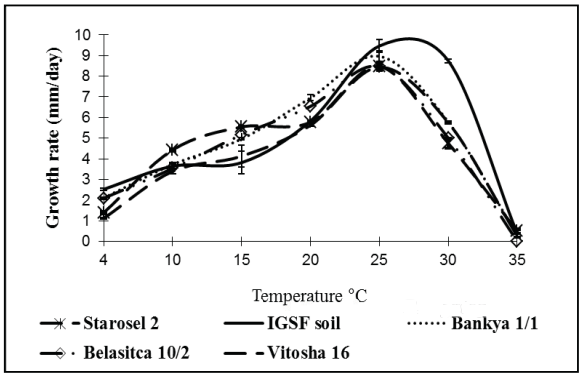

Fig. 2. Temperature–growth relationships of *P. cryptogea* isolates

Among the three isolates of *P. cambivora* tested, two grew best at 25°C, and one (Vitosha 11) – at 30°C (Fig. 3).

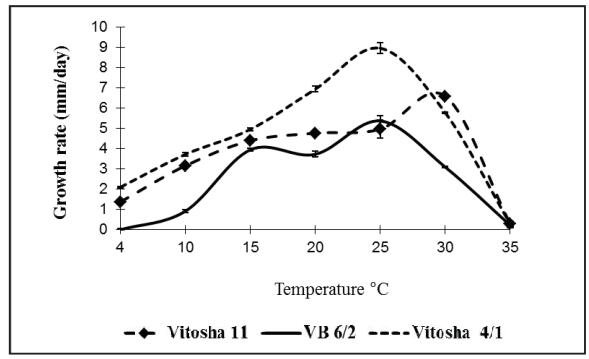

Fig. 3. Temperature–growth relationships of *P. cambivora* isolates

The results obtained for the *P. citricola, P. rosacearum* and *P. megasperma* are consistent with the published temperature range of the growth of referent species [5, 6, 7]. While *P. rosacearum* isolate poses maximum mycelium growth at 25°C, as most of the isolates showed, both *P. megasperma* and *P. citricola* isolates showed maximum growth at 20°C. The *P. rosacearum* isolate is the only one of all tested isolates which shows considerable mycelium growth at 35°C (Fig. 4).

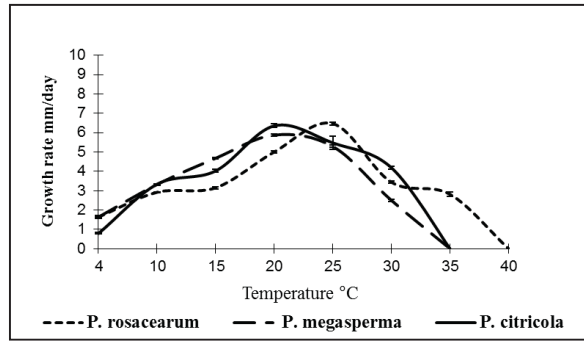

Fig. 4. Temperature–growth relationships of isolates *P. rosacearum, P. megasperma* and *P. citricola*

Discussion

There are several reasons for the dramatically increased distribution of *Phytophthora* tree pathogens in Europe during the last 10-15 years. The increased average of winter and seasonal temperatures combined with a tendency for occasional heavy rains are among the main factors favouring the spread and infection by species of *Phytophthora*.

This study deals with the influence of temperature on the mycelium growth of six plant pathogens species belonging to genus *Phytophthora*.

The presented results confirmed that temperature has considerable effect on the growth of *Phytophthora* isolates with optimum temperatures of highest mycelium growth around 25°C for most of them. At the same time some deviations in the optimal temperatures were observed in the species presented with more than one isolate. For example one of all five presented *P. plurivora* isolates had a maximum growth rate at 20°C and the remaining four - at 25°C. The studied population of this species could exhibit optimal development and eventual spread even in some years with lower average temperature in the vegetation season. At the opposite, one of the studied *P. cambivora* isolates showed a maximum mycelium growth rate at 30°C. It means that among the population of those two species forms exist whose spread will not be suppressed, even more will be facilitated by the higher temperatures.

It seems that optimal temperature of maximal mycelium growth did not depend either of the host species from which isolate is originating, or the region where the isolate is obtained from. For example there are two isolates of *P. plurivora* – Tulovo 7/2 and Tulovo 4/1, both obtained from the region of Tulovska koria and from the same host species – English oak, but Tulovo 7/2 has an optimal temperature for maximal mycelium growth 20°C, and Tulovo 4/1 – 25°C. The similar situation is found with two isolates of *P. cambivora*. The isolate Vitosha 4/1 shows optimal temperature of maximal mycelium growth 25°C and Vitosha 11 – 30°C, nevertheless that both of them are originating from Nature Park Vitosha and are isolated from the same host species – spruce. Results show that the optimal temperature did not depend of the location of the isolate, since the tested isolates are obtained from very diverse locations – mountains, river banks, towns and villages or national parks.

All mentioned above illustrate once again that the studied *Phytophthora* species as all of the pathogens from genus *Phytophthora* expressed high potential for adaptation to the different temperature amplitudes. It is well known that temperature is an important environmental factor with differential effects on each *Phytophthora* growth stage and could have important implications for disease development and disease management in upcoming climatic changes.

Each of the tested *Phytophthora* isolates with Bulgarian origin posses a potential important role in the adaptation of those pathogens and their distribution in future changing climate conditions in various types of ecosystems in the country. Climate changes including changes in the temperature, mainly expected increases in the temperature, will allow the ranges of some *Phytophthora* species to expand in the country in the future.

In conclusion, we found considerable variation among the isolates of six *Phytophthora* spacies related with the minimum, maximum and optimal growth within a relatively small geographic area in the territory of the country. This high level of growth plasticity dependent on the temperature may partially explain the invasion success of these pathogens in recent years. The accumulated knowledge about the variation of this phenotypic characteristic may play an important role in predicting the future spread of the *Phytophthora* pathogens.

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THE EFFECT OF ORGANIC AND CHEMICAL FERTILIZERS ON THE YIELD AND DISEASE RESISTANCE OF TOMATOES – FIELD PRODUCTION

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Abstract

Tomatoes are one of the most valuable and widespread crops in the world.

This work aims to study the effect of different types of fertilizers (organic and chemical), their norms and methods of application, on the yield and disease resistance of Rio Grande.

Materials and Methods: The experiment was performed on alluvial meadow soil in the village Tsalapitsa after pumpkins grown on the same area and according to the same scheme with the following variants: (control – no fertilizer); 100% farmyard manure; 100% chemical fertilizer; 50% farmyard manure + 50% chemical fertilizer), with conventional technology, including irrigation regime and treatment against pests.

The results demonstrate that the combined application of organic and chemical fertilizer is the most effective and results in the highest yield (4307.4kg.da^{-1}). There was a moderate development of economically significant diseases during growth (up to 25%).

Conclusion: This allows us to make the assumption that the combined application of organic and chemical fertilizer increases the crop yield, regardless of the growth of the leaf mass affected by the disease.

Keywords: organic and chemical fertilizer, yield, diseases of tomatoes.

Introduction

Tomatoes (*Solanum licopersicum* L.) are grown in various climate and soil conditions, and the area in which they are grown for economic reasons starts from the northern parts of the globe to reach the cold boundaries of the southern hemisphere. It is assumed that they were brought to Bulgaria by the Bulgarian gardeners from Austria, Hungary and Russia in the 17th century, yet their mass production started in the 18th century.

Whereas in the world there is a trend of steady increase of vegetable production in general and of tomatoes in particular, in Bulgaria there is the reverse trend, namely from the quantities produced in the period 1980 – 1989 (651.108 – 904.784 tonnes) the production decreased steadily in the following years to reach 445.694 tonnes in 1999. In that period of reforms and transition, Bulgaria lost its positions and from a serious producer and exporter of tomatoes became an importer. The main factors contributing to the deepening crisis of tomato production in Bulgaria are the low economic support for producers, the reduced areas, the low production efficiency, the decreased consumption, and the reorganization of the canning industry [1]. Tomatoes are one of the most valuable vegetable crops consumed and spread in the world. They have high taste properties and are rich in vitamins and bioactive substances (antioxidants and polyphenols), important mineral salts and organic acids [2]. There has been identified a connection between their daily consumption as a main source of antioxidants and the reduced risk of cardiovascular and carcinogenic diseases [3].

The knowledge and skilful use of abiotic factors is a prerequisite for the success of tomato production. The important abiotic factors include: light; soil and air temperatures, water, soil and soil pH level, nutrition regime. Tomatoes are a crop of the short (12h) day, but also develop well in a longer such (of up to 14-15h), with the optimal photosynthetic processes taking place at PAR (photosynthetically active radiation) values of around 30.000 – 32.000 lx. For the Bulgarian conditions, the most conducive temperature for the normal development of tomatoes during the day is considered 22-25^{O}C, and during the night – 16-18^{O}C [4]. They are sensitive to water treatment – from the planting of the crops to the formation of the first and second flower head there must be maintained a soil humidity of around 70% of field capacity, and during the fruiting period – 80-85%. Excess moisture is dangerous in the flowering period – it impedes fruit formation, flowers fall off, ovaries are not formed and there is excessive growth of growth mass. The requirements to air humidity are lower, as flowering and fruit formation go well with moderate humidity of 60-65% [5], whereas higher humidity, especially during flowering, may cause pollen to adhere and thus deteriorate pollination and fertilization [4]. The excessively high relative humidity of air is a prerequisite for the development of dangerous fungal diseases in tomatoes), whereas the low such – for the development of mites [6].

Tomatoes are less demanding with regard to soil conditions. The suitable soils are the structured ones, with favourable water and air regime such as the alluvial meadow soil, diluvial soils, carbonate soils, the typical and the leached black earth - soils. The environmental pH level affects the absorption of nutritional substances in plants, and the optimal soil pH range for tomato growth is from 5.5 to 7.0 [7].

Tomatoes extract from the soil a big quantity of nutritional substances, and the export depends on the yield quantity, the soil and weather conditions, the resistance to disease and

infestation, etc. They develop well when the organic substance in the soil is above 1.5% [8]. It serves as storage of nutritional substances and creates conditions for optimal balance in the development of vegetative and reproductive organs of the plants [9].

The economically important diseases during the growth period of tomatoes are *Fusarium oxysporum* Schlecht, *Verticillium* spp., *Fusarium oxysporum* Schlecht f. sp. radicis lycopersici Jarvis&Shoem., *Phytophtora infestans (*Mont) de Bary, *Levilula taurica* Arnaud, *Alternaria solani* Sorauer [10]. The control of these diseases, further to the phytosanitary protection and good care, includes treatment with fungicides, observing the rule of proper alternation of products of different active base and action mechanism.

This work aims to investigate the effect from different types of fertilizers (organic and chemical) and their norms on the yield and disease resistance with paste-type tomatoes, late field production.

Materials and Methods

The experiment was made on alluvial meadow soil after pumpkins were grown on the same area and according to the same scheme of fertilizer application. The data from the agrochemical analysis is baseline for the experiment. There were used 4 variants /4 repetitions as per the Latin rectangle design: control – no fertilizer; 100% farmyard manure; 100% chemical fertilizer; 50% farmyard manure + 50% chemical fertilizer. Cattle farmyard manure was used with contents of general N - 1.45%, P - 2.32% and K - 0.88%. The chemical fertilizers were in the form of ammonium nitrate, triple superphosphate and potassium chloride, in a norm of $N_{30}P_{12}K_{15}$. The farmyard manure together with the potassium and phosphorus fertilizer were applied in the autumn during the deep ploughing, while the ammonium nitrate was distributed into three stages – for nutrition of the plants, during the transplanting and applied two weeks after the planting.

The seeds were sown at the beginning of May (7.05) in an “open" seedbed, and the transplanting was made after 34 days (9.06) in two-row bands with a total area of the plot of 30 m^2 and 80 plants in the plot. The harvesting was one-off, at the end of the second ten-day period of September (18.09). The Rio Grande variety is a mid-early determinate paste-type plant, with growth period of 120 to 125 days, resistant to tobacco mosaic, *Verticillium* and *Fusarium* and giving average yield of 6-7 t/da. The data concerning the weather factors in the growth period were provided by a local weather station. Plant protection treatment was applied as a background in the entire experiment – treatment with: Ridomil Gold 0.25%, Quadris 50g.da, Korsate - 0.25%, Ditan - 0.2% and Equation Pro - 0.2%, twice during growth in the described order, the first one being one week after transplanting and then the interval was 14 days. Against other pests there were applied – three times Decis 10 ml/da for the cotton bollworm; once off Mospilan against the leaf mining moth (tuta absoluta) - 0.02%, as well as two-time treatment with Sencor-50g/da before the transplanting of the tomatoes at a permanent place and after the formation of the first flower head for weed control. The watering was according to the design watering regime with a 75% irrigation norm [11, 12], and the watering norms ($m_{нт}$ mm), by ten-day periods, are as follows: for May, June and September - of 20; July - 20, 40, 60; August – 60, 40, 20.

The following indicators were reported: fruit yield (green and red fruit mass, total yield – kg/da) and growth mass (green mass leaves and stems - kg/da); and the economically significant

diseases through inspection of the experimental areas and macroscopic diagnostics after transplanting; during the 1st, 2nd, and 3rd flowering and after the harvest. For the phytopathological assessment, in case of diseases on the tomatoes there will be used scales for evaluation of the affected area and fruits - respectively grade/% development: a) for *Ph. infestans*: an eight-grade scale – 0/ 0%; 1/0.1; 2/1; 3/5; 4/25; 5/50; 6/75; 7/95 and 8/100 [13]; b) for *A. solani*: a six-grade scale 0/0%; 1/up to10%; 2/11 - 25%; 3/26 – 50%; 4/ 51 – 75% and 5/more than 75% (14), c) for *L. taurica* – 0/1; 2/10; 3/25 and 4/50 (15), d) the other diseases will be reported together, according to the pathogene group (viruses, bacteria and phytoplasms).

Results

The data from the agrochemical analysis characterize the "baseline" soil (no fertilizer) as having low humus content (1.37%) and being slightly sub-alkaline (pH 7.5-7.9). The concentration of mobile forms of phosphorus and potassium is medium to good in the variant without fertilizer (P -14.5; K-14.8), and in the variants with farmyard manure and mixed organic and chemical fertilizer the concentrations of mobile phosphorus compounds are depressingly high (24.7 -98.0). The concentration of mineral nitrogen in all variants is low (8.6 – 14.4), and with the mobile potassium the concentration is medium to good (14.8-29.8). The concentration of mobile forms of phosphorus and potassium is medium to good, as a result of the continuous application of farmyard and poultry manure, and the content of general phosphorus in the sample of the farmyard manure was excessively high - 2.32%. In order to avoid a disproportion of N: P: K with the chemical fertilizing which would affect the outcome, the variants 2, 3 and 4 were leveled only as regards the content of general nitrogen.

The weather during the growth period this year was not any different from the ordinary weather for this region. The data for the period may characterize it as dry, with high maximum values of temperature, which exceeded 25.9^{O}C as early as at the time of sowing the tomato seeds (May) and reached 35 - 36^{O}C in the hottest months (July – August) (Table 1)

Table 1. Average values of the weather factors during the growth period in 2015 in the village of Tsalapitsa

Factors/months	*April*	*May*	*June*	*July*	*August*	*September*	*October*
T m 24h avg.^{o}C	**12.2**	**18.9**	**20.6**	**24.9**	**23.9**	**20.7**	**11.7**
T m.avg.max.^{o}C	**18.3**	**25.0**	**26.5**	**32.2**	**31.1**	**26.4**	**17.2**
T m.avg.min.^{o}C	**5.9**	**12.9**	**14.6**	**17.3**	**17.3**	**14.7**	**8.8**
T abs.max.^{o}C	**25.9**	**29.8**	**33.7**	**36.6**	**35.4**	**34.6**	**24.2**
T abs.min.^{o}C	**0.8**	**8.6**	**10.6**	**12.9**	**13.5**	**8.7**	**3.1**
H.m.24h.avg.(RH,%)	**61**	**73**	**70**	**64**	**68**	**77**	**86**
H.m.avg.min. (RH,%)	**40**	**52**	**48**	**40**	**43**	**57**	**70**
Precip., monthly, mm	**35.4**	**49.6**	**61.0**	**3.8**	**114**	**87**	**30.4**
Precip., days	**9**	**7**	**12**	**4**	**11**	**7**	**7**

T m. 24h avg.°C - monthly 24-hour average air temperature; T m.avg.max.°C – monthly average maximum air temperature; T m.avg.min.°C – monthly average minimum air temperature; T abs.max..°C – absolute maximum air temperature; T abs.min.°C – absolute minimum air temperature; H.m.24h.avg.°C (RH,%) – monthly 24-hour average relative air humidity; H.m.avg.min. (RH,%) – monthly average minimum relative air humidity (RH,%); Precip., monthly, mm – monthly precipitation; Precip,. days – days with precipitation

During vegetation period, rain full were less than the period May – October, optimal for the region, we had a small number of days with precipitation (from 4 to 12), the least being in July, and the most in June and August. In those months there were reported respectively the minimum and the maximum precipitation values in mm. The low levels of precipitation and the high temperatures in the growth period were also a reason for the air humidity (%) to be relatively low. Its values were in the range from 61 during the transplanting (April) to 77% and 86% around the end of the growth (Table 1).

During the inspections of the planted area (at the end of May, the beginning and end of August, the middle of September), two of the economically significant diseases were registered – *Ph. infestans* and *A. solani*.

They affected the growth mass, on which the development of the diseases was registered from their appearance to the end of the growth, for each variant; initially the tomatoes were affected by *Ph. infestans* before the transplanting (end of May), while *A. solani* appeared at the end of August (Table 2).

Table 2. Average values, in grades, of leaf surface affected by *Ph. infestans* and *A. solani*

Variant/ Reporting	*Ph. infestans/A. solani*	*Ph. infestans/A. solani*	*Ph. infestans/A. solani*	*Ph infestans/A. solani*
	1st	2nd	3rd	4th
1	2/0	3/0	4/3	5/4
2	2/0	3/1	4/2	6/3
3	2/0	3/0	4/3	5/4
4	2/0	3/1	4/2	6/3

In the initial period of development of tomatoes in all variants and repetitions single incidences of *Ph. infestans* of minor size were observed. In the course of time, the percentage of affected area gradually increased and around the end of August it reached up to 25% of the leaf mass, and there were not reported any differences in the separate variants and repetitions. At the end of the growth (September) in the separate variants of fertilization, the affected areas reached 50-75%, and a significant difference (of 25%) was observed where organic substance was applied - 2 and 4 (Table 2).

With regard to *A. solani*, despite the later manifestation, the development of the disease expressed as a percentage at the end of the growth period was similar to that of *Ph. infestans*, but was in the range from 3rd to 4th grade (50-75%), with stronger development in the variants without fertilizer and with separate use of chemical fertilizers (Table 2).

The data concerning the yield from the experimental areas of Rio Grande tomatoes, field production, show that the highest yield was obtained in the variant with a combined use of 50% farmyard manure and 50% chemical fertilizer (4.307.4 kg/da^{-1}), and the lowest one

was obtained in the control variant (no fertilizer) – 1.670 kg/da^{-1}. It is worth pointing out the values of the cumulative growth mass (leaves and stems), where the highest values were obtained only with the separate use of chemical fertilizer (2.636 kg/da^{-1}), which significantly exceeded the next variant, the combined use of chemical and organic fertilizer – by 760 kg/da^{-1}. In this case the greater organic mass did not mean higher yield of tomato fruits. The derived differences have been statistically proven (Table 3).

Table 3. Yield of fruits and growth mass at the end of the growth period (kg.da^{-1})

Variant	Leaf mass	Stem mass	Fruit mass		Total yield
			green	red	
Control	752.8	623.2	641.1	1029.0	1670.1
100% farmyard manure	981.6	834.4	1061.9	1280.6	2342.6
100% chemical fertilizer	1470.6	1166.9	1181.8	2366.8	3548.6
50% f. m. +50% chem. fert.	1128.5	746.7	1309.4	2997.9	4307.4
LSD ≤ 0.05	207.13	197.73	195.12	836.76	
LSD ≤ 0.01	301.38	287.71	283.91	1217.54	
average	*1083.367*	*842.8*	*1048.575*	*1918.592*	*2967.175*
median	1032.1	822.6	1095.4	1510.7	2945.6
Standard deviation	265.15	210.58	254.80	851.5	

f.m. – farmyard manure; chem. fert. – chemical fertilizer

Discussion and Conclusions

During the tomato growing period, the plant protection actions were performed on a regular basis and it is assumed to be one of the reasons for the obtained results, and the separate variants do not demonstrate any significant differences as regards disease resistance of Rio Grande.

The data concerning the main climate factors (temperature and humidity) in the experimental period show that there are no favourable conditions for the development of *Ph. infestans* – temperatures of around 18-24^{O}C and presence of water or high humidity of around 90%, whereas the occasional precipitation and temperatures higher than 25^{O}C are not conducive to its development. There is a more favourable environment for the disease development at the end of the growth period and then naturally the percentage of affected leaf mass increased by 25 and 50% compared to the preceding inspection in the different variants. The appearance and development of *A. solani* was in line with the pathogene etiology – its preference to aging plant tissues (Table 1).

The analysis of the results of the total yield of Rio Grande tomatoes and the effect of the reported diseases on it, as well as the connection with the applied types and norms of fertilization in the particular weather environment does not provide a marked connection between the considered yield factors. The biggest incidence of *Ph. infestans* was registered in the variants with organic fertilizer, whereas of *A. solani* in those with chemical substances.

Therefore it can be suggested that the major role is played by the type of fertilizer used, and if a comparison is made between the volume of leaf green mass and the obtained yield of tomatoes, certain dependence may be identified. The affected parts of the plants are practically dry, necrotic tissue, incapable of photosynthesis and of producing organic substance. The following picture is outlined if the useful photosynthesizing area is calculated for the respective variants for 100 kg.da^{-1} and is compared to the obtained yield. In the control variant 100 kg.da^{-1} of leaf mass ensure a yield of 444 for kg.da^{-1}. In the independent use of farmyard manure and chemical fertilizer it is respectively 318 and 440 kg.da^{-1}, whereas in the combined use of those it is 509 kg.da^{-1}.

In this case the organic fertilizer used in this form is less efficient, and even has a negative impact on tomatoes in comparison with the non-treated variant. Its combined use with chemical fertilizer, however, increases its efficiency which is manifested in the crop yield, regardless of the larger growth mass affected by diseases as well.

The obtained results from the experimental period provide use full data to assume that the combined use of organic and chemical fertilizer is the most effective variant in the fertilization of Rio Grande paste-type tomatoes – late field production, which gives the maximum yield of fruits.

With the unfavourable weather conditions and the used standard schemes and products for plant protection, the development of the economically significant diseases *Ph. infestans* and *A. solani* in tomatoes during growth was of medium size (up to 25%).

The variants with organic substance have a higher percentage of areas affected by *Ph. infestans*, whereas those without fertilizer and separate use of chemical fertilizer - of *A. solani*.

The obtained results give grounds to assume that the combined use of chemical and organic fertilizer increases the overall efficiency in terms of crop yield, regardless of the larger leaf mass affected by disease.

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METHODS, APPLICABLE TO THE ECOTOXICOLOGICAL BIOMONITORING OF TERRESTRIAL VERTEBRATES: A REVIEW

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Abstract

Recent years have seen an expansion in the scope of ecotoxicological studies involving the biomonitoring of chemical and physical toxic agents (heavy metals, organic pollutants, radioisotopes). The aim of the current work is to provide an overview of the methods which can be used in this area of study, and to assess their respective strenghts, shortcomings and applicability. Some of them include: morphophysiological and hematological indicators, histopathological and cytogenetic methods, molecular biology techniques like the comet assay and micronucleus test, determination of the radiological and other toxic burden by means of different spectrometric methods, and non-invasive sampling techniques. The methods are assessed critically and their strengths, weaknesses and utility are presented in the context of ecotoxicology. As a review, this article provides an evaluation of these approaches to ecologists and other scientists involved in work in the field.

Keywords: ecotoxicology, biomonitoring, methods, review

Introduction

Ecotoxicological biomonitoring has a rich and complex history; since the First and Second industrial revolution, an increasing number of chemical pollutants, biogens and other chemical, physical or biological agents are screened for and used in biomonitoring studies. The field has developed and expanded to include an ever-increasing scope of agents and a broad array of methods. The biomonitoring of contaminants in terrestrial ecosystems *in situ* is especially relevant when using small mammals, because they are common, convenient for sampling, and are the subject of many past studies and standardized protocols [1, 2, 3].

In Bulgaria the history of biomonitoring started in earnest in the 1970s when background stations were established in Rozhen in the Rhodope Mountains, Strandzha Mountain, Steneto in the Stara planina Mountain and an impact station was established in the Srednogorie region. In the late 1980s the applicable methods, were standardized across the nations of the former COMECON, in part through a comprehensive training course in biological monitoring in 1984 and a publication issued by the Publishing house of the Bulgarian Academy of Sciences [4]. Later, reference point such as the Mussala Peak was established and an impact station was set up near the non-ferrous metallurgical plant in Plovdiv. Both in Bulgaria and abroad, the field of ecotoxicology has expanded to include a variety of methods, which were approved and standardized during the initial development of background and impact stations in Bulgaria in the 1970s and 1980s [4]. Some of the methods, like DNA damage assays (micronucleus assay and comet test) and cytogenetics have been adapted in the monitoring set. Others, such as non-invasive sampling of monitor species and the measurement of chemical bioaccumulation, are particular to the field of ecotoxicology. The methods described below comprise the main instrumentarium for scientists working in the field.

Methods Discussed:

1. Morphophysiological indicators.

a) Classic morphophysiological studies

Morphophysiological indicators (method of Shwartz) are one of the classic tools and one of the first methods applied to ecotoxicological biomonitoring [1, 5, 6]. They are conventionally used in population studies on the effects of environmental factors, including pollution, on individual development. The method enables to access physiological characteristics of animals based on the set of indirect indicators such as the relative mass of animal visceral organs. The following parameters were estimated the animal body mass, index of fatness, liver, adrenal glands, testis index etc. In addition, the progeny of a monitor species can be screened for the presence of anomalies [7]. For example, in heavy metal-polluted environments, where lead is present, the ratio of the mass of the liver to total body mass increases proportionally [8], and, in the case of cadmium intoxication, the kidney-to-liver mass ratio decreases [9]. It is important to mention that, while comparing morpho-physiological indicators, animals of the same gender and approximately the same age are used. To obtain the reliable results a large sample for statistical significance needs or to be combined with other suitable methods.

b) Hematological indicators

Haematological indices are important tools for the status of individuals and populations of wild animals that are affected by toxicants or diseases and must be studied with respect to possible influence of natural or anthropogenic factors. Hematological changes in the peripheral blood are a good indicator of general pathology associated with an environmental contamination. They include hemoglobin, hematocrit, number and shape of erythrocytes and differential counting of white blood cells, as well as other parameters such as creatinine and uric acid. While classical hematology has been supplemented by more modern methods in the case of the screening for genotoxic agents, the above parameters can serve as indicators for both 1) an action of a toxic agent in biologically effective concentrations, and 2) the adaptation of an organism to a particular disturbance in the environment [3]. An example is exposure to ionizing radiation at doses above 500mGy which induce lymphocytopenia, and at higher doses, granulopenia and general anemia. Heavy metal pollution, for instance, exposure to lead, may cause a drop in hemoglobin and hematocrit values, as well as changes in the morphology and function of erythrocytes [8], leading to pernicious aplastic anemia in mammals if blood levels of lead increase above 40 μg/dL [10]. While hematology remains a useful and important diagnostic tool in ecotoxicological studies, molecular methods such as the micronucleus test may provide higher resolution for genotoxic agents because of the appearance of effects at lower concentrations than for hematological studies [11]. Some techniques such as the measurement of white blood cell count need to be supplemented by other methods like toxic body burden concentration, since changes in blood cell count can be due to other factors: infection, the presence of parasites, or other challenges to the immune system. Knowledge on haematological indices of various species small mammals is essential for the examination of the moment physiological status, reproduction, adaptations and scientific management if this species.

2. Histopathology.

Changes, which can be detected by histopathology, include stenosis, cirrhosis, steatosis (fatty degeneration), preneoplastic lesions (foci), focal necrosis and blood vessel occlusion. Histopathological techniques are sensitive, well-developed methods for the biomonitoring and bioindication of toxic stress coming from the environment [8, 12]. According to [12] they are good tools especially in the validation of prognostic biomarkers. One of the most important target organs for histopathological studies is the liver, since it is the tissue where both 1) metabolic activation of certain toxicants occurs, and 2) accumulation of toxicants, especially organic pollutants, is known to occur [13]. The activation of potentially carcinogenic substances can result in neoplastic alterations. Together with the morpho-physiological examination of the liver, a histopathological analysis can be very informative in providing data for the action of a toxic agent. For instance, a study in polluted and reference sites in Poland has indicated that contamination with polymetallic industrial dust inducing whole-body concentrations of >7.6 μg/g cadmium and 1.6 μg/g lead can induce histopathological lesions in the livers of bank voles (*M. glareolus*), including depletion of glycogen, acidosis and focal necrosis [8]. While histopathological techniques are well established in ecotoxicology, they are seldom used nowadays as the sole experimental technique in a given study – they are most often employed in conjunction with other methods such as determination of chemical

body burden by mass spectrometry [8], or molecular biology and biochemical techniques for the determination of prognostic biomarkers of toxic exposure such as enzyme activity [12].

3. Determination of body burden, and bioaccumulation of toxicants

Bioaccumulation is a very significant phenomenon, since it can lead to increased concentrations of a given toxicant at the trophic levels, affecting heavily populations and organisms. The determination of body burden of a particular toxicant plays a key role in ecotoxicological biomonitoring as it provides a link between the physical amount of a given agent in the organism and its biological effects. Some toxic element or compound can increase over time in particular tissues and organs or in the entire organism in a process of bioaccumulation. In this context, it is important to mention target organs and tissues. More than 70% of the total body burden of lead is accumulated in the bones, where it displaces selenium and calcium. Other non-essential elements such as cadmium can be found in the liver and kidneys due to enzymatic pathways and excretion via metallothionein-bound complexes [9, 13]. An example of bioaccumulation is lead's pathway in the mammalian organism [14], which includes a displacement of selenium, and induces a deficiency in the absorption and retention of calcium, iron and zinc. Lead is accumulated in the bones, where it interferes with hematopoietic enzymatic pathways, specifically the ALAD enzyme, and this accumulation is one of the reasons for lead-induced anemia.

The determination of the concentration of a given element can be performed by one of the following methods: mass spectrometry, atomic absorption spectrometry (AAS), inductively coupled plasma mass spectrometry (ICP-MS), and neutron-activation mass spectrometry (NAS). While AAS is a standard technique, ICP-MS provides a more convenient and high-throughput method, which is nowadays more often used for the determination of toxic body burden. NAS is a very precise method, but requires a large neutron source (usually nuclear reactor) and sometimes involves long waiting times.

In the case of measurement of radionuclides, additional techniques are also available. These include external gamma counting, determination of total beta-activity [15] and the measurement of the total burden of a given gamma-emitter by gamma spectrometry.

4. DNA integrity/molecular biology techniques

a) Micronucleus test

The micronucleus test is based on the increase in frequency of extranuclear DNA-containing vesicles (Howell-Jolly bodies), which increase in the presence of clastogens, aneugens and other general cytotoxic agents. It is a standard, sensitive method to detect the effects of environmental toxicants and has been used in wild rodents since 1978. Nowadays the method is increasingly applied especially in the context of only small amounts of blood and sampling with limited invasiveness and fast results even in field conditions.

Micronucleus frequencies (MN) are used to detect the cellular action of agents causing breaks in DNA and chromosomal aberrations. The test has two main variations: the cytokinesis-block micronucleus assay, and the staining of micronuleated erythrocytes from whole-blood smears [11, 16, 17]. The cytokinesis-block micronucleus assay, performed by chemical-induced cytokinesis block, has two main requirements, namely 1) availability of dividing or division-inducible cells, and 2) sample amount in milliliter quantities, which make it hard to apply to small terrestrial vertebrates.

Significant correlations between heavy metal contamination and micronucleus frequency have been detected in wild rodents leaving in polluted areas [2, 16, 18, 19]. Cadmium concentrations inducing body burden above 13.45 μg/g can produce increased frequency of micronuclei in *Apodemus sylvaticus, Apodemus flavicollis* as well as *Microtus arvalis* [11, 19]. Especially in the case of polymetallic industrial contamination, significant correlations between contamination and MN frequencies have been detected [19]. The micronucleus test is relatively sensitive and informative, though it is non-specific (being confounded by factors such as age, diet, metabolic activity and infectious/parasitic disease), which means it must be combined with other techniques such as determination of whole-body burden of a given toxicant, as well as more standard cytogenetic methods or fluorescence *in situ* hybridization (FISH) in order to rule out confounding variables.

b) Comet assay

This is a sensitive method for detecting multiple types of DNA damage, based on the formation of a tail of genomic DNA in lysed cells during electrophoresis, which depends on the amount of DNA breaks present in the cell. The Comet Assay is also used to monitor DNA repair by living cells. It exists in three main variations: neutral comet test, alkaline comet test and enzymatic comet assay, which combines the action on DNA of an alkaline denaturing step with the action of an enzyme which converts DNA base and nucleotide damage to single-strand DNA breaks. All three methods are applicable to ecotoxicological studies and detect DNA fragmentation with different sensitivities [20]. The most sensitive assay is the enzymatic comet test, which is based upon the action of an endonuclease converting base and nucleotide damage into single-strand breaks [20]. While all versions of the comet test are applicable to ecotoxicological biomonitoring, they carry certain limitations. One of the main drawbacks of the method is that so far it has not been conclusively proven that each of the three versions indicates a specific type of DNA damage. While comet assays, especially in the alkaline and enzymatic versions, are sensitive methods for the initial detection of a genotoxic agent, they are compromised by the fact that there is inter-individual variability in DNA fragmentation, compounded by factors such as age and sex differences [21]. Last but not least, the comet assay is not specific for the action of a particular genotoxic agent, meaning that results obtained by this method do not lead to specific conclusions regarding the action of a given chemical or physical agent; results are often confounded by variations in metabolic rates. The drawbacks listed above mean that while this technique remains a useful tool for detecting varieties in DNA fragmentation, it can't replace the older, more standard micronucleus test.

5. Cytogenetic techniques

a) Chromosome aberrations (CA)

These are changes in the cellular chromosome pool, detected during metaphase in dividing cells, and appear as a result of DNA breakage during interphase and cell division. They can be several varieties – dicentrics, ring chromosomes, deletions, insertions, acentric fragments and aneuploidy, and, although present physiologically, increase in frequency after exposure to a clastogen. The scoring of chromosome aberrations is the gold standard in assessing damage from ionizing radiation and the action of genotoxic agents and is even, when calibrated, used quantitatively for biodosimetry since some of the genetic damage stemming from radiation is specific (double-strand breaks). For the purposes of

ecotoxicological biomonitoring this technique is also useful when addressing the effects of clastogenic factors, including ionizing radiation [22, 23]. Due to sampling considerations, for the monitoring of small terrestrial vertebrates bone-marrow cells are used [11, 22]. There have been studies, comparing the effects of ionizing radiation on several species of voles, including *Microtus arvalis, Microtus agrestis, Clethrionomys glareolus,* and *Microtus oeconomus* [22]. The induced background and mutation spectra (c. 5 metaphases with CA) indicate that the *Microtus* species are the most suitable when it comes to biomonitoring for ionizing radiation. In the biomonitoring of terrestrial vertebrates, an exemplary application is the use of monitor species (*Clethrionomys glareolus* and *Apodemus flavicollis*) for the biomonitoring of ground contamination in Belarus in the wake of the Chernobyl accident, producing definitive results with respect to the correlation of a biological parameter (chromosome aberrations and polyploidy) and exposure to a clastogen (ionizing radiation) [24]. One drawback of this method is that the frequency of chromosomal abnormalities degrades over time after the initial exposure to a clastogenic factor. In spite of these shortcomings, the scoring of the above-mentioned aberrations remains a very useful tool in biomonitoring. In Bulgarian research, these data have been correlated with the heavy metal loading of small mammals from different regions, producing spectacular results [25, 26].

b) Sister chromatid exchanges (SCEs)

This technique is another cytogenetic method, based on the exchange of chromosomal material between mother and daughter strands during mitosis, influenced by the action of genotoxic agents [27, 18]. It is not as standard as the counting of chromosome aberrations, but it has a good applicability to ecotoxicological biomonitoring and has certain advantages: 1) it is a fast procedure, requiring almost no waiting time for its application, and 2) it gives rapid results for the detection of initial toxic exposure in a primary culture or an immortalized cell culture line. However, it requires a stable population of dividing cells in order to be effective. Despite this disadvantage, the scoring of sister chromatid exchanges remains a useful tool for the determination of a toxic exposure, although for better estimation of biological effects it needs to be combined with other methods. It is mostly used in variant ecotoxicological studies, where a chemical must be assessed for genotoxicity [22, 27].

In the context of biomonitoring, clastogenicity, DNA damage potential and genotoxicity can be measured fairly conveniently by methods such as the micronucleus assay and the comet test, yet these techniques are seldom used by themselves due to the potential of confounding variables. Cytogenetics includes a more standard and well-established set of methods, yet they too need to be supplemented by other techniques. Neither method by itself can provide conclusive results, which means that in modern ecotoxicological studies these techniques are usually combined with spectrometric measurements of the contaminant under study [8, 25, 28].

Conclusion

The future of ecotoxicological biomonitoring includes the application of non-invasive sampling techniques, the application of which is still in its early stages [5]. These techniques for terrestrial vertebrates include the collection of excreta, hair, feathers, eggs and other materials in a way that will permit the conducting of ecotoxicological studies and the

analysis of parameters such as concentration of a certain pollutant. Tissues such as hair and feathers are particularly good indicators since these tissues are not metabolically active and frequently animals deposit pollutants in them as a means for excretion.

For the biomonitoring of terrestrial vertebrates, the methods described above seldom offer solutions by themselves, therefore an integrated approach is often used. Sometimes two or more methods to measure genotoxicity and toxicity to the organism are combined [11, 18], or a classic histopathological method is combined with measurements of total protein content and activity [13], or a measurement of whole-body burden of a given toxicant [8]. More often than not, the selection of methods includes measurement of toxicant concentration in the body [28, 29].

The above techniques constitute the most popular methods in ecotoxicological studies of terrestrial vertebrates. The measurement of body burden, or another way of estimating the toxic loading, are essential in almost all situations, and classic indicators such as morphophysiological indices and hematological parameters are key in the initial evaluation of a toxicant's action. These methods are nowadays very often supplemented by molecular analyses of the genome (especially in cases of pollutants with marked genotoxic effect), with cytogenetics being the most precise method and micronuclei and comet scoring coming second and third in utility. Although most techniques are not sufficiently informative by themselves, in combination, and depending on the endpoints desired, these techniques can give conclusive results in a study, and provide solutions to scientists in the field well within the 21st century.

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THE CHOLINESTERASES AS BIOMONITORING MARKERS IN CASES OF POISONING OF ORGANISMS IN ECOTOXICOLOGICAL ENVIRONMENT

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Abstract

This research is part of a project for biomonitoring study of the intoxication of various animals in an ecotoxicological environment (carbamate and organophosphate pesticides, nerve poisons, drug overdoses, medicines and others).

The main biomarkers in our work are changes in the activity of acetylcholinesterase (AChE; EC 3.1.1.7.) and butyrylcholinesterase (BChE; EC 3.1.1.8.) compared to normal reference values in various body structures. Cholinesterase activity has been determined.

Enzyme mapping activity and some kinetic parameters of AChE and BChE in the some target animal species and tissues indicate the existence of a correlation between enzyme activityes and structure and functions of these objects. We have also determined the dose-dependent inhibitory concentrations of certain carbamates and organophosphate compounds on AChE and BChE.

AChE and BChE are very good marker enzymes to measure the degree of intoxication of organisms in ecotoxicological environment. It was found that some donors of NO (e.g. L-Arginine) are potent activators of the two enzymes. The implication is that such donors can be successfully used as antidotes in some ecotoxic situations.

Keywords: cholinesterases; biomonitoring; anticholinesterases; L-Arginine; neurotoxicology, antidotes.

Introduction

AChE and BChE are important components of the cholinergic system, which control synaptic processes (AChE and partially BChE) and the content of choline esters, pesticides with anticholinesterase action, some drugs (heroin, cocaine, etc.), medicines (aspirin, diazepam, etc.), toxins of plant and animal origin and others in the blood and haemolymph (invertebrates), extracellular fluid and other body fluids (mainly BChE) [1, 2, 3, 4].

The very low activity of BChE is an indicator for BChE deficit [5, 6]. It is a diagnostic indicator for severe genetic defects, specific for some communities in India, Japan, China, the Caucasian region and others. An example in surgical practice is the reaction of patients to the introduction of succinylcholine. In normal state, muscle relaxant effects of this ester last only for a few minutes (it is very quickly degraded to 90-95% by BchE). In the case of BchE deficiency, the immobilization continues for hours [6].

While the main biological role of AChE is the rapid degradation of acetylcholine (ACh) in the synapses and for maintaining the normal dynamic concentration of this neurotransmitter, the role of the BChE is much more diverse. This enzyme is a kind of an "immune system" of the body providing preventive protection of the nervous system from damaging factors [7].

AChE and BChE play a very important role not only as bio-indicators for eco-toxic damage to the environment, but in some cases of poisoning of humans and animals.

In this work, data are presented for the activity of AChE and BChE (reference values) in various brain parts in rabbits and laboratory white rats, the level of inhibition of cholinesterase activity depending on the concentration of certain carbamate compounds and the role of certain sources of nitrogen oxide (NO) as an antidote in cases of poisoning by anticholinesterase agents.

Materials and Methods

Preparation of enzyme fractions. The isolation of the membrane and mitochondrial fraction of the brain has been performed by the method of differential centrifugation. The isolation solution has been composed of: 0.1 M KCl, 1mM $MgCl_2$, 0.1 mM EDTA-Na salt, 50mM Tris-HCl (pH 7.6). The ratio between the respective tissue (g) and the isolation solution (ml) has been 1:9 or 1:5. The duration of homogenization has been 60 seconds repeated twice. The homogenate has been centrifuged (centrifuge model "Yanetski" K24) at 3000 rpm (1500 g) for 25-30 min. The resultant precipitate has been comprised of cell nuclei and damaged tissues, cells and undegraded particles. The supernatant is the so-called membrane-mitochondrial fraction that has been used in this study [8].

Uses and work with laboratory animals. In our laboratory studies we have used male and female white rats (200-250 g) from the vivarium of the Faculty of Biology of the Sofia University "St. Kl. University" and rabbits from the University's experimental base. The investigation is conformed to the international and national rules and regulations for ethical treatment of animals.

Cholinesterase activity assay. There are many methods for determining the acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity [8]. We have assayed the AChE and BChE activity by the method of Ellman et al. [9]. The esterase activities have been measured by using artificially synthesised substrate of acetylthiocholine iodid (ATChI) or butyrylthiocholine iodide (BTChI). One of the hydrolysis products of ATChI or BTChI is thioholine (TCh). The SH-groups of TCh react with dithio-nitrobensoic acid (DTNB) which is reduced to thionitrobensoic acid (mercaptonitrobensoic acid) – a yellow coloured anion with spectrophotometric density maxima (E) at λ=412 nm. The color intensity is proportional to the concentration of TCh. The reaction is stopped by adding a specific blocker of AChE- or BChE activity. The protein content has been determined by the method of Lowry et al. [10].

Calculation of enzyme activity. One unit of AChE is the amount of enzyme that catalyzes the production of 1.0 μmol of thiocholine per minute under the conditions of the assay (temperature of 37^{O}C and pH 7.6). The principle of measurement of AChE activity is by measuring the contents of the SH-group. Therefore, we used L-Cysteine in different molar concentrations for preparing the standard curve. The initial solution of L-Cysteine is 0.1 μmol/ml.

Procedure. Enzyme activity has been determined in samples containing 3.0 ml 50 mM K, Na-phosphate buffer (pH 7.6); 0.1 or 0.2 ml enzyme fraction (20-100 μg protein/ sample – depends on the level of enzyme activity); the appropriate amount of compensation water, 0.02 ml ATChI (21.67 mg/ml – stock solution) or 0.02 ml BTChI (4.80 mg/ml –

stock solution); 0.2 ml the corresponding reagent; followed by incubation, for example 30 minutes at 37^{O}C; the enzymatic reaction has been stopped by adding 0.1 ml 0.1 mM eserine salicilate; finally. 0.1 ml DTNB has been added (39.6 mg of DTNB with 15 mg $NaHCO_3$ in 10 ml of 0.1 M phosphate buffer – stock solution); the optical density has been measured after 5 min (E) at λ=412 nm.

Statistic calculations and analysis. The statistical significance of differences between control and experimental samples in real terms or as a percentage of the control has been estimated in accordance with Student's t-test [11].

Results and Discussion

Biomonitoring is a complex system for continuous monitoring for evaluation of the condition and for assessing the ongoing changes occurring in natural objects. It is performed by research on groups of organisms, individuals or parts of them, called biomonitors.

In our study, biomonitors, i.e. criteria for eco-toxic and other damage to the environment and the intoxication of the body are two types of cholinesterases – AChE and BChE in vertebrates and AChE in invertebrates. These cholinesterases are presented with numerous variations of isoenzymes.

Biomarkers are substances, their parts, or metabolites, that act specifically on biomonitors and whose impact can be objectively measured.

The object of the study is mainly focused on organophosphates, carbamates and their function as anticholinesterase agents and toxicants in the environment. Three groups of chemicals (pesticides) dominate this part of the chemical industry. They are: herbicides – substances that kill or inhibit growth of unwanted plants (weeds) (47%); insecticides – substances that kill arthropod pests, i.e. insects and mites (24%) and fungicides – substances that destroy or prevent the growth of pathogenic fungi (26%) (Global Markets for Biopesticides (CHM029D, Publishing date: 2012). Pesticides are now more than 1,400 generic products.

Among the insecticides, the most commonly used are organophosphates (22%), pyrethroids (16%), neonicotinoids (15%) and methyl carbamates (11%).

According to a recent technical market report of BCC Research, the global pesticide market has been amounted to $37.5 billion dollars in 2011 and to $46.1 billion dollars in 2012. The total market value is expected to reach $65.3 billion dollars in 2017 after increasing at a five-year compound annual growth rate of 7.2%. All these and many others issues draw the attention towards the development of global programmes for environmental protection against the damaging effects of the different types of pesticides, continuous monitoring on the condition of the environment, prevention of intoxication in humans and animals, etc.

Another reason for the study is the importance of BChE as a defense system to cholinergic synapses from acetylcholine and agonists, metabolites, anticholinesterase agents, pharmaceuticals and others. The progressive increase in the activity of the BChE, for example, is an indicator for the development of Alzheimer's disease. A radical approach to the treatment of drug overdose (heroin, cocaine, etc.) is achieved by introducing enriched BChE [2, 3, 4].

1. Enzyme mapping of AChE and BChE in different brain regions in rats and rabbits

This study has aimed to show the indicative benchmark activity of AChE and BChE in various brain regions; the presence of parallelism between the level of enzyme activity and the content of the cholinergic and/or choline receptive neuronal aggregations; the ratio between the activity of AChE and BChE in the same fractions and some kinetic parameters of the enzyme reaction. The results are shown in Fig. 1.

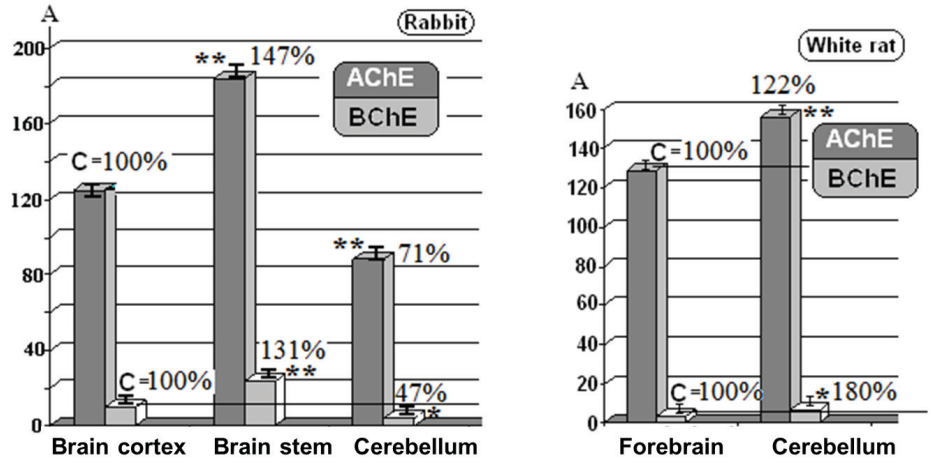

Fig. 1. Activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) in various fractions of the rabbit's and white rat's brain

(A – enzyme activity (μg hydrolyzed acetylcholine/mg protein/min);C – control; n= 8-12; ±m about AChE activity = 5-15 and ±m about BChE activity = 0.65-0.90; significant differences – *-$p<0.05$. **-$p<0.001$. This indication is valid for all figures and tables below.

The data show a general pattern – different level of activity of AChE and BChE in different species, different tissues, and in different target organs (various brain areas, synaptic and functional areas of the skeletal muscles, etc.), in the nervous and other systems. This means that the conduct of biomonitoring testing requires a bank (library) of reference data.

These results also indicate a significant presence of BChE in the brain tissue – 9-10% relative to the true AChE, which is specific for the synaptic structures. This shows the role of this enzyme type (BChE) as a protective mechanism in the nervous tissue against the damaging factors and as an additional participant in the mechanisms of synaptic processes. In this case, part of the BChE is synthesized in BChE-positive neuroglial cells.

Another important fact is the difference in the activities of both types of cholinesterases in the different brain areas. These differences and dependencies are specific for the species. For example, the activity of AChE (respectively for BChE) in the cerebellum of the white rat has been significantly higher compared to the same lobe in the rabbit.

There is also parallelism between the activity of AChE and BChE in the relevant brain areas. For example, the highest activity of both enzymes (in rabbit's brain) is present in the brain stem, where the cholinergic neurons are dominant, and is lowest in the cerebellum which is characterized by different neurochemistry of the neurons.

These data characterize some of the mechanisms of the physiological activity and role of the two types of cholinesterases in the nerve processes and have to be taken into account in biomonitoring studies.

2. Concentration-dependent inhibition of brain cholinesterases in rats and rabbits by anticholinesterase agents – carbamate and organophosphate pesticides

This section presents data on the level of inhibition of the enzyme activity of AChE and BChE in some parts of the brain in laboratory rats and rabbits, depending on the concentration of specific inhibitors of enzyme activity – ezerine salicylate and parathion (Fig. 2).

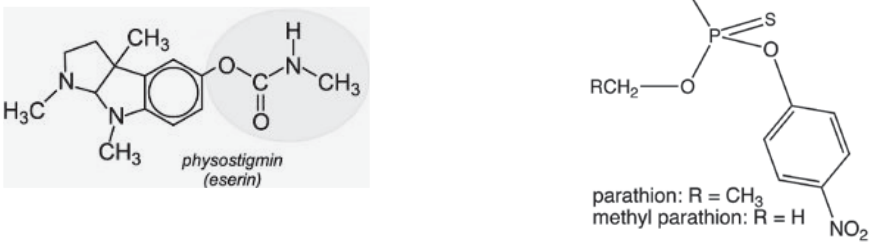

Fig. 2. Eserine (physostigmine) and parathion – potent anticholinesterase agents

Eserine (physostigmine) is a reversible cholinesterase inhibitor. It occurs naturally in the Calabar bean and has been synthesized for the first time in 1935. LD_{50} of physostigmine in mice is about 3 mg/kg. The chemical nature of eserine is N-methylcarbamate. Indirectly, eserine stimulates both nicotinic and muscarinic acetylcholine receptors. It is also used to treat anticholinergic poisoning and also to reverse neuromuscular blocking. It can cross the blood-brain barrier. Many carbamate compounds similar to eserine are applied successfully as insecticides [12].

Parathion or diethyl parathion is an organophosphate compound and a potent insecticide and acaricide. It is highly toxic to non-target organisms, including humans. Its use is banned or restricted in many countries, and there are proposals to ban it from all use. It is an irreversible cholinesterase inhibitor. As a pesticide, parathion is generally applied by spraying. It is often applied to cotton, rice and fruit trees. The usual concentrations of ready-to-use solutions are 0.05 to 0.1%. The chemical is banned for use on many food crops. LD_{50} for parathion is: 5 mg/kg (mouse, oral); 10 mg/kg (rabbits, oral); 2 mg/kg (rat, oral), etc. Absorbed parathion is rapidly metabolized to paraoxon, as described in insecticidal effectiveness.

Exposure to paraoxon (the active metabolic product of parathion) can result in headaches, convulsions, poor vision, vomiting, abdominal pain, severe diarrhea, unconsciousness, tremor, dyspnea, lung-edema and respiratory arrest. Symptoms of poisoning are known to last for extended periods, sometimes months. The most common and very specific antidote is atropine, in doses of up to 100 mg daily. Parathion is toxic to bees, fish, birds and other forms of wildlife.

This section presents data on some parameters of the influence of ezerine salicylate (reversible anticholinesterase; half-life time of about 0.5 h-several hours, carbamate compound) and parathion (a potent anticholinesterase agent; irreversible inhibition of cholinesterase; causing chronic poisoning of the body for several months). Parameters of the

study are: concentration-dependent inhibition (CDI_{50}) of cholinesterase activity in different areas of the brain and different animal species; overview of this dependence; focusing on some influences, caused by carbamate and organophosphate compounds; others.

These cases characterize the behavior of the two types of cholinesterases in respect to carbamate pesticides. Our data indicate that this is a common pattern – BChE has significantly lower sensitivity to anticholinesterase agents. This is comprehensible since the main biological role of this enzyme is to protect the nervous system and the other tissues, organs and systems from damage caused by various exogenous and endogenous agents. This is a type of defense system which protects the synapses from anticholinesterase substances and an enzyme, which effectively neutralizes different poisons, metabolites, etc., by their hydrolysis before they reach the synapses – the most sensitive units of the neural networks.

Herewith, it should be taken into account the reversible inhibition of cholinesterase by carbamate pesticides. This is achieved by competitive relationship between acetylcholine and, for example, ezerine for the active site of cholinesterases and due to the hydrolysis of the carbamate compounds of the BChE.

The influence of organophosphate pesticides (parathion, in this case) on the activity of AChE (cerebral cortex of rabbit's brain) is similar to that of carbamate – concentration-dependent, but with an initiator effective concentration (IEC) = 10 nM, CDI_{50} = 0. 1 μM and CDI_{100} – 10 μM. BChE (like for eserine salicilate) compared to AChE is by an order of magnitude more resistant to parathion, respectively: IEC = 0.1 μM, CDI_{50} = 1.0 μM and CDI_{100} – about 100 μM.

Firstly, we shall make a brief analysis of the concentration-inhibition curve of AChE activity under the influence of a one specific carbamate inhibitor of this enzyme.

The survey data strongly suggests the presence of concentration-dependent inhibition (CDI) of enzyme activity in this fraction. We believe that this is a general pattern in such systems. Like the standard index DL_{50} (50% mortality of individuals when poisoned with certain toxic agents *in vivo* conditions), we have introduced the indexes - CDI_{50} or CDS_{50} (concentration-dependent inhibition or stimulation) by different agents *in vitro* conditions. In our case, the CDI_{50} ratio is in the range of 1.0 μM eserine (Fig. 3, Table 1).

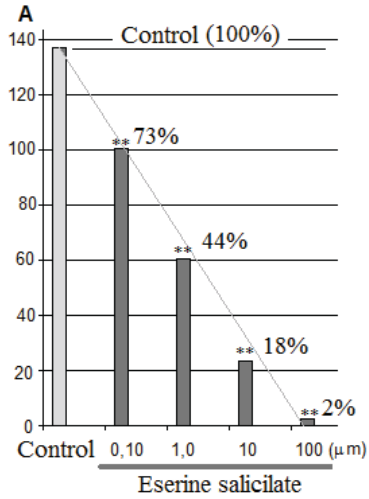

Fig. 3. Influence of eserine salicilate (0.10-100 μM) on the AChE activity of the cerebral cortex of the rabbit's brain

(A – Enzyme activity (μg hydrolyzed acetylcholine/ mg protein/min); n = 10; ± m = 3.50-5.50; **p < 0.001; % – inhibition relative to control = 100%)

The effect of ezerine salicilate on the activity of same brain BChE fractions is similar. The differences are recorded in the higher resistance of this enzyme to methyl carbamate (within a range). In this case, the CDI_{50} ratio is about 10.0 μM and CDI_{100} – 1.0 mM eserine.

Table 1. The influence of eserine salicylate and parathion on the activity of AChE and BchE (A) in fractions of the cortex of the rabbit

(A – μg hydrolyzed acetylcholine/mg protein/min; n = 10; ± m = 4.50-6.50 (AChE) and ±m (BChE) = 0,65-0,90; % inhibition relative to control = 100%; **p < 0.001)

Indicators (Anticholinesterase compounds)	Enzyme activity (A)	Enzyme activity (A)
	AChE	BChE
IEC (Eserine salicilate)	0.1 μM	0.01μM
CDI_{50} (Eserine salicilate)	1.0 μM	10 μM
CDI_{100} (Eserine salicilate)	10 μM	1.0 mM
IEC (Methyl parathion)	10 nM	0.1 μM
CDI_{50} (Methyl parathion)	0.1 μM	1.0 μM
CDI_{100} (Methyl parathion)	10 μM	100 μM

The general conclusion is that organophosphate insecticides are several times more toxic for organisms and humans. BChE also (similar to the effect of carbamate pesticides) is more resistant to parathion as compared to AChE. This is one of the reasons for the gradual ban on the use of organophosphate pesticides in practice.

3. L-Arginine, NO and other donors of NO as modulators of the activity of cholinesterases and as possible antidotes against poisoning by carbamate pesticides

The general theory of living in life-asserting environment is a complex system for assessing, monitoring, prevention, diagnosis, prognosis, resuscitation procedures (reanimation) and clinical activities, lawmaking and administration, science, humanitarian approach, radical solutions, international common policies to protect the environment and biosphere of any type disabilities [13].

Our work has the ambition, if not solve, to put the one of the problems – radical approach in the prevention of animals and human in terms of eco-toxic environment against one group poisons by the methods of monitoring analysis.

Some time ago it has been found that L-Arginine, NO and other donors of NO stimulate cholinesterases in different animals and tissues [7]. This has led to the idea for the application, for example, of L-Arginine as an antidote in cases of carbamate or organophosphate pesticides poisoning, in drug and medication overdoses, against pathogen metabolites, etc. Some of the results from similar studies on the role of L-Arginine (respectively NO) as a possible antidote in cases of poisoning by carbamate pesticides are indicated in Tab. 2 and Fig. 4.

The data from these series of studies have shown that L-Arginine in a wide concentration range stimulates the activity of both AChE and BChE of different brain fractions of rat and rabbit.

This stimulation has been concentration dependent on the respective fraction. This effect is manifested in an interval from 1.0 mM to 50 mM and more inhibition.

Table 2. Stimulatory effect of L-Arginine (50 mM) (in % relative to control = 100%) as a source of NO on the activity of AChE and BChE in fractions of various structures of the rat`s and the rabbit`s brain (% vs control = 100%; n=8-10; ** p <0.001)*

Rat's brain			
Enzymes	Forebrain	Cerebellum	
AChE	130-140%**	50%**	
BChE	850% **	300% **	
Rabbit`s brain			
Enzymes	Cerebral cortex	Brain stem	Cerebellum
AChE	40%**	100%**	150%**
BChE	270%**	40%**	110%**

*The statistical significance differences between control and experimental samples (in %) was calculated based on the actual activity of the enzymes (μg hydrolyzed acetylcholine or butyrylcholine/mg protein/min), see for example Fig. 1.

There are certain characteristics (for more details see [7]). Some of them are: depending on the respective fraction, the initiating effective concentration (IEC) is different. For example, with regard to AChE and BChE (brain fractions of white rat), IEC for forebrain is 1.0 mM, and for cerebllum – 25 mM. This means that the levels of the IEC under the influence of the respective donors of NO on AChE and BChE, depend on their main activity. For example, the stimulating effectiveness of L-Arginin (50 mM) is higher in cases when the IEC values have been lower.

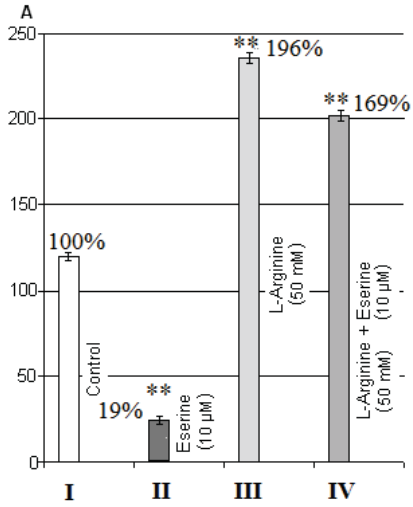

Fig. 4. Influence of eserine salicylate (10 μM) (II), L-Arginine (50 μM) (III) and the combination eserine (10 μM) and L-Arginine (50 mM) (IV) on AChE activity in the mitochondrial-membrane preparation of brain cortex of rabbit (I – control)

(A - Activity of the acetylcholinesterase (μg hydrolyzed acetylcholine/mg protein/min); % – a percentage relative to control = 100%; n = 15; ** - ρ <0.001)

It has been found that the effectiveness of the various inhibitors or stimulators on cholinesterases is dependent on the cytological and biochemical nature of the relevant structures.

An important conclusion from the study is that L-Arginine as a donor of NO and other donors of this radical, as well as certain metabolic products (e.g. L-Citruline and others), as modulators of enzyme activity, are potent regulators of the state and functions of the body in norm and in a pathological state. This means that depending on the degree of poisoning,

L-Arginine can be used as an antidote. This judgment has been confirmed by some of our data on the effects of combined influence of eserine salicylate and L-Arginine on the AChE activity of brain cortex of rabbit (Fig. 4). Naturally, this kind of research should be continued *in vivo* and *in vitro* conditions in other animal species and structures. These data are the basis of the research findings.

The main conclusion from these series of studies (controlling the AChE activity in the brain cortex of rabbit is that under the combined influence of ezerine salicilate (in conc. 10 μM, which alone inhibits about 80% of the enzyme activity) and L-Arginine (in conc. 50 mM, which alone stimulates by about 100% the activity of this enzyme) the total enzyme activity has been about 70% higher compared to the control samples.

This discussion focuses on the role of L-Arginine as a stimulator of the activity of AChE and as an antidote against a partial or complete inhibition of AChE in different brain areas and tissues.

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APPLICABILITY OF INDICATORS OF HYDROLOGICAL ALTERATION TO ASSESS THE CURRENT STATUS OF THE RIVER FLOW

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Abstract

Aim: The aim of the present study was to characterise the natural flow regime of the Veleka River to determine the extent of its alteration and to design environmental flow regimes to protect aquatic habitats and species.

Materials and Methods: A method for hydrologic alteration estimation has been examined and applied to characterize and evaluate the hydrologic alteration of the Veleka River flow regime using streamflow series. The region in concern belongs to the South Eastern climate and is mainly transitional Mediterranean climate. In the study area there are not hydrological perturbations. The used method, referred to as the "Indicators of Hydrologic Alteration" is based upon an analysis of hydrologic data available from existing measurement points.

Results: The hydrological status of the Veleka River was assessed by comparing the two periods. The most significant results suggest changes in low-flows. The current flow regime shows a significant reduction of stream flows.

Conclusions: From this study we conclude that the indicators of hydrologic alteration proved to be a useful approach, capable of focusing, comparing and establishing levels of hydrologic disturbances.

Keywords: River Flow Regime, Indicators of Hydrologic Alteration, Reference Conditions, Veleka River.

Introduction

In recent decades, the importance of natural hydrological regimes in maintaining the integrity of rivers has been widely recognized [1, 2, 3]. The natural regime defines the hydrological variability pattern and reflects the interaction between the climatic regime (precipitation and temperature) and the basin characteristics that regulate runoff (geomorphology, geology and vegetation). The structure and function of river ecosystems are strongly affected by natural flow regimes because the biota is adapted to its components. These components are defined as: magnitude, frequency, duration, rate of change and

predictability of flow events [4]. The relevance of the hydrological regime is recognized by the Water Framework Directive (WFD) (EC, 2000) [5], which explicitly defines the hydro-morphological aspects as quality elements that must be used for the assessment of ecological status/potential. However, as a determinant of ecological status, the hydro-morphological quality elements are fixed only at *High Quality Status*, while, for other status classes, the hydromorphological elements are required to have "conditions consistent with the achievement of the values specified for the biological quality elements" (CIS, 2003) [6]. The hydrological regime of a water body is part of the hydro-morphological quality elements (Annex V, WFD). The revised text of the Bulgarian Water Law, which implements WFD, explicitly states that Basin Management Plans must include the environmental flow regime for each body of water, with priority given to protected zones. The environmental flow regimes will be necessary "to maintain or re-establish the proper state of conservation of habitats or species, meeting their ecological requirements and maintaining the long-term ecological functions they depend on". Additionally, the ecological flow regime must include the time distribution of maximum and minimum flows, flood and drought flows and the rates of flow change. The aim of the present study was to characterise the natural flow regimes of the Veleka River to determine the extent of its alteration and to design environmental flow regimes to protect aquatic habitats and species.

Materials and Methods

The watershed of Veleka River is situated in the Strandzha Mountain in South Eastern part of Bulgaria and covers an area of 1054.6 km^2 of which 266.6 km^2 are located in the territory of Turkey. It includes the natural park "Strandzha" which is one of the biggest protected territories in Bulgaria. The Veleka River flows into Black sea, near the village of Sinemorets and crosses Strandzha Mountain. The topography is characterized as a moderately mountainous area (Fig. 1). The region in concern is a very rural area. The forest types are broadleaf (92%) and coniferous (8%).

According to the climatic classification, the watershed of Veleka River belongs to the Black sea climate sub-type of the continental Mediterranean climate type. The climate is generated from the west and north continental influence, the Black Sea influence from east and the Mediterranean influence from south. The Strandzha climate is mainly transitional Mediterranean. Local climatic differences are due, above all, to the proximity of the Black sea, which warms up the coastal zone in winter and cools it especially in spring. The precipitation features depend on the atmospheric circulation and orography. The average annual precipitation is above 600 mm for the coastal region and in the inner part above 900 mm. The winter precipitation (with seasonal precipitation about 150-271 mm) is the highest precipitation in the South-Eastern part of Bulgaria. During the winter the predominant precipitation is in the form of rain, but about one third of cases the precipitation is snow. The snow cover is of short duration (15-16 days per year). During the spring the mean seasonal amounts of precipitation are 120-180 mm. The summer is sunny, dry and very warm, the amounts of precipitation are 100-120 mm and from June to the middle of September is dry. The drought period is very noticeable in the end of summer when the precipitation for

August it is 30 mm. Hydrological perturbations associated with water abstractions, point discharges and the presence of a dam there are not in the study area.

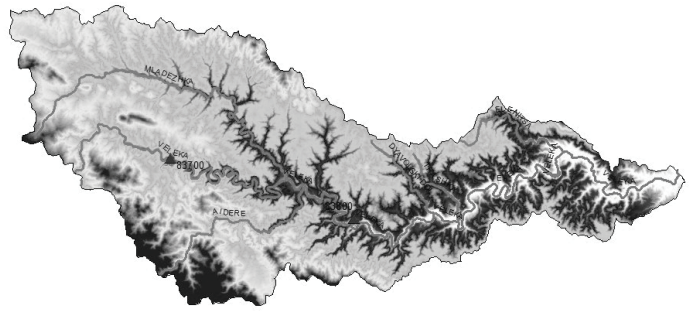

Fig. 1. The Veleka River basin with gauging stations (author: Bernardo Lizama Rivas)

To characterize the hydrological regime it is necessary to have recorded data for both natural and altered (modified) regime for at least fifteen full years of daily water discharges or monthly streamflow; although in the latter case, the parameters used for the characterization of the hydrological regime will obviously be less. The hydrological characterization of the Veleka River was based on the series of monthly mean flow rates gathered from the National Institute of Meteorology and Hydrology (NIMH) for the period 1961-2007 at the gauging station Zvezdets. The hydrological conditions of Veleka River are assessed by comparing the two periods, representing the regime of the reference period (1961-1990) recommended by the World Meteorological Organization and the period of the last current year (1991-2007). During these periods in the studied river has not been established hydrological alterations that are associated with anthropogenic pressures, such as dams, point source discharges, surface water abstractions, and hydropower.

In this study the Indicators of Hydrologic Alteration in Rivers are applied, which are based on Richter's ideas [3, 7]. These indicators summarize the main characteristics of the hydrologic regime, allowing the comparison between natural and modified conditions [8]. Five components are generally used to describe the flow regime: magnitude of discharge (amount of water moving past a fixed location per time unit), frequency (refers to how often a flow above a given magnitude recurs over some specified time intervals), duration (period of time associated with a specific flow condition), timing (regularity with which flows of defined magnitude occur) and rate of change (refers to how quickly flow changes from one magnitude to another) [2]. Modification in these flow components has effects on the ecological integrity of rivers.

Several metrics (also called hydrological descriptors or indicators) have been developed for characterizing the patterns of river flow, and specific hydrological components, which have a direct or indirect influence on biological communities [9]. A general approach for hydrological alteration assessment is based on the analysis of these metrics, which are compared before and after a river has been altered by human activities [3]. This methodology, as well as other methods generally used to analyse the status variations within a system over time, or to compare

an altered system to a reference system, are based on streamflow data, which are referred to as un-impacted and impacted conditions. Both as the reference period regime (period 1961-1990) and the regime of the last current years (period 1991-2007) were analyzed through the computer application IAHRIS, by means of which are calculated as the parameters characterizing both regimes, and hydrological indicators that assess the extent of alteration.

Results

To describe the natural flow regime and evaluate the degree of alteration, the Indicators of the Hydrological Alteration in Rivers were used via the IAHRIS software. This software calculates 24 hydrologic metrics (7 for habitual flows, 9 for high flows and 8 for low flows) that adequately describe the hydrologic regime of the Mediterranean streams. The degree of alteration in each indicator (0: maximum alteration, 1: minimum alteration) is established by dividing its value in regulated conditions by the indicator value in natural conditions. Considering that only the monthly values of the river flow, rather than the daily data are available, the flow regime by assessing the parameters for magnitude, variability and seasonality will be characterized. Before proceeding to the study of habitual values of the flow regime is required to characterize the annual variability. This characterization it is aimed at determining the annual volume thresholds of river flow in order to segregate each year into one of three types of years: wet, dry or normal. To determine the thresholds for "wet", "normal" and "dry" years of runoff volumes as limits are used respectively 25% and 75% percentiles (Fig. 2). The multi-annual variability in river flow volumes of the Veleka River are presented in Fig. 2 below.

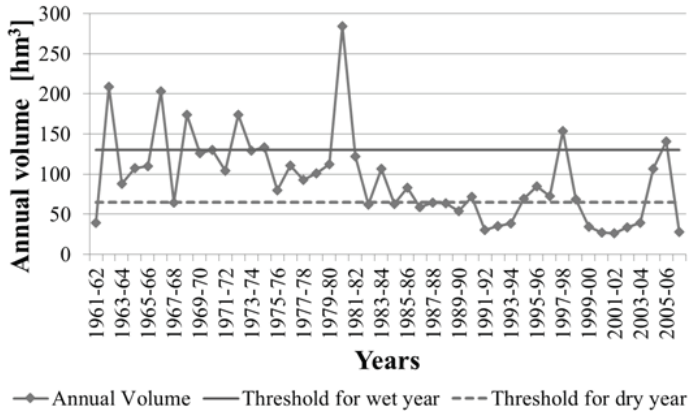

Fig. 2. Multi-annual variability in river flow volumes (hm³) of the Veleka River at Zvezdets

In Fig. 2 can be seen, the magnitude and inter-annual variability of annual flow volumes. As regards the amount of annual flow volumes, one can see the great amplitude (range) of variability from about 26 to 285 hm³, with alternating periods with years of significant flow volumes against very dry periods. The annual variability of the water volume for each annual type is shown in Fig. 3.

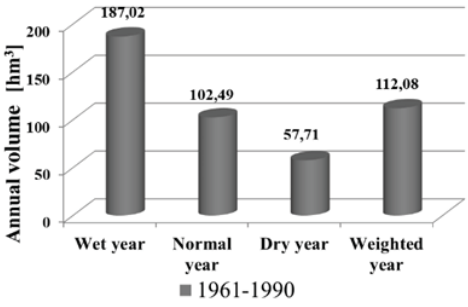

Fig. 3. Magnitude of annual volumes for every type of year

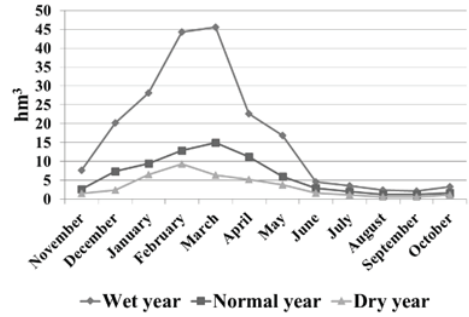

Fig. 4. The intra-annual variability of the water volumes during the reference period (1961-1990)

The intra-annual variability of the water volumes during the reference period for every type of year is shown in Fig. 4. We can see that during the reference period, the largest flow volumes occur on January-April with a maximum in March for wet and normal years, while for dry years the maximum occurs in February. The course of the intra-annual variability over the past 16 years is the same. The hydrological aspects of the flow regime with high environmental significance (magnitude variability and seasonality) for the reference period are presented on Table 1. It can be seen the average annual volumes but also the difference between the maximal and minimal monthly volumes along the year and the month with the maximal and minimal water volume for each type of year (wet, normal, dry and weighted year). The weighted year represents the weighting percentage of each type of year in the series (25% for wet and dry years and 50% for normal years). Table 1 includes the results of the characterization of the natural flow regime of the river with respect to each of the obtained parameters and for each of the specified types of years.

Table 1. Hydrological aspects of the flow regime for the period 1961-1990

COMPONENTS OF THE NATURAL REGIME	ASPECT	PARAMETER			
		DESCRIPTION		VALUE (hm³)	
HABITUAL DATA	MONTHLY OR ANNUAL VOLUMES	**Magnitude**	Average of the annual volumes	Wet year	187.02
				Normal year	102.49
				Dry year	57.71
				Weighted year	112.08
		Variability	Difference between the maximal and the minimal monthly volume along the year	Wet year	72.31
				Normal year	29.59
				Dry year	14.90
				Weighted year	36.35
		Seasonality	Month with the maximal and minimal water volume along the year	Wet year	MAR; SEP
				Normal year	MAR; SEP
				Dry year	FEB; SEP

On Table 2 are given statistical parameters of the Veleka River at Zvezdets to characterize aspects of the flow regime for the period 1961-1990. These parameters are derived based on the annual and monthly volumes river flow. The estimated parameters for the intra-annual distribution for the period 1961-1990 are presented in Table 3.

Table 2. Statistical parameters characterizing the flow regime of the Veleka River at Zvezdets

COMPONENTS OF THE NATURAL REGIME		ASPECT	PARAMETER	
			DESCRIPTION	VALUE(hm³)
HABITUAL DATA	ANNUAL VOLUMES	**Magnitude**	Average of annual volumes	112.08
			Median of annual volumes	106.67
			Coefficient of variation of annual volumes	0.49
	MONTHLY VOLUMES	**Magnitude**	Average of monthly volumes	See Table 3
			Median of monthly volumes	See Table 3
			Coefficient of variation of annual volumes	See Table 3
			Extreme variability	36.41
		Seasonality	Relative frequency of the maximum for each month	See Table 3
			Relative frequency of the minimum for each month	See Table 3

Table 3. Parameters to characterize the intra-annual distribution of the natural flow regime

	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct
Average (hm^3)	16.33	25.96	20.76	13.03	8.20	3.26	2.20	3.15	1.52	2.65	4.21	10.81
Median (hm^3)	10.15	15.81	14.92	11.19	5.97	2.90	1.97	1.23	1.24	1.58	2.59	7.34
Coef. of variation	0.77	1.14	0.69	0.56	0.63	0.44	0.66	2.69	0.77	1.55	1.04	1.31
Relative frequency of maximum	0.17	0.31	0.31	0.03	0.03	0.00	0.00	0.03	0.00	0.00	0.00	0.10
Relative frequency of minimum	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.34	0.45	0.07	0.10	0.00

Below will be discussed the hydrological regime of the Veleka river at Zvezdets for the period 1991- 2007 and will be difined the parameters with highlighted environmental significance that characterize this regime (parameters for magnitude, variability and seasonality). These data will be characterized and will be compared with a reference period (1961-1990). Based on these periods the degree of alteration has been assessed by calculating the indicators that connect the values of the same parameter for both regimes. On Table 4 are given the statistical parameters to characterize the aspects of the flow regime for the period 1991-2007 based on the annual and monthly flow volumes. The estimated parameters of the intra-annual distribution of the river flow for the period 1991-2007 are presented in Table 5.

Table 4. Environmentally significant indicators characterizing the flow regime of the Veleka River at Zvezdets for the period 1991-2007

COMPONENTS OF THE ALTERED REGIME		ASPECT	PARAMETER	
			DESCRIPTION	VALUE(hm^3)
HABITUAL DATA	ANNUAL VOLUMES	Magnitude	Average of annual volumes	61.76
			Median of annual volumes	38.77
			Coefficient of variation of annual volumes	0.66
	MONTHLY VOLUMES	Magnitude	Average of monthly volumes	See Table 5
			Median of monthly volumes	See Table 5
			Coefficient of variation of monthly volumes	See Table 5
			Extreme variability	17.31
		Seasonality	Relative frequency of the maximum for each month	See Table 5
			Relative frequency of the minimum for each month	See Table 5

When comparing the characteristics of the period in natural regime (reference period 1961-1990) with the period for last current regime (1991-2007) a significant change in the flow regime (Table 2 and Table 4) can be seen, that the mean annual volumes significantly have been reduced (around of 44%).

The next stage after the characterization of the two regimes is the comparison of the two periods 1961-1990 and 1991-2007 through indicators of hydrological alteration (IHA). Attending to CIS-WDF recommendations [6, 8] five levels or hydrological status have been established. The alteration in the habitual values will be assessed separately for each type of year. The obtained results are very similar to each year. The values of the indicators of hydrological alteration that correspond to the weighted year are shown on Table 6. The values that show a greater alteration in the magnitude of the monthly volumes (M2=0.36) are an indicator of the poor hydrological status. The magnitude of the annual volumes (M1=0.55), the monthly variability (V2=0.57) and the extreme variability in the flow volume (V4=0.48) have a moderate status.

Table 5. Parameters to characterize the intra-annual distribution for the flow regime (period 1991-2007)

	Nov	**Dec**	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Oct**
Average (hm^3)	6.84	10.83	12.78	8.09	4.97	2.68	4.00	1.06	0.75	1.29	2.62	5.85
Median (hm^3)	5.20	6.88	6.22	5.00	4.17	2.81	1.58	0.95	0.79	0.94	1.64	3.51
Coef. of variation	0.73	1.00	1.15	0.86	0.62	0.43	2.15	0.72	0.38	1.04	0.93	1.12
Relative frequency of maximum	0.13	0.38	0.31	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06
Relative frequency of minimum	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.25	0.31	0.25	0.13	0.00

Table 6. Indicators of the hydrological alteration (IHA) for the habitual values

ASPECT		Indicators of Hydrologic Alteration (IAH)			Level I	Level II	Level III	Level IV	Level V
		Value	Code	Description	0.8<I ≤1	0.6<I ≤0.8	0.4<I ≤0.6	0.2<I ≤0.4	0< I ≤0.2
HABITUAL VALUES	Magnitude	0.55	M1	Magnitude of the annual volumes			**Yes**		
		0.36	M2	Magnitude of the monthly volumes				**Yes**	
	Variability	0.75	V1	Variability of the annual volumes		**Yes**			
		0.57	V2	Variabilityof the monthly volumes			**Yes**		
		0.48	V4	Extreme variability			**Yes**		
	Seasonality	1.00	E1	Seasonality of the maximual values	**Yes**				
		0.83	E2	Seasonality of the minimal values	**Yes**				

Performing a detailed study month by month reveals that in the magnitude of the flow volume the poor status prevails, the indicator M3 (Table 7) varies between 0.2 and 0.4 in nine months (January, February, March, April, May, September and October) and the bad status

is observed in August (M3=0.19). In June, the status is moderate (M3=0.45) and in July it is high or corresponds to the reference period (M3=1). The indicator values show that during 1991-2007 there was a strong reduction in the river flow of the Veleka River at Zvezdets. From the Table 7 can be seen that the indicator V3, which evaluates the variability of the monthly volumes fluctuates between 0.4 and 0.6, indicating that the hydrological status is moderate. In August the indicator V3 is equal to 0.97, which shows that hydrological status is high. In September (V3=0.60) and October (V3=0.65) the status is good. In order to make easier the global analysis for the main elements of the regime, an indicator of the global alteration is calculated for each component. This indicator sums up the values of the indicators selected to assess all aspects considered for this component of the regime [8]. The value of the global alteration indicator for the habitual values (IAG_H=0.41) shows that the hydrological status is good for the Veleka River at Zvezdets (Table 8).

To perform an environmental assessment of river sections in the study area was analyzed both the current situation in which the river is encountered, as well as was compared with the situation which the river would have had if there had not been a change by humans, i.e. in their natural status. Consequently the degree of conservation or deterioration was determined. This assessment is complemented by determining the possible effects on the watershed that can determine its current status, as well as the circumstances that may determine or limit the recovery of the conditions. The obtained mean values for the environmental flow regime in wet, normal and dry years are below the average natural regime (Fig. 5) and have low variability but they follow the patterns of intra-annual variability of the natural regime. The annual volume of the natural regime is around 201.2 hm^3 for wet years, 73.3 hm^3 for normal years and 40.3 hm^3 for dry years (Fig. 6).

Table 7. The indicators of hydrological alteration (IHA) of intra-annual distribution of river flow

Aspect	Month	Indicator M3 - Magnitude of the month volume	Indicator V3 - Variability of the month volume
Habitual values	Nov	0.34	0.57
	Dec	0.30	0.58
	Jan	0.23	0.55
	Feb	0.23	0.57
	Mar	0.34	0.47
	Apr	0.34	0.50
	May	0.33	0.55
	Jun	0.45	0.55
	Jul	1.00	0.34
	Aug	0.19	0.97
	Sep	0.27	0.60
	Oct	0.27	0.65

Table 8. The global alteration indicator for the habitual values of volumes of river flow

Indicators of global alteration			Level I	Level II	Level III	Level IV	Level V
Aspect	**Value**	**Code**	0.64<I≤1	0.36<I≤0.64	0.16<I≤0.36	0.04<I≤0.16	0<I≤0.04
Habitual values (M1, M2, V1, V2, V4, E1, E2)	0.41	IAG_H		**Yes**			

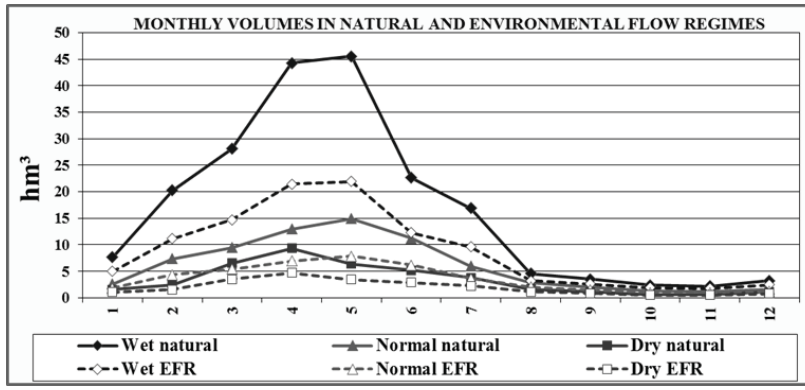

Fig. 5. Natural and environmental flow regime of the river runoff (hm^3)

Under the scenario with a high level of protection for aquatic ecosystems (m = 1.2) the final outcome of the flow volumes of the environmental flow regime was 107.9 hm^3 for wet years, 42.7 hm^3 for normal years and 23.0 hm^3 for dry years. This means that the water which will be available for other purposes is 93.1 hm^3 in wet years, 30.6 hm^3 in normal years and 17.3 hm^3 in dry years. Otherwise, if it do not be complied with these water quantities, the components, functions and processes of the river ecosystem that are associated with the regime of the river flow will be affected by the use and management of water resources in the studied river.

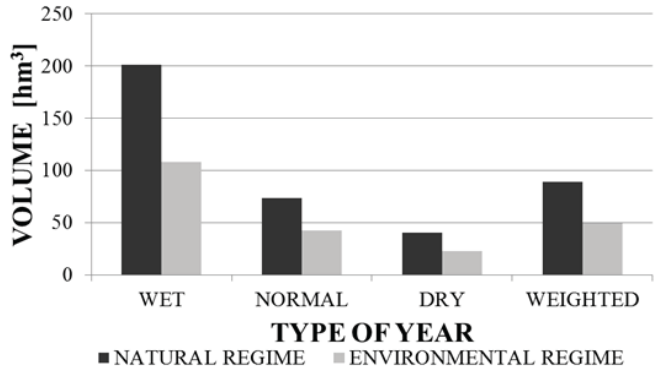

Fig. 6. Average annual volumes of natural and environmental flow regime for different types of years

Conclusion

The following conclusions can be drawn:

1. The employed methodology objectively allowed evaluating the hydrological flow regime of Veleka River.

2. The values of the indicators of hydrological alteration for the average year showed greater alteration in the monthly flow volumes corresponding to the presence of poor hydrological status. The magnitude of annual volumes and extreme variability of the river flow volume indicate moderate hydrological status in concerned stretch of the river.

3. The global alteration indicators for habitual values of flow volumes for the Veleka River show good hydrological status. Compared to the monthly runoff volume prevails a poor status. The indicator values show that during 1991-2007 there was a strong reduction in river flow of the Veleka River notably in August.

4. A significant change in the flow regime of Veleka River can be observed, the mean annual volumes have been reduced which about 44%.

It could be concluded that the results of this study showed that the indicators of the hydrologic alteration are useful to focus, compare and define the levels of the hydrological alterations.

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EVALUATION OF THE STATE OF THE WATERS OF THE STRUMESHNITSA RIVER ON BULGARIAN TERRITORY ACCORDING TO PHYSICO-CHEMICAL ELEMENTS FOR QUALITY

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Abstract

Aim: The aim of the report is to analyze the data from the monitoring points and to assess the state of the Strumeshnitsa River on the territory of Bulgaria.

Materials and Methods: The paper focuses on the research of the Strumeshnitsa River during the period 2010-2015. It is based on the existing information from the monitoring carried out by statistical and comparative analyses.

Results: Based on the gathered data from the monitoring programs, an analysis has been made of the data from the monitoring points situated along the Strumeshnitsa River. In the period of research the Strumeshnitsa River flows into Bulgarian territory in a not very good condition. The values of most indicators for organic materials are much higher than the norms of a good condition. The content of $N\text{-}NO_2$, N-total, $P\text{-}PO_4$ and P-totalis the above the norm and do not suit the requirements of a good condition. On Bulgarian territory the quality of the waters of the river is additionally deteriorated.

Conclusion: The Strumeshnitsa River was assessed in moderate condition according to the physico-chemical elements for quality.

Keywords: Strumeshnitsa River, monitoring point, physico-chemical elements of quality, classification system

Introduction

The Strumeshnitsa River (Republic of Macedonia in Strumica) springs from the mountains Plachkovitsa in the Republic of Macedonia. Its length is 114 km, of which 81 kilometers in the Republic of Macedonia and 33 km in Bulgaria [1]. The catchment area of the Strumeshnitsa river is 1900 km^2, of which 442.6 km^2 on Bulgarian territory [2]. In Zlatarevo (at checkpoints Zlatarevo) it enters Bulgarian territory, it runs about 2 km north of the town of Petrich and flows right into the river Struma at 93 m altitude, 2 km northeast of the village Mitino [1]. Along the Strumeshnitsa River there are 12 settlements, including 9 settlements in Macedonia - in the municipalities of Radovish, Vasilevo, Strumica and Novo Selo (1 town and 8 villages) and in Southwestern Bulgaria - 3 villages of Petrich Municipality [1].

Materials and Methods

The paper focuses on the research of the Strumeshnitsa River on the territory of Bulgaria during the period 2010-2015.

According to the Management Plan of River basins in the West Aegean Sea District, the Strumeshnitsa River is determined as an independent water body with code BG4ST400R072

„The Strumeshnitsa River from the Bulgarian-Macedonian border to its flowing into the Struma River“ [2], while the type of the water body is R5 „Semi-mountain rivers“ [3].

During this period the Strumeshnitsa River has two monitoring points from the program for operative monitoring of surface waters - Strumeshnitsa River at the border and Strumeshnitsa River before estuary, the bridge to the village of Mitino [4, 5].

Based on information available from monitoring programs for surface water it presents an analysis of the data from monitoring points and assesses the state of the Strumeshnitsa River on physico-chemical elements for quality classification system for assessment of physico-chemical quality elements, in accordance with Appendix №6 of Ordinance № H-4 from 14.09.2012 on the characterization of surface water, amended and suppl., SG. 79 of 09.23.2014, effective from 23.09.2014 [6].

Indicators of the classification system are studied: pH (BS EN ISO 10523: 2012); Dissolved oxygen, mg/l (BS EN ISO 5814: 2012); Conductivity, μS/cm (BS EN 27888: 2000); BOD_5, mg/l (BS EN 1899-2: 2004); N – NH_4, mg/l (BS ISO 7150-1: 2002); N – NO_2, mg/l (BS EN 26777: 1997); N – NO_3, mg/l (BS ISO 7890-3: 1998); N-total, mg/l (BSS EN12260: 2004); P - ortho -PO_4, mg/l (BS EN ISO 6878: 2005); P - Total phosphorus, mg/l (BS EN ISO 6878: 2005).

Criteria used for the application of the classification system for evaluation of the physico-chemical quality elements are in accordance with the requirements of Regulation H-4 to characterize surface water [6].

Results

The condition of the Strumeshnitsa River at the Bulgarian-Macedonian border and prior to infusion into the Struma River in the examined indicators are presented in Figs. 1 -10.

1. pH:

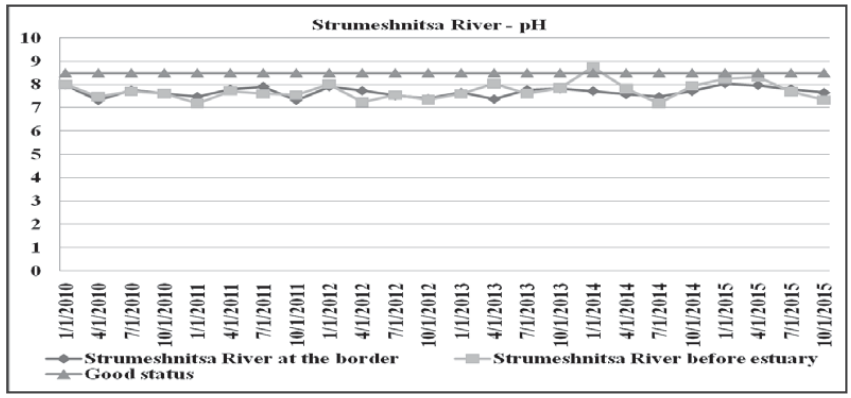

Fig. 1. State of the Strumeshnitsa River according to the pH indicator

The measured values of the indicator of the Strumeshnitsa River on the border are in the range of 7.32 to 8.05, and before the estuary - 7.19-8.75. One excess of the Strumeshnitsa

River before the estuary, at the bridge of the village of Mitino in January 2014 (measured pH value is 8.75 with an upper limit for good condition 8.5) has been found.

2. Dissolved oxygen, mg/l:

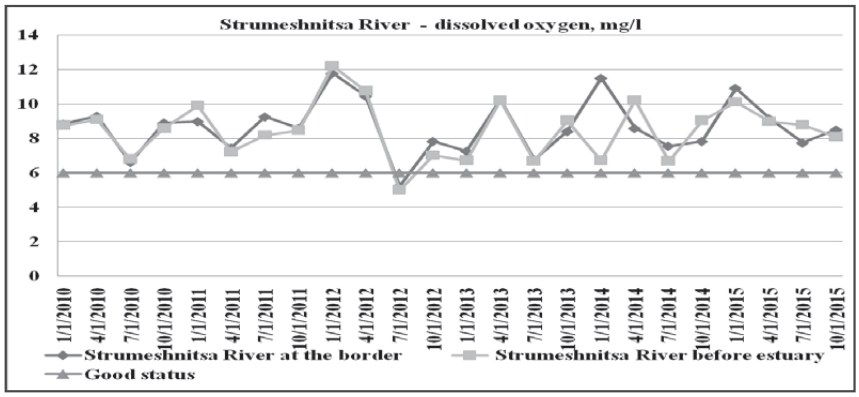

Fig. 2. State of the Strumeshnitsa River according to dissolved oxygen indicator

The values of the indicator of the Strumeshnitsa River on the border are in the range of 5.22 - 11.78 mg/l and before the estuary in Mitino 5.00 to 12.23 mg/l. At the two points one deviation in July 2012 was found - the measured values of the indicator dissolved oxygen were 5.22 mg/l at the border and 5.00 mg/l before estuary in the lower limit of condition 6.0 mg/l.

3. Conductivity, µS/cm.

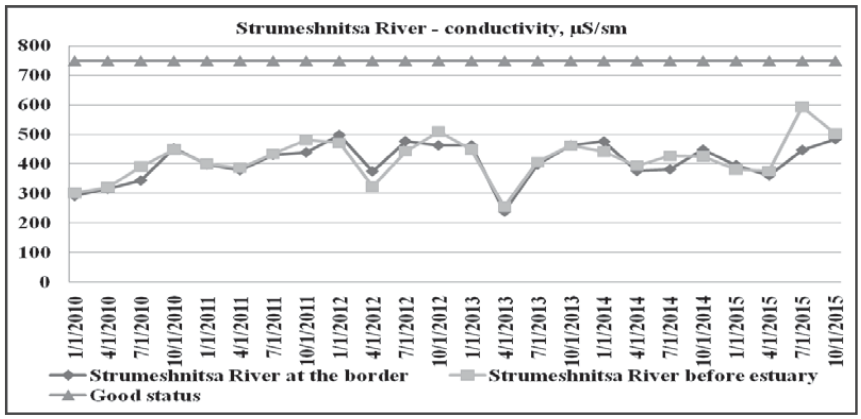

Fig. 3. State of the Strumeshnitsa River according to the conductivity indicator

According to the conductivity indicator, at both points no concentrations exceeding the norm have been measured indicating the good condition throughout the research. At the Strumeshnitsa River on the border the measured values are in the range 239-498 µS/cm, and before the estuary are from 256-593 µS/cm.

4. BOD_5, mg/l:

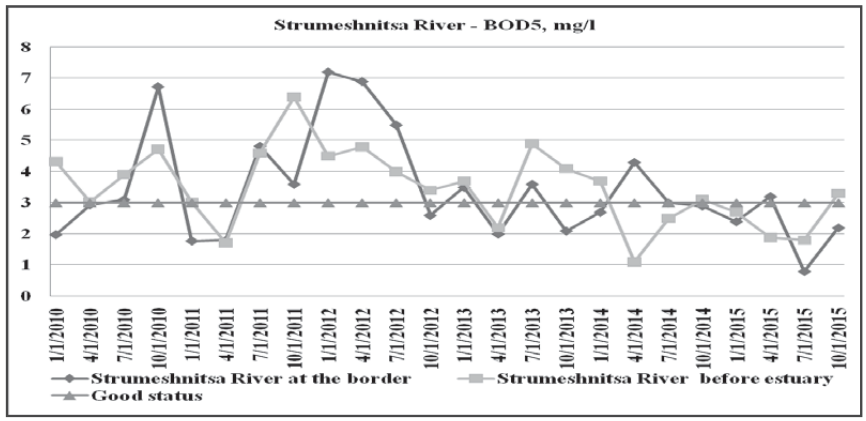

Fig. 4. State of the Strumeshnitsa River according to the BOD_5 indicator

This indicator registered multiple excesses at both monitoring points. The measured concentrations of the Strumeshnitsa River at the border are in the range of 0.8 - 7.2 mg/l, before the estuary - 1.71 - 6.4 mg/l. At the point of the Strumeshnitsa River on the border more significant exceedances were recorded in October 2010, July and October 2011 and the first nine months of 2012. Since the end of 2012, the trend has been to reduce the measured concentration of BOD_5. On the Strumeshnitsa River before the estuary multiple excesses for the period 2010 – 2013 have been measured. Since 2014 the trend has been to reduce the concentrations measured.

5. $N – NH_4$, mg/l:

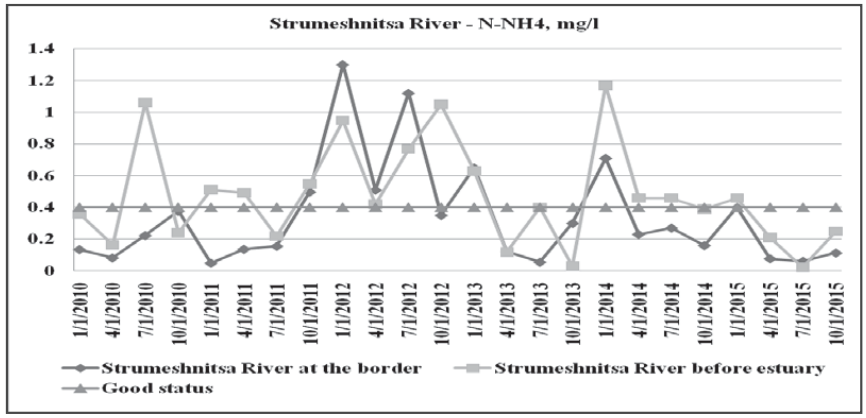

Fig. 5. State of the Strumeshnitsa River according to $N – NH_4$ indicator

The measured values of $N – NH_4$ of the Strumeshnitsa River at the border are in the range 0.049 - 1.3 mg/l. The established excesses are between October 2011 and July 2012, as well

as single exceedances in January 2013 and 2014. The measured values of the indicator on the Strumeshnitsa River before the estuary are in the range of 0.12 to 1.17 mg/l, with multiple exceedances are registered in the period from 2011 to January 2013, in the first nine months of 2014 and in January 2015. The measured concentrations of N – NH_4 on the Strumeshnitsa River before the estuary are significantly higher than those at the border.

6. N – NO_2, mg/l:

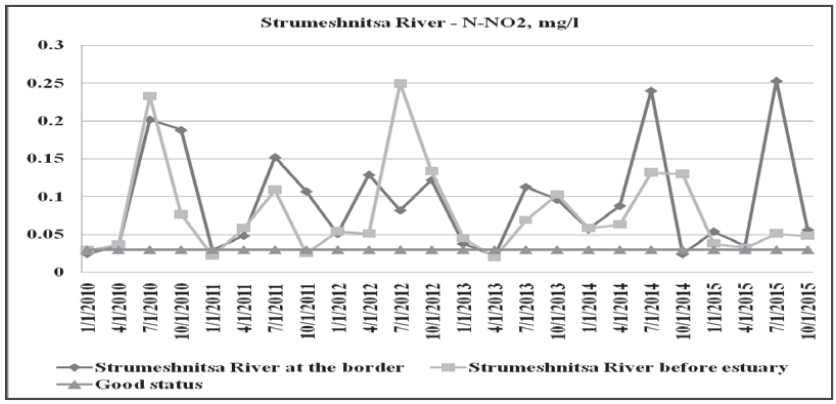

Fig. 6. State of the Strumeshnitsa River according to the N – NO_2 indicator

By the N – NO_2 indicator, multiple excesses throughout the study in both monitoring points have been measured. The measured values of the indicator of the Strumeshnitsa River at the border range from 0.022-0.253 mg/l and before the estuary from: 0.02 to 0.25mg/l.

7. N – NO_3, mg/l:

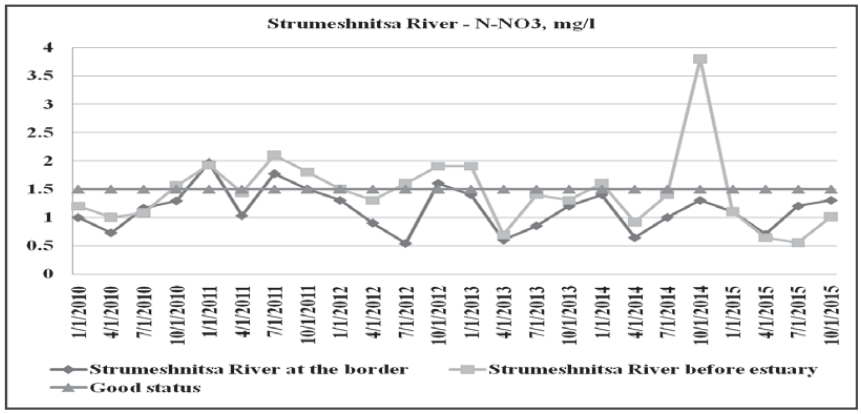

Fig. 7. State of the Strumeshnitsa River according to N – NO_3 indicator

The measured values of this indicator at the point of the Strumeshnitsa River at the border are in the range 0.54 - 1.97 mg/l. Repeated excesses from the norm have been measured

for good condition in January and July 2011 and October 2012.The Strumeshnitsa River before estuary the measured values of the indicator are in the range 0.55 - 3.8 mg/l. Multiple excesses during the period December 2010 - January 2013 and in January and December 2014 have been registered. In 2015 the measured concentrations are in a standard good state.

8. N-total, mg/l:

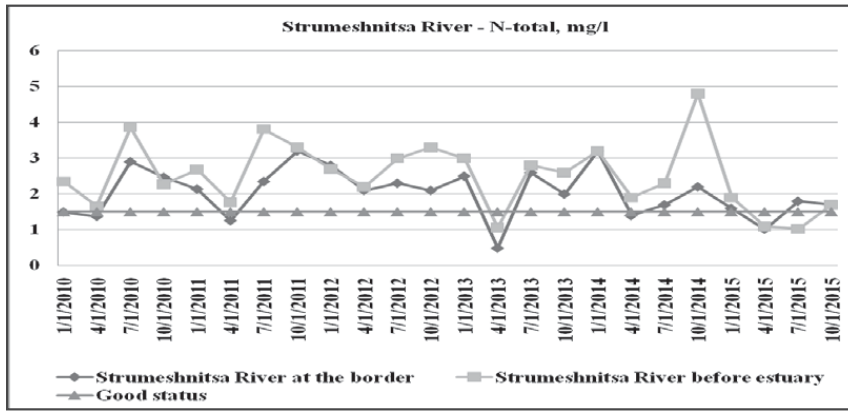

Fig. 8. State of the Strumeshnitsa River according to N-total indicator

According to the indicator of the N-total, multiple excesses throughout the study in both monitoring points were measured. The measured values in the point of the Strumeshnitsa River at the border are in the range 0.495 - 3.2 mg/l and in the point of the Strumeshnitsa River before the estuary 1.03 - 4.8 mg/l. The lowest concentrations of the N-total were measured in 2015.

9. P - ortho -PO_4, mg/l:

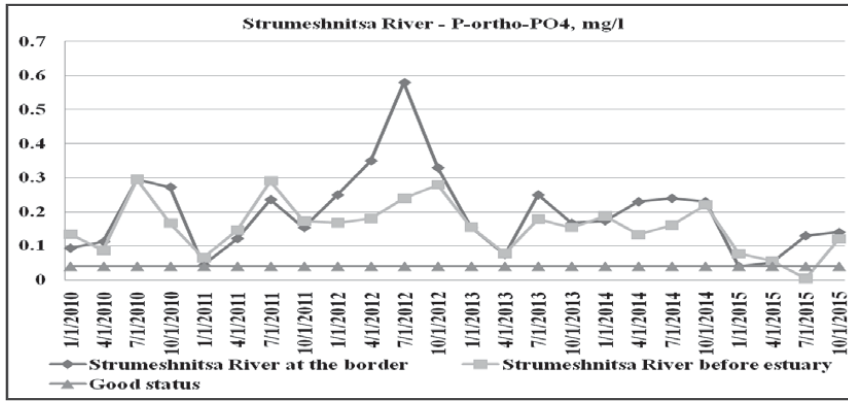

Fig. 9. State of the Strumeshnitsa River according to P - ortho -PO_4 indicator

The measured values of P - ortho -PO_4 in the point of the Strumeshnitsa River at the border range from 0.04 to 0.58 mg/l, and before estuary: 0.0035 - 0.296mg/l. The registered exceedances are throughout the study period. The lowest concentrations in both points were measured in 2015.

10. P - Total phosphorus, mg/l:

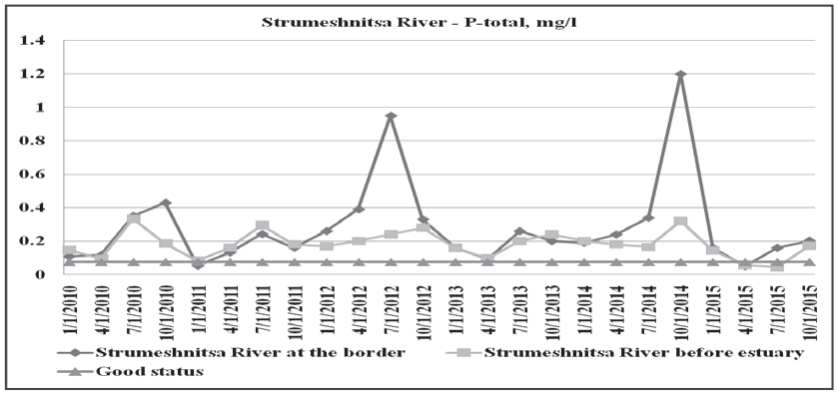

Fig. 10. State of the Strumeshnitsa River according to P - Total phosphorus indicator

Throughout the study period in both monitoring points of the Strumeshnitsa River, exceedances in the indicator of P - Total phosphorus have been registered. The measured concentrations of the Strumeshnitsa River at the border are in the range of 0.05 - 0.95 mg/l and before the estuary: 0.043-0.334 mg/l. The lowest concentrations of the indicator were measured in 2015 in both monitoring points.

The assessment of the state of the Strumeshnitsa River at the border (at checkpoint Zlatarevo) on the above physico-chemical quality elements are provided in Table 1:

Table 1. Evaluation of the physico-chemical elements for the quality at the point of the Strumeshnitsa River at the border

Year	pH	Dissolved oxygen, mg/l	Conductivit, µS/sm	BOD_5, mg/l	N-NH_4, mg/l	N-NO_2, mg/l	N-NO_3, mg/l	N-Total, mg/l	P - ortho -PO_4, mg/l	P - Total, mg/l	Final status
2010	Good	High	High	Moderate	Good	Moderate	Good	Moderate	Moderate	Moderate	Moderate
2011	Good	High	High	Good	Good	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
2012	Good	High	High	Moderate	Moderate	Moderate	Good	Moderate	Moderate	Moderate	Moderate
2013	Good	Good	High	Good	Good	Moderate	Good	Moderate	Moderate	Moderate	Moderate
2014	Good	Good	High	Moderate	Good	Moderate	Good	Moderate	Moderate	Moderate	Moderate
2015	Good	High	High	Good	Good	Moderate	Good	Moderate	Moderate	Moderate	Moderate

The assessment of the state of the Strumeshnitsa River before the estuary, at the bridge to the village of Mitino on the physico-chemical quality elements is provided in Table 2:

Table 2. Evaluation of the physico-chemical elements for quality at the point of the Strumeshnitsa River before the estuary, at the bridge to the village of Mitino

Year	pH	Dissolved oxygen, mg/l	Conductivity, µS/sm	BOD_5, mg/l	$N-NH_4$, mg/l	$N-NO_2$, mg/l	$N-NO_3$, mg/l	N-Total, mg/l	P - ortho $-PO_4$, mg/l	P - Total, mg/l	Final status
2010	Good	High	High	Moderate	Moderate	Moderate	Good	Moderate	Moderate	Moderate	Moderate
2011	Good	High	High	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
2012	Good	High	High	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
2013	Good	High	High	Moderate	Good	Moderate	Good	Moderate	Moderate	Moderate	Moderate
2014	Good	High	High	Good	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
2015	Good	High	High	Good	Good	Moderate	Good	Good	Moderate	Moderate	Moderate

Based on the assessment, the state of the Strumeshnitsa River in the two points for monitoring of the water body code BG4ST400R072 „The Strumeshnitsa River from the Bulgarian-Macedonian border to its flowing into the Struma River“ for the period 2010 - 2015 has been evaluated in moderate condition according to the physico-chemical elements of quality.

Discussion

In the studied period the Strumeshnitsa River flows into the Bulgarian territory in not a very good condition. The values of most indicators of organic substances are much higher than the norms for a good state.

The content of N – NO_2 and N-total, P - ortho-PO_4 and P - Total phosphorus is above the norms and does not meet the requirements of a good state.The registered higher concentrations of these indicators are mainly due to the discharge of untreated sewage from the settlements in Macedonia and as a result of the agricultural activities carried out along the river.

On Bulgarian territory the water quality of the Strumeshnitsa River is further exacerbated due to the discharge of waste waters formed from the sewer system of the town of Petrich and external sewer collector of the village of Mihnevo and the village of Karnalovo directly without purification and the development of the green house production.

The condition of the Strumeshnitsa River on Bulgarian territory will improve after the construction of a treatment plant for the waste waters of Petrich.

Conclusions

The presented data on the condition of the waters of the Strumeshnitsa River according to physico-chemical elements for quality require us to take measures for improving the quality of the waters of the Strumeshnitsa River not only on Bulgarian territory but on the territory of the Republic of Macedonia (due to the fact that it is an international river basin situated between Bulgaria and Macedonia). In order to improve the quality of the waters in the Strumeshnitsa River a cross-border coordination and exchange of information between both countries are needed. A coordination of the objectives of environmental protection (on

items which need an improvement) should be carried out as well as a coordination of the necessary measures to improve the condition of the Strumeshnitsa River.

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INVESTIGATION OF THE ZOOPLANKTON AND THE CHLOROPHYLL *A* LEVEL IN "DOSPAT" DAM

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Abstract

The zooplankton and the chlorophyll *a* are important trophic links in the ecological food chains of all limnic systems.

Purpose: The aim of this study was to investigate the dam ecological status. We use the zooplankton and the chlorophyll *a* levels for this research.

Materials and Methods: The chlorophyll *a* quantity of the phytoplankton was determined according to ISO-1/1980 and ISO 5667-2/1991. The zooplankton numbers and the biomass were calculated.

Results: According to the chlorophyll *a* level the major part of the dam was determined as mesotrophic. The ratio between the basic three zooplankton taxons was untypical for a dam ecosystem. A cladocerans' prevalence over the other two groups was detected. It was accompanied by a good trophic level of the phytoplankton.

Conclusions: The results indicate a low number of planktivorous fish and a possible risk for the dam ecological balance at low oxygen level (including the area with fish farming cages).

Keywords: chlorophyll *a*, zooplankton, ecological status.

Introduction

Recently a lot of hydrobiological studies use the chlorophyll *a* content as a factor to determine the biomass and phytoplankton production [1]. Many authors [2] have found a correlation between the chlorophyll *a* in the microalgae and the nutrient's presence in the water, especially phosphorus. Based on this correlation the determination of the assimilation pigment concentration has been used successfully as a fast method for the estimation of the eutrophication rate and the water quality in the natural ponds [3]. A zooplankton analysis in the ecosystems and the ways for guiding the zooplankton to the desired direction are the most important tool in the transformation of the autotrophic production to the next trophic link – the fish. When there is a prevalence of small zooplankton forms the clearance is lower despite of the comparable total biomass [4] to populations with a presence of the large forms (*Daphnia pulicaria, D. longispina* and *D. magna*).

Furthermore, the fish density reduces the zooplankton population, especially when certain conditions are available [5]. The high eutrophic level sometimes is related to the prevalence of consumption of unfitted Cyanophyta forms. The main implications of the high eutrophication are the large fluctuations in the base environmental factors (accompanied with the respective risks) and the reduction of the fish production [6]. Then the effectiveness of phytoplankton along the trophic chain phytoplankton – zooplankton – fish is very low [7].

Because of the geographic location of the Dospat dam, it is a preferred place for fishing during the summer. The zooplankton has been investigated by Naidenov and Sais [8], and Terziyski [9]. Additionally, there is scarce data available for the chlorophyll *a* [9]. The investigation of the actual dam statement about the trophicity is very important because the trends for the social and economic development of the Dospat dam are of great public interest.

Materials and Methods

The investigation has been conducted in the period between September 2014 and July 2015 in the Dospat dam. The geographic coordinates (Table 1) of each sampling station were localized by GPS (GARMIN 76CSx).

A vertical closing zooplankton net "Juday" (mesh size 50 μm, net mouth opening 36 cm, 0.1 m^2) was used for the sampling. The zooplankton numbers were calculated by Dimov's method [10].

The biomass quantity was obtained by Prikryl's volume-weight method [11]. To get more conclusive results the biomass of some samples was determined by the standard individual weight for each biological species and the developmental stage [12].

The chlorophyll *a* quantity in the phytoplankton was determined using a spectrophotometric analysis after ethanol extraction according to ISO-1/1980 and ISO 5667-2/1991.

Table 1. Geographical coordinates of the sampling stations

Sampling station №	North latitude	East longitude
1	41°43.635'	024°01.903'
2	41°43.061'	024°03.125'
3	41°42.499'	024°04.239'
4	41°49.959'	023°51.780'
5	41°39.45'	024°09.06'
6	41°38.69'	024°09.13'

Results

The conducted studies of the chlorophyll *a* (Table 2) characterized the ecological status of the Dospat dam as eutrophic (10-35 $\mu g.l^{-1}$) and mesotrophic (4-10 $\mu g.l^{-1}$). Due to the typical seasonal phytoplankton development an increased chlorophyll *a* level was reported during the autumn of 2014 and the early spring of 2015. There were no substantial differences between the different sampling stations data.

A maximal chlorophyll *a* level during the longest investigation period (2015) was reported at the sampling station 2. A possible reason for this could be a combination of the effects of several factors, such as the local sources of anthropogenic influence and the optimal depth for the photosynthetic processes (about 10 m) giving relatively small water temperature fluctuation. In ecological aspect these conditions characterize the area around the sampling station 2 between the mesotrophic and the eutrophic type. According to the analyzed factor (chlorophyll *a*) the area around the rest of the sampling stations is mesotrophic.

During the spring and the beginning of the summer of 2015, the average chlorophyll *a* levels were mesotrophic. An oligotrophy was registered at the end of April and during May 2015 at sampling stations 2, 3 and 6. This trend combined with the typical peak in the zooplankton development for this period is a note of caution regarding the phytoplankton ability to provide the oxygen necessary 24 hours a day (Table 3). If certain factors are available, an oxygen deficiency is possible in this situation.

The Dospat dam is narrow and deep. It is located in a valley, high above the sea level and there is a short period of sunshine. Similarly to Naidenov and Sais [8], in our research this is the main reason for the limitation in the number of phytoplankton species in the Dospat dam.

Table 2. Chlorophyll *a* level for the investigation period, µg.l^{-1}

Sampling station №	Year 2014		Average value for a station	Year 2015				Average value for a station
	Sampling date			Sampling date				
	28.09.	23.10.		04.04.	24.04.	29.05.	02.07.	
1	13.81	7.70	10.75	8.88	5.43	2.22	2.96	4.87
2	15.29	7.10	11.20	18.25	4.44	1.48	3.70	6.97
3	13.81	7.10	10.46	12.33	1.48	1.85	3.70	4.84
4	-	-		12.58	2.47	2.22	3.33	5.15
5	-	-		9.99	3.95	2.07	2.96	4.74
6	-	-		11.47	1.48	2.96	4.37	5.07
Average value	14.31	7.30	10.80	12.25	3.21	2.13	3.50	5.27

Table 3. Zooplankton biomass for the investigation period, g.m^{-3}

Sampling station, №	Year 2014		Average value for a station	Year 2015				Average value for a station
	Sampling date			Sampling date				
	28.09.	23.10.		04.04.	24.04.	29.05.	02.07.	
1	0.53	0.27	0.40	0.09	0.11	0.90	0.49	0.40
2	0.70	0.12	0.41	0.16	0.13	0.82	0.60	0.43
3	0.55	0.02	0.28	0.25	0.13	0.91	0.86	0.54
4	-	-	-	0.24	0.08	1.03	1.00	0.59
5	-	-	-	0.29	0.28	1.12	1.04	0.68
6	-	-	-	0.30	0.21	1.12	0.95	0.64
Average value	0.59	0.14		0.22	0.16	0.82	0.82	

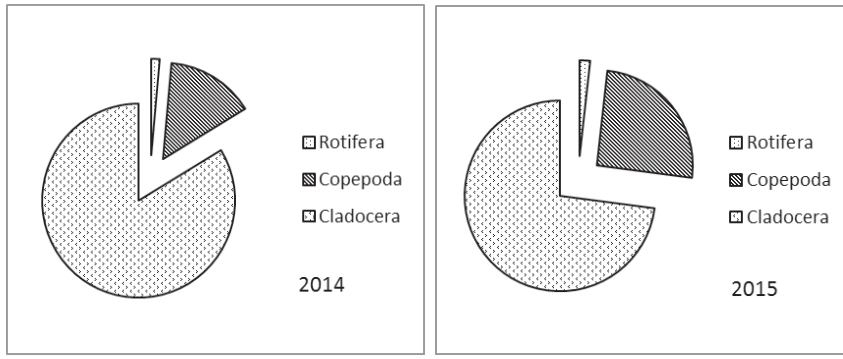

Fig. 1. Percentage distribution of the zooplankton by groups (2014 year on the left and 2015 year on the right)

The zooplankton investigation in September 2014 detected an undeniable prevalence between 87% and 95% of the Cladocera zooplankton group (Fig. 1) with major species *Bosmina coregonii* (Baird) and *Daphnia galeata* (G. O. Sars), (Table 4). The mentioned species are relatively small in size (less than 1.5 mm). Therefore, an efficient transfer of the phytoplankton production through the zooplankton to the next trophic link (the fish) could not be expected [7]. A similar situation has been observed during October 2014. The only exception is the sampling station 3, which is located near the inlet of the dam (about 400 meters) and it is highly influenced by the flowing Dospatska river. Thus, the better development of Copepoda is related to seasonal reasons. The zooplankton biomass (Table 3) of both of the samplings in 2014 was relatively low (I sampling - 0.525-0.699 $g.m^{-3}$; II sampling - 0.271-0.018 $g.m^{-3}$). There was one more powerful filtrator identified in the zooplankton species – the rotatorian *Asplanchna sieboldi*.

During 2015 (Table 3) a peak in the zooplankton development was registered. The maximum was at the beginning of the summer. The biomass increased 4 times, but actually the increase was below 1 $g.m^{-3}$. There is a trend of relatively high values of the zooplankton factor at the sampling stations located in the middle and at the end of the dam. The dominant species were *Bosmina coregonii* (Baird) and *Daphnia longispina* (O. F. Müller) of the Cladocera group (Table 4). They form 70 - 81% of the biomass (Fig. 1), with an exception when the Copepoda species reached about 42% from the biomass at the end of April 2015. Therefore all Cladocera species determined the zooplankton biomass for the entire period of the analysis. Thus Cladocera prevalence over Copepoda in the zooplankton community was untypical for the dam ecological system. According to Uzunov and Kovachev [2], the zooplankton species in the dams are mainly rotatorian and a small number of filtrating crustaceans. The ratio between the different taxons is favorable for better utilizing of the phytoplankton primary production along the „grassland“ food chain. On the other hand this is a signal for a lack or an insufficient presence of ichthyofauna species feed on zooplankton. The main reason for this is the poaching, especially by gillnets, leading to reducing of the fish species like rudd, carp, crucian. Smaller fish like perch and common sunfish survive and manage to reproduce successfully at a relatively small size (7-9 cm for the perch). Considering the fact that all fish eat zooplankton in some part of their development, an untypical structure of the fish population with a prevalence of carnivore species has been observed. These carnivores have a strong influence on the typical zooplanktonophages resulting in a great reduction of the large zooplankton species. The extensive development of small filtrating crustaceans inhibits the phytoplankton and leads to the presence of small forms. The crustacean prevalence in the epilimnion combined with an oligotrophy (regarding chlorophyll *a*) at some sampling stations and time periods conceals risks for the aerobic organisms especially for species with hidh demands on the level of dissolved oxygen (like the trout, including the raising trout in cages).

Table 4. Zooplankton taxonomic composition

Taxons	2014		2015			
Rotatoria	28.09.	23.10.	04.04.	24.04.	29.05.	02.07.
Keratella cochlearis cochlearis (Gosse, 1851)	-	+	-	+	-	+
Keratella quadrata (Müller, 1786)	-	+	-	+	-	+
Kellicottia longispina longispina (Kellikot, 1879)	+	-	-	+	-	+
Asplanchna sieboldi (Leydig, 1854)	-	+	-	-	+	+
Filinia major (Colditz, 1914)	+	+	-	-	-	-
Fillinia longiseta (Ehrenberg, 1834)	-	+	+	-	+	-
Polyarthra vulgaris Carlin, 1943	+	+	+	-	+	+
Cladocera						
family Daphnidae						
Daphnia pulex Leydig, 1862	+	-	-	-	+	+
Daphnia longispina O. F. Müller, 1785	+	+	+	+	+	+
Daphnia curvirostris Eylman, 1886	-	+	-	-	+	+
Daphnia sp. juv.	+	+	+	+	+	+
family Bosminidae						
Bosmina longirostris O. F. Müller, 1785	-	+	-	+	-	+
Bosmina coregonii Baird, 1857	+	+	+	+	+	+
Copepoda						
order Cyclopoida						
Cyclops vicinus Uljanin, 1875	+	+	+	+	+	-
Acanthocyclops robustus (Sars, 1863)	-	+	-	+	+	+
order Calanoida						
Eudiaptomus gracillis (Sars, 1863)	+	+	-	+	+	+
Copepodites	+	+	+	+	+	+

Conclusions:

1. The sampling stations and the bigger part of the dam are determined as mesotrophic.

2. Seasonally, during the autumn of 2014 and the beginning of the spring of 2015, the Dospat dam is determined as eutrophic by chlorophyll *a*. In the rest of the vegetation period it is mesotrophic.

3. In the beginning of the summer of 2015 there was a maximum of the zooplankton biomass. The increasing was 4 times, but below 1 $g.m^{-3}$.

4. Probably due to a poaching a reduction of the zooplanktonophage ichthyofauna was found. A prevalence of small zooplankton of the Cladocera group was detected and this conceals risks of oxygen deficiency.

5. A low inefficient transfer of the phytoplankton production along the food chain phytoplankton – zooplankton – fish was reported.

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SHORT COMMUNICATIONS

Topic:
BIOTIC AND ABIOTIC IMPACT ON THE LIVING NATURE AND MECHANISMS OF ADAPTATION

DNA PROTECTION BY *CLINOPODIUM VULGARE* AGAINST ZEOCIN-INDUCED DOUBLE-STRAND BREAKS - ROLE OF GENOTYPE AND EXPERIMENTAL DESIGN

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In recent years, the interest of using herbal medicine in health care is growing rapidly worldwide [1]. It is of a great importance to gain enough data on the biological properties of the studied plants in order to evaluate possible effects on DNA depending on the experimental design, genotype, etc. Plants with long-term use in folk medicine such as wild basil *Clinopodium vulgare* L. are of a particular interest.

In the present study, the potential DNA protection of *Clinopodium vulgare* extract against DNA damaging action of zeocin is evaluated based on the reduction of double strand breaks (DSBs). DSBs in nuclear DNA are considered as the most critical DNA damage. When DSBs are misrepaired, mutations, genomic instability, cancer development, etc., may occur. In the case that they are not repaired this may result in cell death [2].

The aim of the study was to evaluate the role of the genotype and experimental design for revealing the DNA protective potential of *Clinopodium vulgare* leaf extract.

Materials and Methods:

Two Saccharomyces cerevisiae strains were used:

- Diploid strain D7ts1 (*MATa/α; ade2-119/ade2-40; trp5-27/trp5-12; ilv1-92/ilv1-92; ts1/ts1*)
- Haploid strain 551 (*MATα ura3-167 his3Δ200:TymHIS3AI sec53 rho*$^{+}$).

Chemical: Zeocin, purchased from Invitrogen, was used as a standard radiomimetic capable of inducing DNA DSBs.

Plant extract: Aerial parts of *Clinopodium vulgare* L. were collected in Lozenska Mountain (Sofia Region, Bulgaria), near Monastery "Saint Spas", open grass area with shrubs, 850 m a.s.l., Bulgaria. Date: 01/07/2014, Leg.: Krum Bardarov, Det. Anely Nedelcheva. Aqueous *Clinopodium vulgare* leaf extract was kindly provided by the colleagues from Chromana LTD.

Treatment procedures:

Cell suspension with a density of $1x10^6$ cells/ml was treated as follows:

Single treatments:

- *Clinopodium vulgare* extract at concentrations 10, 100 and 1000 µg/ml - for 30 min at t=30^{O}C, on a shaker (200rpm).

- Zeocin at concentration 100µg/ml - for 1 min on ice during centrifugation.

Several experimental designs were also applied:

• Treatment with extract and zeocin without washing the cells between both treatments:

- at optimal for cell growth conditions - t=30^{O}C, without recovery time (RT) given after zeocin treatment

- on ice (to prevent DNA repair processes), without RT.

- with 30 min RT on ice.

- with 30 min RT at optimal conditions (design 1)

Two additional designs were applied on strain D7ts1:

• Pretreatment with extract, washing the cells, treatment with zeocin, and 30 min RT (design 2);

• Pretreatment with extract, washing the cells, 45 min inter-treatment time (ITT), treatment with zeocin, and 30 min RT (design 3);

After that, cells were centrifuged, the pellet was embedded into agarose plugs and constant field gel electrophoresis (CFGE) was performed as described in [2].

Results:

Role of genotype: DNA susceptibility of both strains was compared based on the spontaneous and primary induced DSB levels. No statistically significant differences were obtained between the spontaneous DSBs levels. Around 2.5-fold increase in DSBs levels was measured in both strains after single zeocin treatment in comparison with the control untreated cells.

Additionally, the repair capacity of both strains was evaluated based on comparison of the DSBs levels without and with RT. Relatively similar repair capacity was calculated for both strains - 1.199 for 551 and 1.405 for D7ts1. Although the two-way ANOVA revealed a slight influence of the genotype.

In order to evaluate whether *Clinopodium vulgare* extract would possess some DNA damaging properties, single treatment experiments were performed with the three concentrations of the extract. Treatments with any of the concentrations did not increase the DSBs in a statistically significant way to levels higher than that in the control untreated cells.

Role of experimental design: In order to obtain information concerning the role of the experimental design, cells were subjected to combined treatment at optimal for cell growth conditions and on ice.

In samples subjected to combined treatment at optimal conditions, statistically significant decrease in the DSB levels compared to that after single zeocin treatment was measured for both strains. No effect of extract concentration was observed.

Differences were obtained between strains when combined treatment was performed on ice. In the diploid strain D7ts1 DSB levels remained similar to those induced after single zeocin treatment. Statistically significant minor decrease was obtained in the haploid strain. Such result suggests that although the treatment was performed on ice to prevent DNA repair processes, some processes could be still active in strain 551.

Analyzing the dynamics of repair capacity depending on the extract concentration, relationship between increased extract concentrations and decreased DSB levels was observed only for D7ts1. Such effect was not obtained for strain 551. Difference in repair capacity of both strains after combined treatment with the highest tested concentration *Clinopodium vulgare* extract and zeocin was found. The repair capacities of the strains were calculated as 3.081 for D7ts1 and 1.226 for 551.

It is well known that DNA susceptibility of organisms could depend on the levels of primary induced DSBs or the repair capacity. Our results show that despite the genotype differences between both strains the levels of primary induced DSBs are relatively similar. The obtained differences in repair capacity suggest that repair capacity plays significant role for the susceptibility of *S. cerevisiae*.

In order to provide insights concerning the role of experimental design, the diploid strain was further subjected to treatment following design 2 and 3.

Although decrease in DSBs levels was observed in all the designs, the most pronounced repair capacity was obtained in design 1 - combined treatment with extract concentration 1000 μg/ml and zeocin. The repair capacity was more than 2.5-fold higher than that calculated in design 2 and 3.

Conclusion: Evaluating DNA susceptibility of *S. cerevisiae* it might be supposed that DSBs repair capacity could play more significant role than primary induced levels of DSBs. The role of the genotype and concentration is well expressed when combined treatment with *Clinopodium vulgare* L extract and zeocin is performed. The most pronounced DNA protection of *Clinopodium vulgare* L extract is observed after combined treatment with 1000 μg/ml extract and zeocin in the diploid strain D7ts1. Based on our data it could be suggested that the experimental design and genotype of this organism should be always taken into consideration when applying treatment with natural products.

Keywords: *Clinopodium vulgare* extract, zeocin, double strand breaks

Acknowledgements: This study was supported by project "Ecological and genetic risk: methods and strategies for overcoming" – BAS.

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DOES COMBINED TREATMENT WITH *CLINOPODIUM VULGARE* EXTRACT AND ZEOCIN PROTECT NUCLEAR DNA FROM ZEOCIN-INDUCED DAMAGES?

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Various biological activities of a large number of plants like wild basil *Clinopodium vulgare* have been screened. Chemical analysis, purification and investigation of plant constituents such as flavonoids (quercetin, kaempferol, luteolin) and polyphenols have already revealed not only their antioxidant properties but also DNA protective effect via stimulation of DNA repair processes and antimutagenic activity [1].

Aim: To evaluate the protective potential of aqueous *Clinopodium vulgare* leaf extract in combined treatment with zeocin

Materials and Methods:

Test-system: *Two Saccharomyces cerevisiae* strains - 551 (haploid) and D7ts1 (diploid). Strain 551 was designed for the evaluation of the potential carcinogenic effect by measurement of the Ty1 transposition levels [2]. Strain D7ts1 was designed for the simultaneous detection of three genetic events - the mitotic crossing-over, the reverse mutations and gene conversion [3].

Clinopodium vulgare aqueous leaf extract was kindly provided by the colleagues from Chromana LTD.

Experimental designs:

Cell suspension with a density of 1x 10^6 in the beginning of stationary phase was treated as follows:

Single treatments:

Clinopodium vulgare extract at concentrations 10, 100 and 1000 µg/ml was added to the cell suspension and samples were incubated for 30 min at t=30^O, on a shaker at 200 rpm.

Cells were treated with zeocin at concentration 100 µg/ml for 1 min on ice during centrifugation.

Combined treatment:

Cell suspensions were incubated with different concentrations of extract and subsequently treated with zeocin. No cell washing between both treatments was performed. After zeocin treatment cells were washed, resuspended in fresh YPD medium and 30 min recovery time was given at optimal for cell growth conditions (t=30^O, on a shaker at 200 rpm). Further, several endpoints were studied following the procedures described in detail in Todorova et al. [1, 4]:

• Pro-oxidative/antioxidant activity - quantitative measurement of superoxide anions in living cells in strain D7ts1. It is a spectrophotometric method, which is based on the color change of the reduced tetrazolium dye XTT from pale yellow to orange-red. XTT enters only in live yeast cells.

• Genotoxic/antigenotoxic activity - as percentage of visible colonies survived in treated samples divided by that in untreated samples.

• Constant field gel electrophoresis (CFGE) was used for the evaluation of double strand breaks (DSBs) levels. The advantage of this method is that intact cells are included in

agarose plugs prior to cell lysis, thereby avoiding the risk of further fragmentation of DNA in the processing of the samples.

• Mutagenic/antimutagenic potential - gene conversion, reverse mutation, mitotic crossing-over, measured by the Zimmermann's test. This test allows determination of both mutagenic and recombinogenic activity.

• Carcinogenic/anticarcinogenic potential - Ty1 retrotransposition events measured by Ty1 retrotransposition test - microbiological test for the quantification of carcinogenic effect of various substances. This test allows distinguishing mutagens with and without carcinogenic effects.

Results: Data from the single zeocin experiments showed well-expressed pro-oxidative, DNA damaging, mutagenic/recombinagenic activities and genotoxic potential of zeocin. An increasing of the levels of superoxide anion - 3-fold, DSBs - 2.5-fold, reverse mutations - 10-fold, gene conversion - 8-fold, transposition - 4-fold and 40% reduction of cell survival were documented.

No pro-oxidative, genotoxic, DNA-damaging, mutagenic and recombinogenic effect was found after single treatment with 10, 100 and 1000 μg/ml *Clinopodium vulgare* extract.

After the application of the combined treatment, an antioxidant, antigenotoxic, DNA protective and antimutagenic effects were well recognized. Statistically significant reduction of the superoxide anion levels, increase of cell survival and decrease of the levels of DSBs, reverse mutations and mitotic crossing-over compared to those after single zeocin treatment were measured.

Statistically insignificant antirecombinogenic effect in terms of gene conversion was observed for all the concentrations.

A correlation between the extract concentration and the frequency of transposition was established - with increasing of the extract concentration, the frequency of transposition decreases. Well-expressed anti-carcinogenic effect was observed after combined treatment with 100 and 1000 μg/ml extract concentrations and zeocin. The transposition levels were comparable to the control levels. These results are in accordance with previous results in our laboratory concerning the anticarcinogenic potential of *Papaver rhoeas* extract towards zeocin [1].

Conclusion: The well expressed pro-oxidative, genotoxic, DNA damaging, mutagenic and recombinogenic potential of zeocin was confirmed. No statistically significant pro-oxidative, genotoxic, DNA damaging, mutagenic and recombinogenic effects of *Clinopodium vulgare* L. concentrations tested by us were revealed. All of the selected concentrations of *Clinopodium vulgare* extract have a protective effect after combined treatment. Relationship was established between extract concentrations and the antimutagenic and anticarcinogenic potential. The most promising protective effect was observed for concentrations 100 and 1000 μg/ml *Clinopodium vulgare* extract. The results allow us to believe that the extract is suitable for further pharmacological researches.

Keywords: DSBs, antimutagenic, anticarcinogenic, zeocin, *Clinopodium vulgare*

Acknowledgements: This study was supported by project "Ecological and genetic risk: methods and strategies for overcoming" – BAS.

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ISSR PRIMER SELECTION FOR GENETIC VARIABILITY ANALYSES WITH THE BULGARIAN ENDEMIC *VERBASCUM DAVIDOFFII* MURB. (SCROPHULARIACEAE)

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Introduction: Plant diversity on the territory of Bulgaria is remarkable compared with the relatively small territory of the country – about 4,100 species. An alarming fact is that around 20.5% of them are threatened with extinction [1]. *Verbascum davidoffii* Murb. (Scrophulariaceae) is one of the rarest plant species in Bulgarian flora. It is a Bulgarian endemic, protected by the National Biodiversity Act, included in the Red List of vascular plants in Bulgaria and in the Red Data Book of the Republic of Bulgaria with conservation status "Critically endangered" [1].

Successful adaptation of plant species to environmental changes depends on their genetic diversity. Thus, its maintaining has a central role in the conservation programs. Inter Simple Sequence Repeat (ISSR) markers are dominant markers used in the genetic diversity studies of various plant species [2]. An important step prior to genetic diversity analysis is selection

of primers in order to avoid those that either fail to amplify or generate only few fragments and low polymorphism levels.

The aim of the present study is to select appropriate ISSR markers applicable to the evaluation of natural single world population of *Verbascum davidoffii*, in order to estimate the genetic diversity of such an endangered and economically important plant species.

Materials and Methods: Fourteen *Verbascum davidoffii* individuals were sampled from the population of the species between the valleys of river Bunderitsa and Razlozhki Suhodol, Pirin National Park. Genomic DNA was extracted from young leaves following the modified CTAB – procedure [3]. Eleven ISSR primers (Microsynth, Balgach, Switzerland) were selected, after the screening of 35 primers on this small subset of samples. Polymerase chain reactions were performed in a volume of 25 µl, containing a final concentration of 1 × PCR buffer (Fermentas, Vilnius, Lithuania), 1 U Taq DNA polymerase (Fermentas, Vilnius, Lithuania), 100 µM of each dNTP, 1 µM of each primer and 50 ng of extracted DNA. PCR cycling conditions were as follows: 5 min initial denaturation at 95^{O}C, 35 cycles of amplification [45 s at 94^{O}C, 1 min at the annealing temperature (T_a), 2 min elongation at 72^{O}C] and a final elongation step of 5 min at 72^{O}C. PCR experiments were performed with a TC-5000 gradient thermal cycler (Techne, Staffordshire, UK). To determine the optimal annealing temperature for each primer, an interval of 10^{O}C around the melting temperature (T_m) was tested. The temperatures leading to clear patterns were then repeated until the optimal T_a was selected for each primer for routine ISSR fingerprinting. The reproducibility of the technique was tested by replicating each amplification reaction twice. To further ensure the quality, PCR reactions were performed with one positive and one negative control. The PCR products were analysed on 2 % agarose gels (Fermentas, Vilnius, Lithuania) in 0.5 × TBE buffer. A 100-bp plus DNA ladder size standard (Fermentas, Vilnius, Lithuania) was used to estimate the length of PCR products. The gels were stained by incorporating 1.5 µl of ethidium bromide (0.5 mg/ml) in 100 ml agarose. Electrophoresis was run for 1.5 h at 150 V, the ISSR - profiles were visualised with a UV transilluminator (TFP-M/WL, Vilber Lourmat, Eberhardzell, Germany) and further analysed with a video image analyzer.

Results: The ISSR marker system has the following main advantages over other markers: ISSRs are reproducible due to their better stringency (high annealing temperature), no gene sequence information and no prior genetic studies are required for these analyses [2]. Our study shows the importance of knowing the profile of bands of a certain ISSR primer before the study of genetic variability of a given species. The assignment of ISSR bands to genetic loci was done semi-automatically using GelAnalyser 2010a image analysis software (http://www.gelanalyzer.com/). Of the 35 primers tested in the screening procedure, the following eleven primers were informative:

ISSR 1: GAG AGA GAG AGA GAG AT
ISSR 2: GAG AGA GAG AGA GAG AA
ISSR 3: CAC ACA CAC ACA CAC AG
ISSR 4: ACA CAC ACA CAC ACA CT
ISSR 5: ACA CAC ACA CAC ACA CC
ISSR 6: ACA CAC ACA CAC ACA CG

ISSR 7: AGA GAG AGA GAG AGAGYT
ISSR 8: AGA GAG AGA GAG AGA GYC
ISSR 9: GAG AGA GAG AGA GAG AYG
ISSR 10: ACA CAC ACA CAC ACA CYT
ISSR 11: ACA CAC ACA CAC ACA CYA

The optimal annealing temperature T_a = 60^{O}C was identified for primers ISSR 1 ÷ ISSR 6 and ISSR 11. Four of the ISSR primers (ISSR 7 ÷ ISSR 10) produced bands at T_a = 55^{O}C. The selected ISSR primers amplified 111 fragments, ranging from 200 – 3000 bp in size. They had a high level of polymorphism, repeatability and scorability. The total number of amplified fragments in each reaction ranged from 7 to 16, with an average of 11 bands per primer. The primer ISSR 4 showed the highest number of amplified fragments, generating 16 bands, 100% of which were polymorphic.

Conclusion: The primers indicated in the present study are informative for the genetic diversity analysis of *Verbascum davidoffii* and therefore can be used in the future investigations aimed at the diversity characterization and conservation of natural population of this endangered plant species.

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MONITORING OF VIRAL DISEASES IN INDUSTRIAL VINEYARDS IN BULGARIA DURING THE PERIOD 2011 – 2015

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Introduction: The viruses Tomato ringspot virus (ToRSV), Tobacco ringspot virus (TRSV), Grapevine fanleaf virus (GFLV), Grapevine fleck virus (GFkV), Arabis mosaic virus (ArMV), Grapevine leafroll virus 1, 3 (GLRV 1, 3) cause serious diseases of grapevine (*Vitis vinifera* L.), spread all over the world in almost all viticultural regions [1]. Their significance is determined by the way of spreading through vectors [2] or in the production of grapevine planting material [3]. ToRSV and TRSV belong to the group of Nepoviruse and are quarantine viruses for Bulgaria and the EU [4]. During the last few years new industrial vineyards have been planted with European and traditional Bulgarian varieties [5]. In order to clarify the phytosanitary status and prevent a possible spread of quarantine pests, the Plant Protection Directorate at the Bulgarian Food Safety Agency (BFSA) is implementing a programme for grapevine quarantine pests. Along with the study of quarantine pests, including viral infection by Tomato ringspot virus (ToRSV) and Tobacco ringspot virus (TRSV), the economically dangerous grapevine viruses GFLV, GFkV, ArMV, GVA, GLRV1 and GLRV3 have also been studied.

The purpose of this study was to determine the presence of ToRSV, TRSV and the state of economically significant grapevine viral diseases in the main viticultural regions in Bulgaria.

Materials and Methods

Period of examination. Samples of 20 Regional Food Safety Directorates (RFSD) were analyzed at the laboratory twice annually (in the spring and in the autumn). The samples of a possible viral infection were analyzed at the Central Laboratory of Plant Quarantine (CLPQ). According to the preliminary plan of the Monitoring programme for quarantine pests of the BFSA 854 examinations of vineyards, produced by imported and local grapevine planting material, were performed from inspectors.

Plant samples. During the period from 2011 to 2015 629 plant samples were analysed for ToRSV, TRSV and others viruses, of which 370 were from vineyards with imported grapevine planting material (France, Italy, Germany, Serbia). The others 291 samples from vineyards with Bulgarian grapevine planting material were analysed only for GFLV, GFkV, GLRaV1, GLRaV3, ArMV and GVA. Samples were collected as follows from the regions (for ToRSV, TRSV/and for other viruses GFLV, GFkV, ArMV, GVA, GLRV1, GLRV3): Blagoevgrad (6/19), Bourgas (69/28), Dobrich (14/10), Haskovo (48/0), Montana (45/10), Pazardjik (27/3), Pleven (47/4), Plovdiv (63/33), Russe (52/0), Shumen (33/22), Silistra (8/4), Sliven (27/22), Stara Zagora (13/9), Targovishte (20/22), Varna (44/37), Veliko Turnovo (28/31), Vidin (31/8), Vratsca (27/29), Yambol (52/0). The samples consisted of 3 to 5 annual or biennial shoots with leaves from symptomatic vine. They were taken from a

grapevine and represented a sample. The length of every petiole was about 35 to 40 cm with minimum 5-6 nodes. Upon examination and identification of the similar symptoms, we took samples by grouping together several plants but not more than ten grapevines. The size of the sample for detection was from 1 to 1.2 g. We cut 3-4 circles with thickness of up to 5 mm from each petiole and when analyzing the leaves we took samples from their base and near the leaf stalk.

DAS ELISA method. Samples were analyzed through extraction of the viruses from the vegetation material (leaves, shoots and petioles) in Grapevine Extraction Buffer \pH-8.2\ in proportion 1/10 (w/v). The laboratory analysis for possible infections with ToRSV, TRSV, GFLV, GFkV, ArMV, GVA, GLRV1 and GLRV3 were performed using the DAS-ELISA method. Polyclonal antibodies in optimal concentration (Ag/Ab) were used as recommended by the manufacturer (SEDIAG S.A.S, France). When determining the final result, we calculated the average value of both repetitions and accepted for positive those with values of absorption of the staining reaction twice and more than the negative control.

Results: More than 2400 laboratory analyses for possible viral infection were performed. They were distributed as follows: 1104 analysis were performed for determining the presence of ToRSV, TRSV in 19 regions (Blagoevgrad 10, Bourgas 137, Dobrich 21, Haskovo 88, Montana 70, Pazardjik 51, Pleven 94, Plovdiv 126, Russe 81, Shumen 66, Silistra 10, Sliven 46, Stara Zagora 22, Targovishte 20, Varna 88, Veliko Turnovo 47, Vidin 45, Vratsca 39, Yambol 43) and 291 analyses were performed for GFLV, 171 analyses for GFkV, 302 analyses for GLRV-1, 288 analyses for GLRV-3, 126 analyses for ArMV and 124 analyses for GVA in a 16 grapevine regions of Bulgaria (Blagoevgrad 84, Bourgas 120, Dobrich 40, Montana 45, Pazardjik 15, Pleven 20, Plovdiv 147, Shumen 100, Silistra 20, Sliven 99, Stara Zagora 40, Targovishte 102, Varna 165, Veliko Turnovo 137, Vidin 35, Vratsca 133).

TRSV and TRSV, which are quarantine for Bulgaria, were not found during the study. In 2011 mixed infection of GFLV and GFkV was identified in one sample and GLRV-1 and GLRV-3 in a two samples in Plovdiv region. In 2012 the GFLV and ArMV were found in two samples in Shumen region. In the spring and autumn of 2013 viral infections were found in the regions of Stara Zagora (GFLV in 1 sample), Yambol (GFLV in 1 sample), Pazardzhik (GLRaV-1 in 1 sample) and Haskovo (GFkV in 1 sample and GLRaV-1 in 1 sample). In 2015 GFkV and ArMV in two samples were found in Sliven region. In four samples the established infection was in material imported from France; in five samples the established viral infection originated from Italy; and in two samples the origin of the grapevine planting material was unknown and unconfirmed.

Discussion and Conclusions

Based on the obtained results, it is confirmed that there is no ToRSV and TRSV on the territory of Bulgaria.

The low level of viral infection can be considered as a new significantly better phytosanitary and healthy state of the newly created vineyards with local planting material in Bulgaria. This will be confirmed by continuing the studies for determining the status of a possible viral infection.

The results confirm that the viral infections found during the period of this study do not differ from the well-known and widespread viruses in the industrial viticultural regions. The viral infections found in 2011-2015 are in vineyards with imported grapevine planting material from France and Italy. As a result of the monitoring, two regions are established with sources of grapevine viral infections – Thrace region (Plovdiv, Stara Zagora, Yambol, Pazardzhik and Haskovo) and North-East region – Shumen. These regions are at risk and it is necessary to pay attention to vine-growers and plant nurseries that they should not use uncertified grafts material for production of grapevine planting material.

Keywords: grapevine, monitoring, ToRSV, TRSV, viruses, Bulgaria.

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FRAGARIA VESCA L. – A NEW FOODPLANT TO THE IMAGO OF *ORCHESTES FAGI* L.

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Introduction and Aims: The beech weevil, *Orchestes fagi* (Linnaeus, 1758) (Coleoptera: Curculionidae), is the only leafmining beetle among the leafminer complex on the beech trees. The feeding of its larvae takes place in leaf parenchyma, where it forms characteristic mines facilitating the process. Such a kind of damage is monitored during unfolding of beech leaves until the end of May [1].

The imago has leaf-chewing mouthparts and it makes round holes in the fragile beech leaves. The feeding of a new generation in June and supplementary feeding of pre-wintered imago in early spring is important to the pubescence of the insects. The new emerging adults are visible from the end of the May to the beginning of June. The young weevils prefer grass and bush species which have soft leaves in comparison with the crude and hard covered beech leaves in this period [2]. The probable reason is the lignification and biochemical processes in the leaves which have a seasonal nature [3]. In Denmark, this species causes serious damage in orchards [4]. Feeding of *O. fagi* has been detected on *Tilia europaea* L., *Acer pseudoplatanus* L., *Malus sylvestris* Mill., *Rosa canina* L., *Rubus fructicornis* L., *Rubus idaeus* L., *Sorbus aucuparia* L., *Ribes nigrum* L., *Ribus silvestre* Lam., *Ribus uva-crispa* I., *Ulmus procera* Salisb., *Betula pubescens* Ehrh., *Corylus avellana* L., *Quercus robur* L., *Salix fragilis* L. and *Salix nigricans* Sm. [4]. Feeding of the adults on the leaves of these species, has been established when beech is not available at a certain time of the year. It is of interest which micro- and macro-elements in the leaves are suitable for adults of beech weevil as well as the chemical changes in the leaf tissues during the vegetation period.

The beech weevil spends the winter in the soil litter, in the cracked bark of trees nearby or cones of conifer species, where they are being protected from the unfavorable winter conditions. The feeding after hibernation is necessary for the maturation of oocytes in females [1]. It occurs in the young beech leaves, close to the places of the subsequent oviposition. While in Europe foodplants and feeding preferences of the *Orchestes fagi* L. have been well studied [1], no such observations have been performed in Bulgaria. Such studies are of great importance to the better interpretation of processes such as recognition, acceptance and suitability of food by insects.

The objective of the study was to collect information about the preferences of the beech weevil to the other forest plants (except of common beech).

Materials and Methods: The experiment was conducted in May-June 2015 in the Laboratory of Entomology of the University of Forestry with new emerged individuals from leaves under laboratory conditions.

Newborn weevils (20 specimens) were fed in a Petri dish with leaves of other forest plants (except of common beech), such as *Aesculus hippocastanum* L., *Prunus cerasifera* Ehrh., *Carpinus betulus* L., *Crataegus monogyna* Jacq., *Fragaria vesca* L. The choice of the

proposed plants was motivated by the natural form in Bulgaria of mixed stands of common beech and common hornbeam, with the participation of hawthorn and cherry plum. The wild strawberry is a part of natural grass cover. We have used existing plants in the dendrarium of the University of Forestry, which in our opinion have a suitable consistency to be tasted by the insect.

The leaf material was gathered from the Botanical garden of the University of Forestry. The leaves were replaced with fresh ones in every 24 hours. The experiment was similar to that made by Bale & Luff [4].

At the same time the insects were provided with old and young leaves of live beech saplings in order to verify the preference of adult individuals to leaves with different consistency.

Results and Discussion: The experiment showed that the new emerged imago of *O. fagi* prefers the leaves of wild strawberry *Fragaria vesca* L. On each part of wild strawberry leaf were observed about 12 number of holes, and on the young beech leaves - up to 20 numbers. At the leaves of two plant species the area of one hole is on the average of 0.03 cm^2. In Bulgaria there is no real danger for fruiting of orchards and strawberry fields because of the significant difference in the distribution of the beech forests and agricultural crops and differences in the phenological development of species.

Bale & Luff [4] reported *Crataegus monogyna* as a food type for the pre-wintered imago. In our studies were not found any holes on hawthorn leaves.

Laboratory experiments with beech-tree saplings showed that *O. fagi* doesn`t eat leathery beech leaves at the end of the growing season. Subsequental analyzes of the content of mineral nutrients in the leaves from beech and different plants will clarify the reasons for these food preference by the *Orchestes fagi* L.

Conclusions: In conclusion it must be noted that in this study *Fragaria vesca* L. was established as a new foodplant to new-born imago of *Orchestes fagi* L.

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REPORTS

Topic:
ECOSYSTEM RESEARCH, SERVICES AND ECOLOGICAL AGRICULTURE

MODELING OF FOREST ECOSYSTEM SERVICES

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Abstract

Aim: Summary description of the models included in ForEco DSS for smart forest management.

Methods: statistics, dendrochronology, Decision making theory, Utility theory and stochastic approximation technic used as machine learning.

Results: The developed ForEco DSS system consists of three original models: (A) mathematical model for ecosystem asset, supply and services assessment and valuation; (B) Web based model for forest ecosystem state and climate impact assessment by SP-PAM application and (C) Value based model (VBM) for integrated assessment.

Conclusion: ForEco was developed to assess quantity and quality of ecosystem services, to evaluate them and to extract human knowledge and decision making patterns. ForEco system supports the adaptive ecological management of forest areas to achieve their sustainable development and operation as a natural source of ecosystem services. Potential ForEco users are policy makers, governmental and municipal organizations (forest and landscape planners), experts from the Green Economy, Agricultural and Forestry sector, as well as the research and innovation organizations. The described models are under further development.

Keywords: ForEco DSS, Ecosystem services function, SPPAM model, Value based model

Introduction

The study deals with development of formal models for ecosystem assets and supply assessment [1], as well as ecosystem services valuation and adaptive usage management [2]. Many different mathematical models for valuation of ecosystem services have been developed. The main objective in ecosystem services assessments should be to provide decision makers with macro-level information that supports understanding the relative state and importance of different ecosystems. Usually, there will be a limited set of resources available to influence ecosystem condition and capacity and hence choices must be made among a range of investment options [3]. One of the latest attempt to create a web- and

software based decision-making system that integrates two models – supporting forest management model and supporting forest state assessment was published by Lyubenova et all [4, 5, 6] that was realized during the ForEco project to FIWARE [7].

We accepted the following terms in our developments: natural resources (NR), natural capital (NC), ecosystem unit (EU) and basic spatial units (BSU). NR are the material source of wealth, such as timber, fresh water, or a mineral deposit, that occurs in a natural state and have the economic values [8]. Other words NR are the asset or material resources that constitutes the natural capital of a nation. Natural resources require application of capital and human resources (mental and physical labor) to be exploited (extracted, processed, refined) for the realization of their economic value [9]. NC can be defined as the world's stocks of natural assets which include geology, soil, air, water and all living things. It is from this NC that humans derive a wide range of services, often called ecosystem services, which make human life possible. EU are the spatial areas that form the conceptual base for assessment and accounting and the integration of relevant statistics. This approach allows various ecosystems in scope depending on the purpose of analysis. BSU support the delineation of EU and the integration of multiple datasets. For ecosystem accounting, BSUs are assumed to be internally homogenous in terms of their biophysical properties. BSUs may be delineated through the formation of a spatial grid covering the extent of a country. Accounts will generally be produced for relatively large administrative areas, such as provinces, states or countries; or in relation to large ecological areas such as bioregions or river basins. The larger areas that are used for accounting and reporting are referred to as geographical aggregations. The delineation of EU will require the usage of multiple data sources. As a starting point for delineation, land cover data, grouped into relatively broad land cover types may be appropriate. Testing of methods for the delineation of EU and the formation of BSUs is required. A focus should be placed on determining the appropriate scale of information required for integration of data and for accounting and policy use [10].

Detailed models that are web-based, gived possibilities to calculate the available assets of ecosystems and cash equivalents as well as support environmental management of forest resources (DSS) are extremely necessary. The published models cover a part of material ecosystem services assessment and evaluation.

Material and Methods

The developed ForEco system consists of three original models: (A) Mathematical model for ecosystem asset, supply and services assessment and valuation; (B) Web based model for forest ecosystem state and climate impact assessment by SP-PAM application and (C) Value based model (VBM) for integrated assessment. The model (B) is based on dendrochronological analyses and statistics with SP-PAM software. The model (C) represents a multiattribute utility function, developed on the Decision making theory, Utility theory and stochastic approximation technic used as machine learning [11]. The utility functions were calculated using the preferences of the Decision maker (DM), professional in forest ecology and environmental protection. The detailed methodology was published by the authors [4, 5].

Results and Discussion

The set of formal definitions and functions are developed in model (A) related with evaluated ecosystem unit (EU): Ecosystem services function (E), Natural capital value function ($\bar{C}$) and Natural resources function (Φ).

$$E: \Phi^{y} \times X \rightarrow \Phi^{y+1} \times I, \quad (1)$$

where the state of natural resource for EU at y^{th} year is denoted with Φ^{y}, the expenses in ecosystem cervices are denoted with $X \subset R^{+}$ and the income after their application as $I \subset R^{+}$.

$$\bar{C}: \Phi \rightarrow R^{+}, \quad (2)$$

where $$\Phi = \Xi \times \{V\} \times B \times \{W\} \times M \times \{\overline{W}\} \times \Pi \times \{\overline{\overline{W}}\} \times K \times \{\underline{W}\} \times S \times \{\overline{V}\} \times R \times \{\overline{\overline{V}}\} \quad (3)$$

The elements of function (3) are the following separate sub functions: Total tree value function ($\bar{T}$), Herbal value function ($\bar{H}$), Mushroom value function ($\bar{M}$), Game value function ($\bar{G}$), Fruit value function ($\bar{F}$), Soil value function ($\bar{S}$) and Rock value function ($\bar{R}$). The Total tree value function evaluates tree stand in an EU:

$$\bar{T} = T^{*} + C^{*} + F^{*} \quad (3.1)$$

The formal definitions related with (3.1) for EU define the functionality of this sub function.

Definition 1: Tree species $T = \{t_i : i = 1,\ldots,n_1\}$, where t_i is a tree species and n_1 is the total number of all possible tree species.

Definition 2: Tree reserve of an Ecosystem unit $\Xi = \{\xi_i : i = 1,\ldots,n_1^{*}, \xi_i = \{t_i, k_i, b_i\}, t_i \in T\}$ where n_1^{*} is the tree species richness of a specific EU, k_i are their weights. b_i are the respective bonitos of that tree species. A graded scale presents the tree bonitos. The volume of all trees in a EU was denoted with V and with R^{+} the set of all positive real numbers. V is measured in m^3.

Definition 3: Timber value function $T^{*}: \Xi \times \{V\} \rightarrow R^{+}$. The Timber value function evaluates the timber fraction in an EU.

Definition 4: Wood chips value function $C^{*}: \Xi \times \{V\} \rightarrow R^{+}$. The Wood chips value function evaluates the wood chips fraction in an EU.

Definition 5: Firewood value function $F^{*}: \Xi \times \{V\} \rightarrow R^{+}$, where R^{+} is the set of positive real numbers. The Firewood value function evaluation evaluates the firewood fraction in an EU.

In all previous definitions, the variable t_i implicitly presents the price per cubic meter for that specific tree species.

Definition 6: Total tree value function $\bar{T}: \Xi \times \{V\} \rightarrow R^{+}$, where R^{+} is the set of positive real numbers.

Herbal value function evaluates herbs in an EU. The biomass (kg) of all herbs in an EU was denoted with W.

$$\bar{H}: B \times \{W\} \rightarrow R^{+} \quad (3.2)$$

$\overline{H}$ is a tabular function and has concrete values for each year. It can be presented as a regression polynomial depending on values of h_i, k_i. h_i implicitly presents the price per kilogram for that specific herb species.

The formal definitions related with (3.2) for EU define the functionality of this sub function.

Definition 7: Herb species $H = \{h_i : i = 1,\ldots, n_2\}$ where h_i are all possible herb specie and *n* is the total number of all possible herb species.

Definition 8: Herbal reserve of an Ecosystem unit $B = \{\beta_i : i = 1,\ldots, n_2^*, \beta_i = \{h_i, k_i\}, h_i \in H\}$, where n_2^* is the number of different herb species in an EU and k_i are their weights.

Mushroom value function evaluates mushrooms in an EU:

$\overline{M}$: M x $\{\overline{W}\} \rightarrow R^+$, (3.3)

where $\overline{W}$ is the weight of biomass of all mushrooms. The formal definitions related with (3.3) for EU define the functionality of this sub function.

Definition 9: Mushroom species $M = \{m_i : i = 1,\ldots, n_3\}$ where m_i are all possible tree species and *l* is the number of all possible mushroom species.

Definition 10: Mushroom reserve of an Ecosystem unit $M = \{\mu_i : i = 1,\ldots, n_3^*, \mu_i = \{m_i, k_i\}, r_i \in R\}$, where n_3^* is the number of different mushroom species in an EU and k_i are their weights.

Game value function evaluates game in an EU:

$\overline{G}$: Π x $\{\overline{\overline{W}}\} \rightarrow R^+$ (3.4)

The formal definitions related with (3.4) for EU define the functionality of this sub function.

Definition 11: Game species $G = \{g_i : i = 1,\ldots, n_4\}$ where g_i are all possible animal species and n_4 is the number of all possible game species.

Definition 12: Game reserve of an Ecosystem unit $\Pi = \{\pi_i : i = 1,\ldots, n_4^*, \pi_i = \{g_i, k_i\}, g_i \in G\}$ where n_4^* is the number all possible game species in an EU, and k_i are their weights. The total weight of the game was denoted with $\overline{\overline{W}}$.

Fruit value function evaluates fruit in an EU:

$\overline{F}$: K x $\{\underline{W}\} \rightarrow R^+$, (3.5)

where the total weight of fruits was denoted as $\underline{W}$. The formal definitions related with (3.5) for EU define the functionality of this sub function.

Definition 13: Fruit type $F = \{f_i : i = 1,\ldots, n_5\}$ where f_i are all possible fruits and n_5 is the number of all possible fruits.

Definition 14: Fruit reserve of an Ecosystem unit $K = \{k_i : i = 1,\ldots, n_5^*, \pi_i = \{f_i, k_i\}, f_i \in F\}$ where n_5^* is the number all possible fruits in an EU, and k_i are their weights.

Soil value function evaluates soil in an EU:

$\overline{S}$: S x $\{\overline{V}\} \rightarrow R^+$, (3.6)

where the total volume of soil was denoted with $\overline{V}$. The total volume of soil is measured in m^3. The formal definitions related with (3.6) for EU define the functionality of this sub function.

Definition 15: Soil type $S = \{S_i : i = 1,\ldots, n_6\}$ where S_i are all possible soil types and n_6 is the number of all possible soil types.

Rock value function evaluates rocks in an EU:

$\overline{R}$: R x $\{\overline{\overline{V}}\} \rightarrow R^+$, (3.7)

where the total volume of rock was denoted with $\overline{\overline{V}}$. The total volume of rocks is measured in m^3. The formal definitions related with (3.7) for EU define the functionality of this sub function.

Definition 16: Rock type R = $\{r_i : i = 1,\ldots, \eta_7\}$ where r_i are all possible rock types and η_7 is the number of all possible soil types.

The application of the model (A) was reported for the first time by Assenov [12].

For model (B) the set of indicators were involved for the forest state assessments: number of eustress periods; their duration, frequency and depth; eustress years (unfavorable climatic type of years), reactive tree functional type and eustress-climatic predictive patterns.

The authors perceive eustress as a repeating state of diminished radial growth rate of tree stems within a period of one or multiple years and caused by unfavorable factors in the environment. This state encompasses numerous other reactions of the tree species. The level of radial stem growth (or tree ring width) is the main parameter that the developed application operates with, as well as the growth index, which is the main indicator for the statistical determination of low growth threshold (categorized as eustress) under the unfavorable climatic years. The growth index is calculated as a relation between the measured and the approximated values of ring width for each year:

$$It = MW/AW, \tag{4}$$

where MW is the measured value of radial growth for a given year and AW is the value computed through approximating polynomial equation. The calculation of growth index helps to eliminate the tree age as a main factor influencing the tree ring width. The confidence interval of indexes for each trustworthy tree ring sequence at level of significance - α = 0.05 is computed. The study of the forest ecosystem assets state is based on an assessment of eustress depth (A) duration (D) - the number of adjacent eustress years, and frequency (F) - the number of stress years for a period of 100 years, and the creation of eustress nomenclature by 5-graded scale (Table 1).

$$A = \frac{1}{s}\sum_{i=1}^{s}(1 - It_i) \tag{5}$$

Table 1. Fife-graded scale for *Q. cerris* L. stands eustress nomenclature

F			D			A		
Group		Value	Group		Value	Group		Value
1	Very rarely	≤ 34.3	1	Very short	≤1.67	1	Very Small depth	≤0.182
2	Rarely	>34.3 - ≤ 38.15	2	Short	>1.67 - ≤1.936	2	Small depth	>0.182 - ≤0.208
3	Normal	>38.15 - ≤ 45.85	3	Normal	>1.936 - ≤2.469	3	Normal depth	>0.208 - ≤0.259
4	Often	>45.85 - ≤ 49.7	4	Long	>2.469 - ≤2.736	4	deep	>0.259 - ≤0.284
5	Very Often	>49.7	5	Very Long	>2.736	5	Very deep	>0.284

Performance evaluation of the eustress in particular localities allows the expression of reactive functional type of tree species, its behavior to climatic factors for decades. For example, the functional type F4D5A4 means that in particular locality typical for trees of that species there are frequent, very long and deep eustresses that behavior puts the existence of the forest under some risk.

The next group of analyses conducted relate the eustress with climatic conditions. Therefor a climatic year (CY) is defined as the calendar year with relevant average temperature and yearly sum of precipitations, and then a climatic type of year (CTY) is introduced as the climatic year with specific climatic conditions. These conditions are determined by deviations from the proposed climatic norms of temperature (dT) and precipitation (dP). The climatic norms are considered as the mean values of temperatures and precipitations with their confidence intervals (μti, μpi) for every 30 years that the studied period was divided. The used climatic types are: HD - hot and dry (T>Tav+μti and P<Pav-μpi); CW – cold and wet (T<Tav-μti and P>Pav+μpi); HW – hot and wet (T >Tav+μti and P>Pav+μpi); CD – cold and dry (T<Tav-μti and P<Pav–μpi). Confidence intervals (μti, μpi) are calculated at a significance level $\alpha = 0.05$. Adverse climatic years are all climatic years that correspond to eustress year (SY).

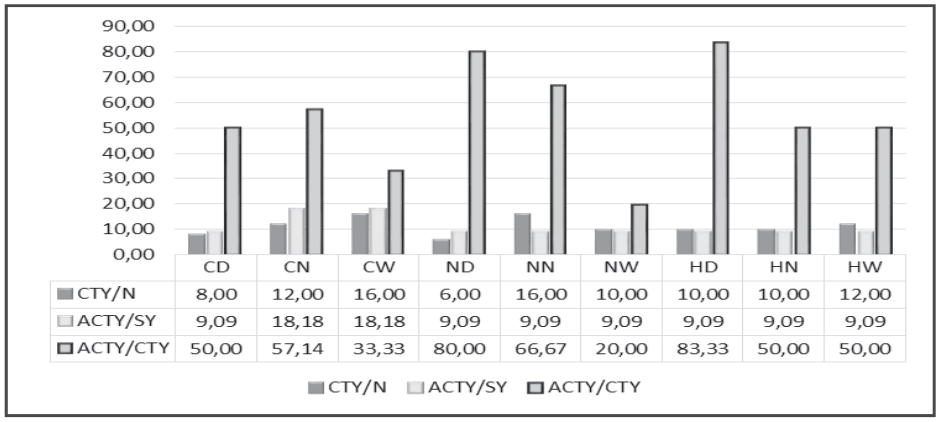

Fig. 1. The relative importance of climatic types for the presence of durmast oak eustress (%):

a) CTY / N - the number of years of climatic type to the total number of years included in the analysis; b) ACTY / SY - the number of years of climatic type, which is set to eustress, to the total eustress years of different climatic types; c) ACTY / CTY - the number of years of climatic type, which is set eustress, to the total number of years of relevant climatic.

The main objective of the analysis is to manifest the importance of different CTY to the eustress occurrence (Fig. 1). This analysis also examines the importance of climatic patterns for the presence of eustress - consistent CTYs for a period of two years before the eustress year. This approach allows predicting how the maintaining of certain adverse climatic regime or its sudden shift to another will affect the production of wood (Fig. 2).

Table 2. CTY changes by three years period for the species locations

№	Scots Pine	Beech
1	D/W/ v.v.	W/D v.v
2	H/C v.v	C/H v.v
3	HD/CW/ v.v	CW/HD v.v
4	HW/CD v.v	HW/CD
5	**DDD***/DD/**D-D**/-DD	DDD/**DD**/D-D/-DD
6	**WWW**/WW/**W-W**/-WW	**WWW**/WW/**W-W**/-WW
7	CCC/CC/C-C/-CC	CCC/CC/C-C/-CC
8	HHH/**HH**/**H-H**/-HH	HHH/**HH**/**H-H**/-HH

**The presence of eustress years in the corresponding climate type is denoted by Bold letters*

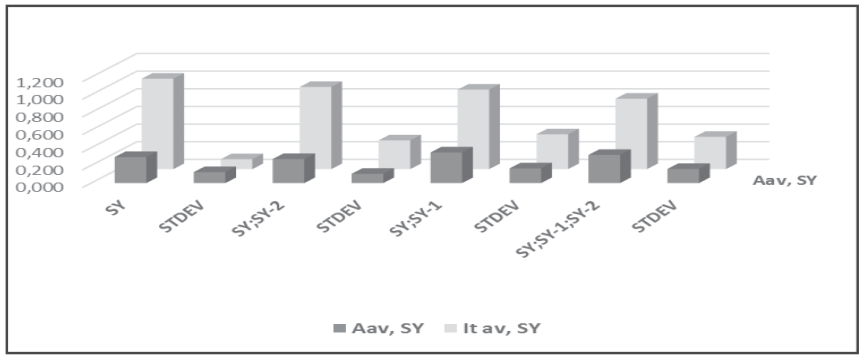

Fig. 2. It_{av} and A_{av} variations for Beech locations by stress patterns

The application of the model to five coniferous and four deciduous trees has been published [13].

For the model (C), we accepted tree indicators (sub-objectives or factors) adequately describing the main objective of investigation. These indicators are: X_1 - timber reserves ($m^3.ha^{-1}$) as representing criteria for the assessment of economic effects or material services; X_2 - species richness ($n.ha^{-1}$) as representing criteria for the assessment of ecological effect, or regulating and supporting services and X_3 - percentage of population employed in the forestry sector as representing criteria for the assessment of the social effect or services. The model is developed as a multiattribute utility function with the three factors mentioned. The coefficients of the function are calculated using the preferences of ecology and environmental professional. In the process of investigation of the independence by utility was found by the DM between the following factors: 1) X_2 from X_1; X_2 from X_3 and 2) X_3 from X_1; X_3 from X_2. The preferences of DM for x2 at different values of x1 and x3 do not change, suggesting independence of x2 from the changes of other two factors. Whatever the reserves of wood in the forest ecosystem and the employment of the population in the forestry sector may be different. In any cases, the preferences are directed to the presence of a large species richness of the forest, i.e. great variety of species of trees, grasses, moss, lichen, algae, and animal

species etc. that form the ecosystem and ensure its greater stability. The preferences of DM for x3 at different values of x1 and x2 do not change, suggesting independence of x3 from the changes of the other two factors. This means that whatever the reserves of wood are and at the different species richness of the forest ecosystem, in any case, the preferences are aimed at the increasing of the number of workers in the forestry sector. At low timber reserves and a poor species composition, the increasing of employment in the forest sector is motivated by the need of reforestation, regeneration, cultivation of existing forests, optimization of forest-related natural resources, development of alternative uses and others. At high timber reserves and rich species composition, the increasing of employment is motivated by the opportunities of multifaceted use of forests and the need of environmental management and balanced utilization of forest resources. Using the theory for decomposition of multiattribute utility to simpler functions given in (Keeney, 1999; by Pavlov et al., 2013) we determine the following multiattribute utility structure:

$$u(X) = k_1 u(X_1;X^o_2;X^o_3)+f_2(X_1)x[u(X^o_1;X_2;X^o_3)]+f_3(X_1)\,x\,[u(X_1^\circ;X_2^\circ;X_3)]+ \\ +f_{23}(X_1)x[u(X^o_1;X_2;X^o_3)]x[u(X^o_1;X^o_2;X_3)], \text{ were } u(X^o_1;X^o_2;X^o_3)=0 \quad (4) \\ \text{and } u(X^*_1;X^*_2;X^*_3) = 1$$

In the formula above $X^o = (X^o_1; X^o_2; X^o_3) = (10,1,1)$ and $X^* = (X^*_1; X^*_2; X^*_3) = (300,200,30)$. The functions f_2, f_3 and f_{23} have the forms:

$$f_2(X_1) = [u(X_1;X^*_2;X^o_3)]-k_1 u(X_1;X^o_2;X^o_3), \\ f_3(X_1) = [u(X_1;X^o_2;X^*_3)]-k_1 u(X_1;X^o_2;X^o_3), \quad (5) \\ f_{23}(X_1) = [u(X_1;X^*_2;X^*_3)]-f_2(X_1)-u(X_1;X^o_2;X^*_3)$$

Each of thise sixth functions is evaluated based on the DM's preferences. For example, the form of function $u(X_1; X^o_2; X^o_3)$ is given in Fig. 1.

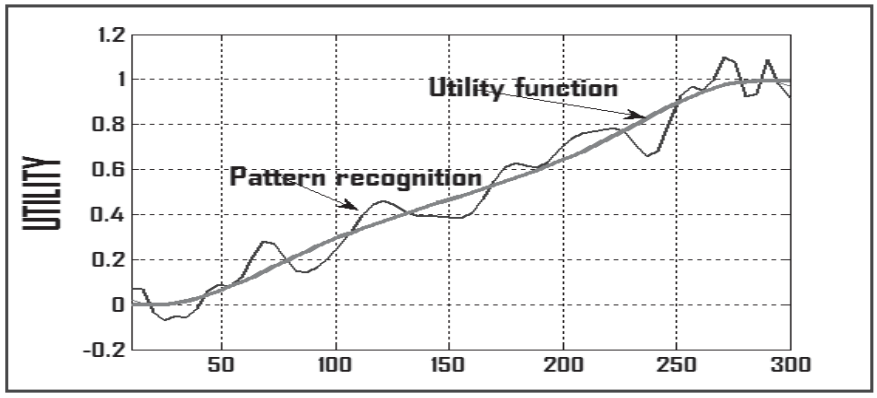

Fig. 3. Utility function $u(X_1;X^o_2;X^o_3)$

The blue seesaw line is pattern recognition of the positive or of the negative DM's preferences. The solid line is the evaluated Utility function polynomial approximation

$u(X_1; X^o_2; X^o_3)$. In Fig. 3 is shown the comparison betwen the evaluated Utility function $u(10; X_2; X_3)$ and the evaluated utility function $u(300; X_2; X_3)$. The results of model (C) application have been reported [4, 5].

Conclusion

ForEco was developed to assess the quantity and quality of ecosystem services, to evaluate them and to extract human knowledge and decision making patterns. The models are applied to simulate the decision making process of ecology and environmental professional to maximize the utility function for a clearly defined goal. ForEco system support adaptive ecological management of forest areas to achieve their sustainable development and operation as a natural source of ecosystem services. Potential ForEco users are policy makers, governmental and municipal organizations (forest and landscape planners), experts from the Green Economy, Agricultural and Forestry sector, as well as the research and innovation organizations. The access will provide to citizens, educational organizations and NGOs, who are interested and engaged in the project area of applications. The described models are under further development.

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MAPPING OF ECOSYSTEMS IN BULGARIA BASED ON MAES TYPOLOGY

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Abstract

According to Action 5 of the EU Biodiversity strategy the member states have to map and assess the state of ecosystems and their services in their national territory. A Working Group on Mapping and Assessment on Ecosystems and their Services the (MAES) provided methodological framework for mapping of ecosystems at the European scale.

Aim: The main objective of this paper is to analyze the spatial distribution of the ecosystems in Bulgaria based on the MAES typology and their dynamics for a 20 year long period. We utilize the CLC data to delineate and map ecosystems in Bulgaria. The information of the ecosystems was organized in a GIS database which includes data about four time series between 1990 and 2012 and the ecosystems' dynamics for this period were analyzed.

Results: The results show that Cropland and Woodland and forest ecosystems are the most spread in the country followed by Grasslands and Urban ecosystems. There are mixed trends in ecosystems change during the period 1990 – 2012. The area of urban ecosystems show a steady increase until 2006 while the area of Woodland and forest ecosystems gradually decrease during the whole studied period. Cropland ecosystems show small changes in the first two periods and a rapid increase during the third period.

There are different reasons for such changes which are both subjective and objective.

Conclusion: The CORINE Land Cover is an appropriate data source to delineate the ecosystem types on the base of the MAES framework at the national scale but at larger scales finer sources are required.

Keywords: Biodiversity strategy, MAES, ecosystem types, CLC, ecosystem dynamics, GIS

Introduction

According to Action 5 of the EU Biodiversity strategy to 2020 the member states have to map and assess the state of ecosystems and their services in their national territory. The results of this mapping and assessment should support the maintenance and restoration of ecosystems and their services. A Working Group on Mapping and Assessment of Ecosystems and their Services (MAES) was set up to underpin the effective delivery of the strategy with objective to support the implementation of Action 5 by the EU and its Member States. The results of its activities have been summarized in three reports that have been issued since 2013. The reports provide a methodological framework for the mapping and assessment of ecosystems and their services at the European and national scales. Section 4 of the first report proposes a coherent typology to be used for the different types of broad ecosystems to be considered in the assessment to ensure consistency across Member States [1]. These main classes are designed for the consistent assessments of state and services from local to national, regional and European scale. Information from a more detailed classification at higher spatial resolution could be combined with the European-wide classification and could be aggregated in a consistent manner [1]. The typology is organized in two main levels and its structure enables applying CORINE Land Cover (CLC) data for spatial delineation. It is also adjusted with the European Nature Information System (EUNIS) habitat types where necessary in order to ensure that further subdivision in the countries would be performed in a uniform and compatible manner. The adoption of this typology led also to the adaptation of other EU biodiversity activities including the EEA biodiversity baseline, which revised its reports in order to provide the relevant facts and figures on the state and trends of the different biodiversity and ecosystem components [2].

The mapping of ecosystems and their services has been mentioned as one of the main challenges for the ecosystem service concept's implementation into decision making [3]. The modern GIS technologies provide different tools and techniques for spatial analyses and database development, which can be used to investigate the relationships and influence of the different spatial units, delineation of ecosystems and assessment of ecosystem services. They should be directed to landscape pattern analyses, incorporation of land cover data and possibilities for spatial statistics and landscape change detection [4].

The main objectives of this paper are:

- to delineate the ecosystem types in Bulgaria following the MAES typology;
- to develop a GIS database of the ecosystem types;
- to reveal the changes in ecosystem areas during the period 1990 – 2012.

Materials and Methods

The research approach in this study was based on the use of free available CORINE Land Cover data and utilization of GIS techniques for aggregation of the MAES typology scheme, development of a GIS database, generation of ecosystems data series and elaboration of ecosystem maps (Fig. 1 – personal development). The workflow of presented

manipulations and analyses ensures appropriate frame for spatial data manipulation, spatial analyses and visualization of the results for ecosystem types at the national level.

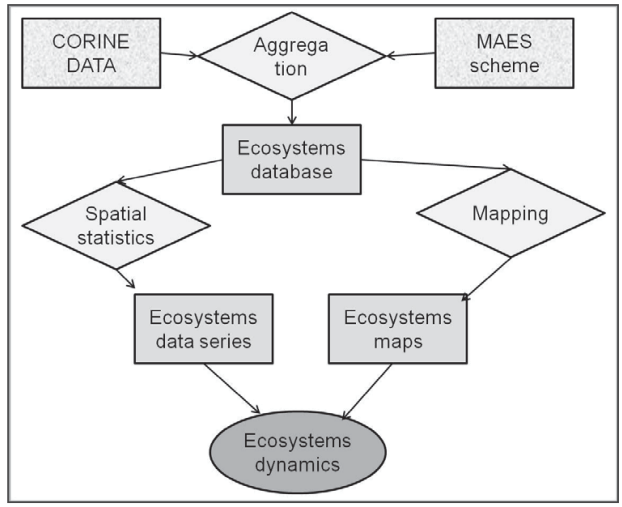

Fig. 1. Conceptual scheme of the research approach (Nedkov, Bratanova-Doncheva, Markov)

The main source of information for this study was the CORINE Land Cover (CLC) dataset. It is a geographic land cover/land use database encompassing most of the countries in Europe. In 1985 the CORINE program was initiated in the European Union. CORINE means coordination of information on the environment and it was a prototype project working on many different environmental issues. The CORINE databases and several of its elements have been taken over by the EEA. One of these is an inventory of land cover in 44 classes organized hierarchically in three levels, and presented as a cartographic product, at a scale of 1:100 000. The first level (5 classes) corresponds to the main categories of the land cover/land use (artificial areas, agricultural land, forests and semi-natural areas, wetlands, water surfaces). The second level (15 classes) covers physical and physiognomic entities at a higher level of detail (urban zones, forests, lakes, etc). The third level is composed of 44 classes dividing the previous entities into more detailed subdivisions. For instance the forests are divided into three classes - deciduous, coniferous and mixed. CLC was elaborated based on the visual interpretation of the satellite images (SPOT, LANDSAT TM and MSS). Ancillary data (aerial photographs, topographic or vegetation maps, statistics, local knowledge) were used to refine the interpretation and the assignment of the territory into the categories of the CORINE Land Cover nomenclature. The smallest surfaces mapping units correspond to 25 hectares. Linear features less than 100 m in width are not considered. The scale of the output product was fixed at 1:100000, therefore the location precision of the CLC database is 100 m [5].

The general MAES typology of ecosystems is organized into two levels. The first level is defined as “major ecosystem category” and includes three main classes: 1) Terrestrial; 2) Fresh water; 3) Marine. At the second level the major categories are subdivided into more detailed subclasses according to the character of their biophysical features. The terrestrial

ecosystems are subdivided into seven subclasses: 1) Urban; 2) Cropland; 3) Grassland; 4) Woodland and forest; 5) Heathland and shrub; 6) Sparsely vegetated land; 7) Wetlands. The fresh water ecosystem class contains only one subclass – Rivers and lakes. The marine ecosystems are subdivided into four subclasses: 1) Marine inlets and transitional waters; 2) Coastal; 3) Shelf; 4) Open ocean. Annex 2 of the MAES report [1] provides a table that links the ecosystem types at level 2 with the CORINE Land Cover classification. Some ecosystem types fully correspond to the CORINE classes from the entire category. For instance, urban ecosystems fully correspond to category 1. *Artificial surfaces* and include all classes from level one to three and sparsely vegetated lands correspond to category 3.3 *Open spaces with little or no vegetation.* Other ecosystems correspond to classes from different levels of CORINE classification. For instance, the ecosystem type grassland correspond to categories 2.3.1 *Pastures* and 3.2.1 *Natural* grasslands which are at different groups at level two of the CORINE classification.

The CORINE database in Bulgaria contains 36 out of 44 classes in the CORINE classification [6]. The correspondence of each class to the MAES typology was analyzed and distributed accordingly. The aggregation of the polygons was performed in two steps. Firstly, a look-up-table including the CORINE classes and the MAES ecosystems was prepared. At the second step, indices corresponding to the ecosystem types were assigned to all CORINE polygons. Thus, a new dataset containing both the CORINE and MAES typology information was created. The procedure was applied to all four CORINE datasets time series – 1990, 2000, 2006 and 2012. They were incorporated as feature classes in an ArcGIS geodatabase, which contains both spatial and tabular data of the two types of information. It was used as a source to generate maps of ecosystems in Bulgaria. The mapping was performed using the MAES typology at second level for terrestrial the ecosystems and freshwater ecosystems and first level for marine ecosystems. The distribution of the ecosystem types was performed using ArcGIS summary statistic tool, which enables to extract parameters for every particular cluster of objects. In our case, the area was used as a statistic field and the record with ecosystem types was used as a case field. The tool enables to generate spatial distribution of the ecosystem types measured in area units (km^2) for respective time series. The ecosystem dynamics for the period 1990 – 2012 was analyzed through integration of ecosystem maps and area distribution tabular data.

Results

There are nine main ecosystem types according to MAES typology that can be delineated in Bulgaria on the base of CORINE dataset (Fig. 2; Table 1). Cropland ecosystems occupy almost half of the counties' territory with about 535000 km^2. The database contains 18419 polygons of this ecosystems and their average size is 2.9 km^2 (the figures here and further on are based on the CORINE 2006 data). The largest polygon is 4546 km^2, which exceeds the total area of some other ecosystem types. They are the dominant type in the north part of the country (especially in the Danube plain) and in lowlands of the central and eastern part of South Bulgaria (Upper Thracian lowland, Tundzha and Burgas plains). Continuous croplands are typical for the areas of low elevation and predominantly plain topography,

while in the mountains they are less and have a patchy distribution. Woodland and forest is the second largest ecosystem type with about 430000 km^2, which comprises almost 38% of the country's territory. There are 19190 forest polygons, which is the highest number of all ecosystems. Their average size is 2.2 km^2, while the largest one is 2218 km^2. They are the predominant ecosystems in the mountain areas especially in the south and south-western part of the country as well as in the central parts. These two ecosystem types (cropland and forest) occupy about 86% of the country and thus dominate the landscape in almost all of its territory.

Grassland and Urban ecosystems are also well represented in the country occupying about 8100 and 5500 km^2 respectively. Both ecosystems are distributed almost evenly in the whole country with slight differences determined by the local conditions. Grasslands have 7754 polygons in the whole country and their average size is 1.01 km^2. Urban ecosystems are presented by 5939 polygons with average area of 0.5 km^2. The other five ecosystem types have limited extend covering about 2% of the countries' territory (Table 1). River and lakes ecosystems have about 1100 km^2 (about 1% of the country), located in the whole country. There are 447 individual polygons with an average area of 2.5 km^2. Sparsely vegetated lands cover about 590 km^2 (0.5%) located predominantly in the high mountains and along the Black sea coast. There are 560 individual polygons with an average area of 1.01 km^2. The largest area is located in Chepan Mountain near Dragoman, which covers 23 km^2. Heathland and shrubs occupy 300 km^2, which is less that 0.3% of the country. They are presented mainly in the high parts of Rila, Pirin and Stara Planina mountains. The total number of polygons is 90 and their average size is 3.5 km^2. Marine ecosystems occupy about 0.3% of the country located in the Black sea and its coastline, but the figures on their area are in very rough approximation because the water bodies class in the CORINE data is delineated as a 1 km strip along the coastline. Wetlands are the ecosystem type with the lowest area in the country with 105 km^2, which is less that 0.3%. There are only 7 polygons with average size of 0.6 km^2. The reason for these low figures is that the wetlands are usually small and most of them have dropped from the CORINE dataset as they are below the 25 ha threshold.

Table 1. Area of the ecosystem types for the period 1990 – 2012

Ecosystem type	1990		2000		2006		2012	
	area km^2	%	area km^2	%	area km^2	%	area km^2	%
Urban	5426.8	4.8	5544.0	4.9	5584.0	5.0	5333.7	4.7
Cropland	53457.1	48.0	53523.7	47.5	53506.8	47.4	53805.0	47.9
Grassland	8310.6	7.4	8188.3	7.3	8168.1	7.2	8083.4	7.2
Woodland and forest	43092.8	38.2	42995.0	38.1	43004.0	38.1	42718.2	38.0
Heathland and shrub	324.6	0.3	321.7	0.3	317.5	0.3	266.2	0.2
Sparsely vegetated land	599.1	0.5	579.0	0.5	569.9	0.5	564.1	0.5
Wetlands	104.9	0.1	104.1	0.1	105.8	0.1	104.2	0.1
River and lakes	1098.9	1.0	1158.4	1.0	1158.2	1.0	1157.7	1.0
Marine	378.6	0.3	379.2	0.3	379.0	0.3	375.1	0.3

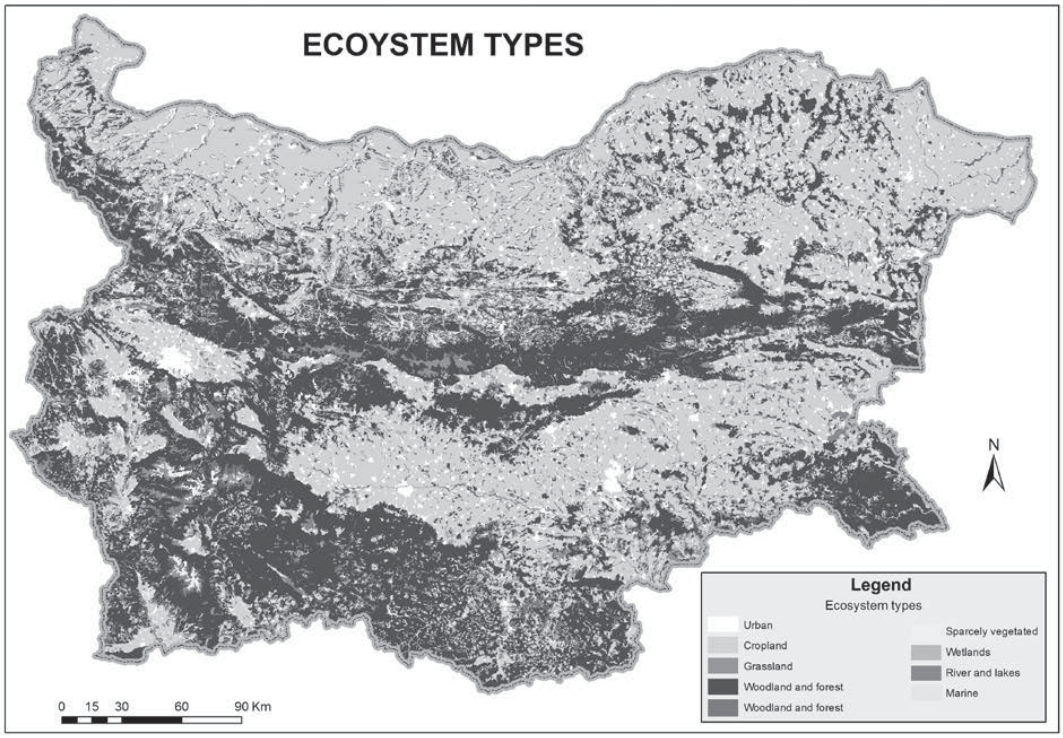

Fig. 2. Map of the ecosystem types in Bulgaria based on the MAES typology (Nedkov, Bratanova-Doncheva, Markov)

The analyses of ecosystem dynamics during the period 1990 – 2012 shows different trajectories for the different ecosystem types as well as during the periods between the CORINE mapping years (Table 2). A stable decreasing trend during the whole period is observed for Grassland, Heathland and shrub and Sparsely vegetated lands. The areas of these three types show negative values for all three periods of observation that are cumulated to reach the figures given in the last column of Table 1. The most significant change is the increase of the Cropland ecosystems, which is 347 km^2 for the whole period. This is mainly due to the increase after 2006, while for the previous periods there are slights increase (1990-2000) and decrease (2000-2006). Urban ecosystems show increase during the first two periods until 2006, but in the last period (2006-2012) there is significant decrease which leads to a negative result for the whole period. The changes in the Forest ecosystems for the whole period are also with negative values, which is mainly due to the significant decrease during the last period (285.9 km^2). During the first period there is slow decrease, while in the second period the area of forest slightly increases. The changes in Wetland and marine ecosystems are relatively small and insignificant for the studied period. Both areas have positive and negative figures during the different periods and a slightly negative figure for the whole period. River and lakes ecosystems have similar pattern except for the first period (1990-200) when their area decreases with 59.5 km^2.

Table 2. Changes in the area of the ecosystem types for the period 1990 – 2012

Ecosystem type	Change (km^2)			
	1990-2000	2000-2006	2006-2012	1990-2012
Urban	117.2	40.0	-250.3	-93.1
Cropland	66.6	-16.9	298.2	347.9
Grassland	-122.3	-20.1	-84.8	-227.2
Woodland and Forest	-97.8	9.1	-285.9	-374.6
Heathland and shrub	-2.9	-4.1	-51.3	-58.4
Sparsely vegetated land	-20.1	-9.1	-5.8	-35.1
Wetlands	-0.8	1.7	-1.5	-0.7
River and lakes	59.5	-0.2	-0.6	58.8
Marine	0.6	-0.3	-3.8	-3.5

Discussion and Conclusion

The territory of Bulgaria is dominated by two types of ecosystems - forest and cropland. The first are determined by the geographical location within the temperate climate zone, which characteristics favors the development of forest vegetation. They are better preserved in the mountain areas where the human impact is not so intensive. The croplands are the most spread ecosystem type due to the favorable for agriculture nature conditions and the resulting human activities. Urban and grassland ecosystems are also well presented in the country and their spread is determined by the human activity. Heathland and shrubs and sparsely vegetated areas have limited extend mainly in the mountains. All these ecosystems are well delineated from the CORINE database and the results could be determined as representative for the country's territory. River and lakes and wetland ecosystems have also limited extend in the country, but the results should be treated as rough estimation because the CORINE database do not include most of the real objects because of their small size. Mapping of rivers and wetlands at a finer scale is necessary for more representative results.

There are mixed trends in the ecosystems change during the period 1990 – 2012. Cropland ecosystems show small changes in the first two periods and a rapid increase during the third period, which could be explained with the result of the EU agriculture policy that was introduced in Bulgaria after 2007 when the country joined the EU. This process could be explained partly as a reason for the decrease of grasslands and heathlands, which were replaced in some areas by croplands. The urban ecosystems show a gradual increase during the period 1990 – 2006 which is in relation with the political and economical changes after 1989. The decrease during the last period (2006-2012) is more difficult for explanation. There are some small villages that were depopulated and excluded from the settlements register during this period but this could not explain such a decrease of urban areas therefore further analyses are necessary. There is a negative change in forest areas with a peak during the last period (2006-2012), which is caused most likely by more intensive forestry. The changes in river and lakes ecosystems during the first period (1990-2000) is dramatic and hardly to explain with the existing processes. The reason is rather “subjective” and is related

to the CORINE data acquisition methods which were significantly changed between 1990 and 2000. For more precise analyses the results should be correlated with other data sources in order to validate them and define possible sources of uncertainties.

The CORINE Land Cover is an appropriate data source to delineate ecosystem types on the base of thr MAES framework at the scale of 1:100 000. It provides easily available and comparable data at the EU scale that could be used for both ecosystem delineation and further ecosystem services assessment [7]. Its applicability at a finer scale is not so effective so for more precise studies other sources of spatial data are required. The ecosystem types database is an appropriate source of spatial information towards the fulfillment of the requirements of the EU biodiversity strategy in Bulgaria.

Acknowledgments: The work for this study has been supported by the EU HORISON 2020 funded project "Enhancing ecoSysteM sERvices mApping for poLicy and Decision mAking" (ESMERALDA) and the EEA funded Predefine project BG03.PDP 2 “Methodological Support for Ecosystem Services Mapping and Biophysical Valuation (MetEcoSMap)”.

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IMPACT OF LAND USE ON SURFACE AND GROUND WATER QUALITY ASSESSED IN THE WATERSHED LEVEL OF PARVOMAITSI VILLAGE – VELIKO TARNOVO REGION

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Abstract

The aim of this study is to present the results from surface water and ground water quality monitoring on the territory of a small watershed.

Materials and Methods: The pilot site is Parvomaitsi village (near V. Tarnovo) in Northern Bulgaria. The fluctuation of the groundwater table was monitored at permanently build pipe wells and home wells. The samples for analysis of the surface water were taken at different sites of the Yantra River. The investigation has been conducted in 2013.

Results and Discussion: It was established that surface- and ground- water sampled had neutral to alkaline reaction. The content of nitrate in the shallow ground-water varies in a large range. The calcium and magnesium concentrations in the surface and ground water do not exceed the maximum permissible level of the Bulgarian standards for drinking water. In this case, it was monitored a significant variation in the data for hydro-carbonate content, but clorine and sulphate contents could be characterised with considerably low variation.

Conclusion: The information will be used as a methodological approach in future environmental impact assessment on a watershed level.

Keywords: land use, surface- and ground- water, chemical elements, environmental protection

Introduction

The groundwater chemical composition depends on the natural factors such as hydrological characteristics of the terrains, the type of soils, geology profile and climate, as well as the anthropogenic loading of the watershed [1, 2]. A lot of groundwater quality monitoring programs are developed in order to prevent water contamination due to the intensive anthropogenic loading of the catchments [3, 4 and 5].

Many attempts have been done for assessing the influence of different land use on the groundwater quality at field experiments and natural conditions on a watershed level [6]. Agricultural practice is considered to be one of the main non-point sources of surface- and ground- water contamination by nitrate and other chemical elements [7, 8]. However, it is not correct to charge all agricultural activities without determining the vulnerable components of the biological cycle of nitrogen in plant-soil-groundwater system. It is important to evaluate the factors, which lead to nitrate accumulation in the root zone and their movements to the surface water and groundwater.

The objective of this study is to assess on a watershed level the impact of different land use activities and natural factors on the surface- and ground- water quality.

Materials and Methods

The study is part of the surface- and ground- water quality monitoring in the region of Parvomaitsi village, V. Tarnovo district (Bulgaria). The site of investigation is situated in the central part of the Yantra river basin (42^{O} 40' and 43^{O} 40'N and 24^{O} 40' and 26^{O} 30' E, and 94-110 m above the sea-level) on a well defined small watershed with climate, soils, municipal economy activities and practices, representative for this part of the country. The pilot site is situated on the first flooded terrace in the middle part of the Yantra river's catchment basin. The details of surface- and ground- water monitoring scheme, and some soil characteristics of the pilot site were presented in our earlier publications [9, 7, 10, 11].

The main agricultural practices in this region included intensive vegetable rotation, irrigation, manuring, and frequent soil cultivation, and they are described in the publication of Stoichev et al.[1].

The water properties are determined using the following methods: pH – potentiometrically [12]; nitrogen - direct distillation with 10 % Fe_2SO_4 and 0.5% Ag_2SO_4 reducing agents [13]; potassium and sodium - flame photometer, calcium and magnesium – atomic absorption spectrometry (AAS) [14], hydro-carbonate - titration with 0.02n H_2SO_4 to pH 4.4, and chloride - Moor method [12]. European and Bulgarian drinking water standards were used for the evaluation of water quality [4, 15].

Results and Discussions

It shows that in all cases the pH values have a neutral to alkaline reaction (Table 1). The pH values in surface - (N 23 - 25) and ground- water (N 5-21) in monitoring well varies from 8.05 to 8.35.

Table 1. Monitoring of pH values of Ground- water (Wr and Wh), Yantra River and Central Water Supply System (CWSS) in the area of Parvomaitsi village (V. Tarnovo district)

Source	No of wells	Depth,m	pH values
CWSS	1		8.05
Wr	5, 6	11-12	8.15
Wh	11, 15	5-12	8.35
Wh	16,17,18,19,20,21	2-5	8.25
Yantra River	23-25	-	8.25

Wr – reference pipe wells; Wh – home wells.

The potassium content in the surface- and ground- water is presented on Fig. 1. The data show that the potassium concentration is higher in surface water (4.45 – 5.45 mg l^{-1}) than

in groundwater, where it varies in a wide range from 0.56 mg l^{-1} to 10.27 mg l^{-1}. During the 2013 year, the highest concentration of potassium was observed in the groundwater sampled from the deep well N 6 (10.27 mg l^{-1}). Shallow groundwater from home wells has lower K+ concentrations compared with the studied deep wells. In general, potassium is an element, which is characterized with very low mobility coefficient, because of its physico-chemical fixation in the soil.

Sodium is an element with a high geochemical mobility and transitional status in the geochemical cycle of the elements, and due to this reason it is highly influenced by the changes occurring in anthropogenic loading. The concentration of Na^+ in the surface- and ground- water varies from 23.57 mg l^{-1} to 50.82 mg l^{-1} (Fig.1). The dynamics of sodium content in the groundwater is close to that of the potassium (Fig.1).

Calcium concentration (Fig.1) varies from 48.50 mg l^{-1} to 143.50 mg l^{-1} in all wells from the monitored 2013 year and does not exceed the MPCL for drinking water (150 mg l^{-1}).

The magnesium content in the groundwater (Fig.1) shows three times lower concentration in comparison with the absolute calcium values. The concentration of Mg^{2+} in the groundwater varies from 33.00 mg l^{-1} to 54.00 mg l^{-1} (Fig.1). The magnesium content in the surface water (Yantra River) from the three points, which is located at the lower part of the watershed, varies from 11.50 mg l^{-1} to 13.50 mg l^{-1}. Values of Mg^{2+} content, like that of calcium do not exceed the MPCL for this element for drinking water (50 mg l^{-1}). The data show that the magnesium content in surface- and ground- water varies in a narrow range.

Data received for the pilot site show that nitrate nitrogen concentration in the shallow groundwater varies in a wide range between 7.30 – 27.80 mg l^{-1}, depending on the locations of the investigated wells. Only in the several cases (5, 17, 18, 19 and 20), the measured nitrate-nitrogen content (Fig. 2) was higher than the maximum permissible concentration level for drinking water (MPCL = 11.3 mg l^{-1}).

It was established that the household gardens are a subject of high nitrogen loading, creating potential sources for nitrate nitrogen pollution of shallow groundwater. Data for 2013 year (Fig. 2) confirm the tendency of decreasing the nitrate nitrogen concentration, in general, with values around and higher than the MPCL. The lowest nitrate nitrogen concentration (1.97 – 4.90 mg l^{-1}) was measured in the samples taken from the Yantra River.

The PO_4^- concentration in the groundwater varies from 1.50 mg l^{-1} to 16.10 mg l^{-1}. The content of hydrocarbonates in the groundwater (Fig. 3) is characterized with a significant dynamics. The high contents of hydrocarbonates in the groundwater of the pilot watershed determines its hardness, which in the more monitored wells exceeds the standard level for drinking water. The sulfate content in the groundwater varies from 36.00 mg l^{-1} to 173.00 mg l^{-1}.

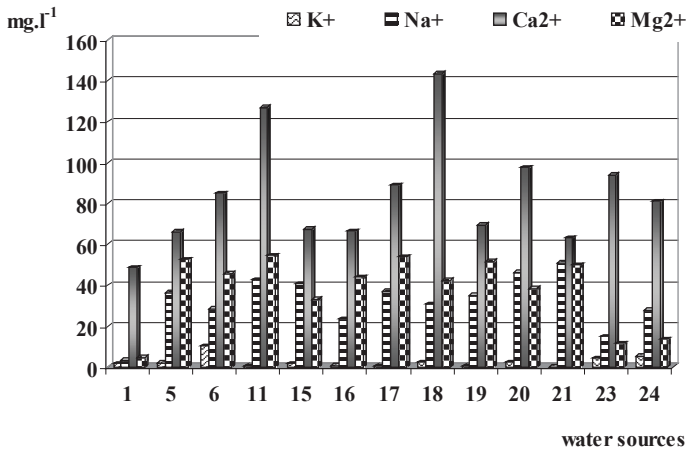

Fig. 1. Potassium, sodium, calcium and magnesium contents in the surface - and ground- water (v. Parvomaitsii, near V. Tarnovo), 2013 year

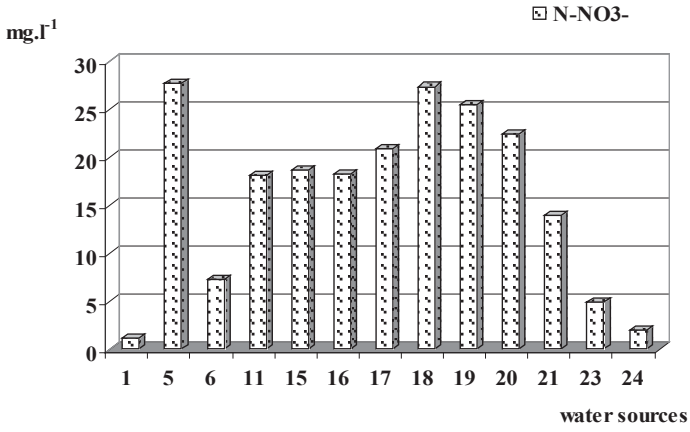

Fig. 2. Nitrate nitrogen content in the surface- and ground- water (v. Parvomaitsi, near V. Tarnovo), 2013 year

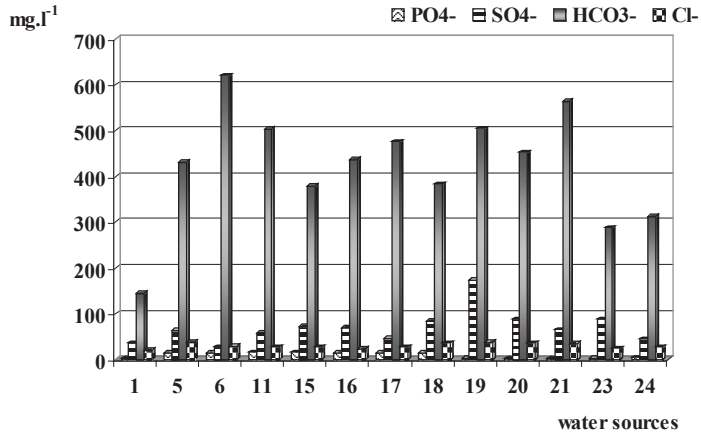

Fig. 3. Phosphate, sulfate, hydrocarbonates and chlorine contents in the surface- and ground- water (v. Parvomaitsi, near V. Tarnovo), 2013 year

Chlorine content (Fig. 3) in the groundwater could be characterised with a considerably low variation in the space and time. The observed data are similar, which is not typical for this element due to its high mobility and status in the geochemical cycle of elements. The chlorine concentrations in the monitored wells do not exceed the MPCL for drinking water (250 mg l^{-1}). Chlorine has a transitional status in the biological cycle of nutrients.

Conclusions

It was established that surface- and ground- water in the studied watershed have neutral to alkaline reaction and a hydro-carbonate-calcium-magnesium chemical composition.

Data show that the nitrate nitrogen content in the shallow groundwater varies in a wide range, depending on the location of the studied wells.The calcium and magnesium concentrations in groundwater do not exceed the maximum permissible concentration for BG's drinking water standard. It was monitored a significant variation in the data for hydro-carbonates in the groundwater, which are slightly influenced by the anthropogenic loading. Chlorine and sulphate contents in the groundwater could be characterised with considerably low variation.

The received information will be used as a methodological approach in further environmental impact assessment on a watershed level.

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ROTIFER COMPLEXES IN THE PLANKTON OF THE SREBARNA LAKE (BULGARIAN DANUBE FLOODPLAIN)

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Abstract

Aim: The aim of this study was to describe the patterns of spatial distribution of the rotifer plankton community in the Srebarna Lake in respect to the seasonal changes of its composition and abundance.

Materials and Methods: Investigations cover the period from 1999 to 2011. Sampling was performed seasonally at 4 to 12 sites covering representative plankton habitats within the lake. Zooplankton samples were collected seasonally from the surface water layer with a depth up to 0.8 m. Some relevant parameters of the water were measured simultaneously. In the laboratory processing after the species identification of organisms their number was determined. The abundance was calculated as the number of individuals per 1 m^3.

Results: The maximal species richness of rotifers occurred in late spring and the minimal – in winter when the maximum abundance occurred. Within the rotifer community were identified relatively compact groups of 2 and more genera and species called "rotifer complex". The spatial distribution and species composition of these complexes changed throughout the seasonal succession of plankton community under the influence of the seasonal dynamics of water depth, temperature and transparency and of other environmental factors.

Conclusion: Rotifer complexes including 2 or more genera and species occur within the zooplankton community of the Srebarna Lake. They change throughout the seasonal succession of plankton community which generally follows the PEG model.

Keywords: Srebarna Lake, zooplankton, Rotifera, seasonal dynamics

Introduction

After the reconnection of the Srebarna Lake with the Danube the flooding regime was identified as the main complex driving factor for the succession of the lake ecosystem. Data of different investigations [1, 2, 3] suggests that the flooding from the Danube influence directly both the species composition and dynamics of the zooplankton community by the transfer of organisms (biological drift) and indirectly – inducing changes of the environmental parameters. However, the different patterns of this influence still insufficiently studied.

The aim of the present study was to follow the seasonal changes in the composition and spatial distribution of the rotifer plankton community in the Srebarna Lake in relation with the environmental factors.

Material and Methods

Investigations cover the period from 1999 to 2011. Sampling was performed monthly or seasonally (emphasizing on the flooding periods) at 4 to 12 sites covering the representative biotops of plankton within the lake (Fig. 1).

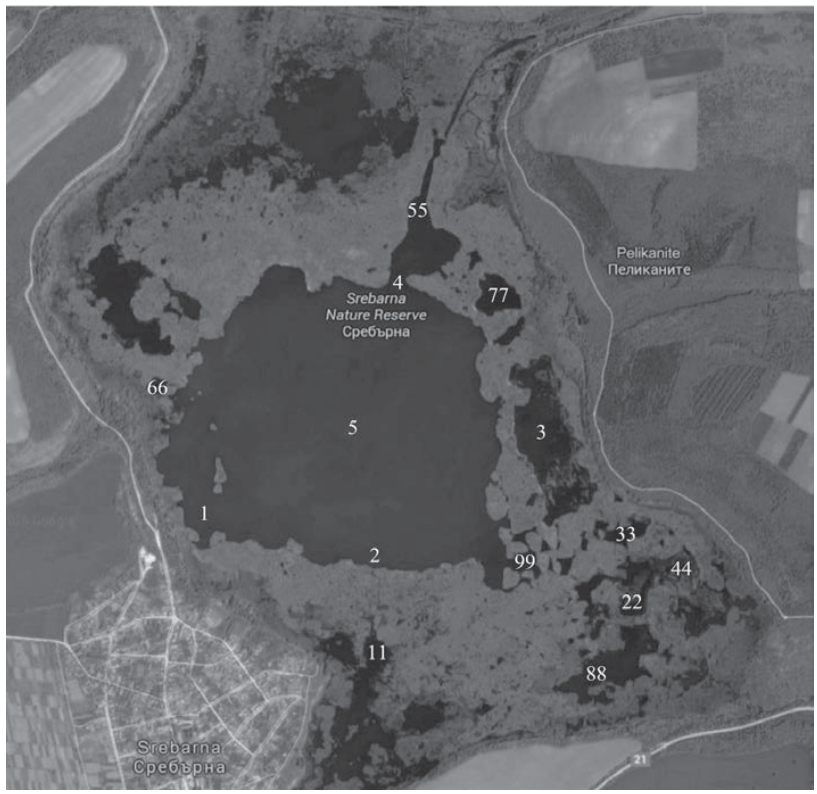

Fig. 1. Sampling points in Serbarna Lake
(source: Google-maps, sampling points: Miro Tsavkov)

Zooplankton samples were collected from the surface water layer with depth to 0.8 m by filtering of 50 dm^3 of water trough plankton net with mesh size of 90 mkm. Samples were fixed and preserved in 4% formaline. Some relevant parameters of the water (transparency, temperature, dissolved O_2, pH, conductivity) were measured simultaneously. Samples for the measurement of the dissolved nitrogen and phosphorus forms were also taken. In the laboratory processing species identification of organisms was done under light microscope after Kutikova [4]. For the species determination of representatives of the genera *Asplanchna, Cephalodella, Conichiloides, Dicranophorus, Encentrum, Notommata, Polyarthra* and *Synchaeta* the structure of their jaws was examined after decomposition of the soft tissues with 30% KOH.

The number of items was determined using binocular lens and Bogorov's counting camera. The abundance was calculated as the number of individuals per 1 m^3.

Species richness and abundances for Rotifera were calculated based on averages of the five sampling points. To be able to extract both seasonal and yearly patterns, the empty spaces in the dataset were filled by interpolation. Analyses were performed in R (2.10.0), time series analyses were performed with the Package for Analysis of Space-Time Ecological Series "pastecs" (version 1.3-11). Correlations between the extracted seasonal and yearly

trends in water depth, rotiferan zooplankton species richness and rotiferan zooplankton total abundance were tested with STATISTICA 7.

Results and Discussion

A total of 163 rotifer taxa (genera, species and subspecies) were described for the whole period of investigations, of which 87 were listed in the Catalogue of the Bulgarien Rotifera fauna [5]. The difference could be explained first with the more extensive studies providing new data and secondly with the identification of more subspecies in a range of species. In the present study many of the recorded rotifers were identified to the subspecies level what was necessary for the analysis of formation of rofifer complexes. Pronounced annual variations of the species composition were recorded whereupon high similarity in the species composition of rotifer community within the periods of low water level and high water level respectively against the significant distinction between the years with high and low waters in the lake [2].

Both Rotifera and Crustacea richness follow very similar trends. The highest number of Rotifera species is observed the May-June, and after that strongly declines during the summer months, with a small temporary peak in late summer (around September), to further decline afterwards and reach the yearly minimum in October to February. The highest richness in Crustacea is also observed in May- June, and after that strongly declines during the summer, with a small temporary peak in summer (around August), further decreases afterwards in a more temperate way to reach the yearly minimum in late winter (January to March) (Fig. 2).

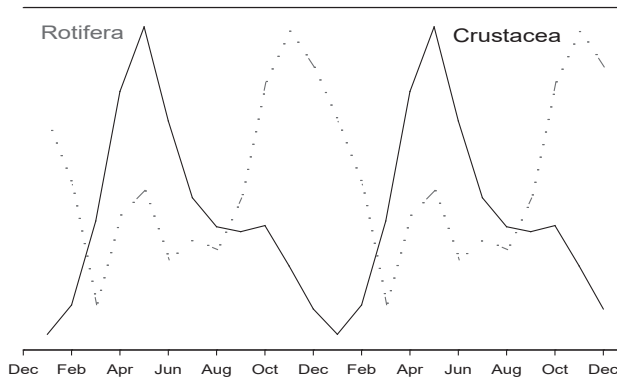

Fig. 2. The extracted seasonal cycle of crustacean zooplankton abundance (black full line) and rotiferan zooplankton abundance (red dotted line)

On the contrary, the maximum of total annual abundance of Rotifera occurred in winter (December-February). At the same time Crustacea densities climb to the highest value during the year in February. Both Rotifera and Crustacea densities strongly decrease in March (the point of the highest water level) and remain relatively low during the rest of the summer. In late summer, in the beginning of fall both densities of Crustacea and Rotifera increase (Fig.3 and 4).

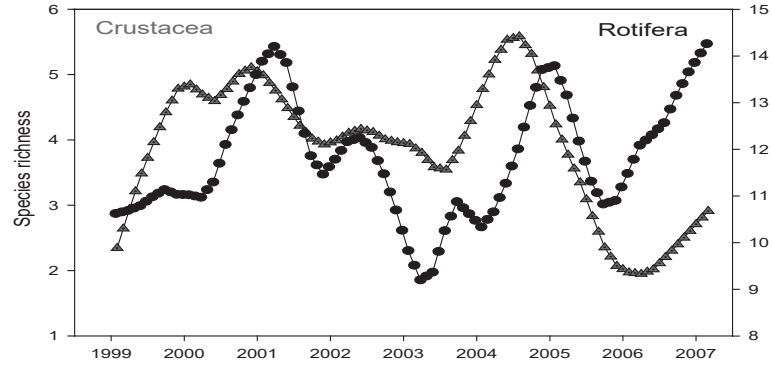

Fig. 3. Extracted trend in Crustacea (triangles) and Rotifera (circles) species richness every month over seven years, after removal of the seasonal component

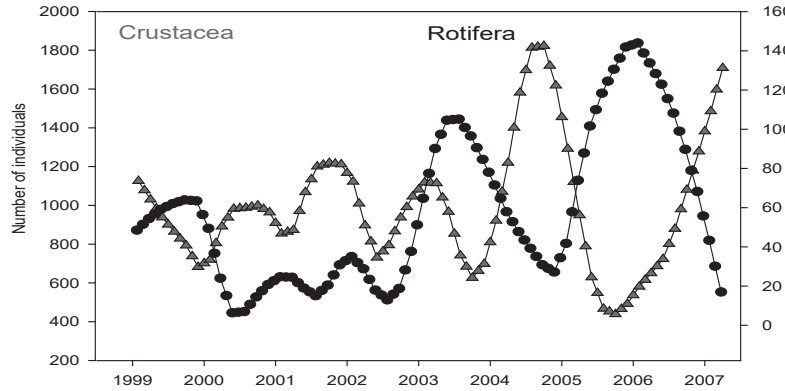

Fig. 4. Extracted trend in Crustacea (triangles) and Rotifera (circles) abundance every month over seven years, after removal of the seasonal component

In a seasonal perspective a positive correlation (r = 0.49, P = 0.001) was found between the species richness of rotifers and water depth as an expression of the flooding regime but a strong negative (r = - 0.57, P = 0.001) correlation between the abundance of rotifers and water depth. The seasonal variation in the crustacean community composition did not correlated with water depth.

In the annual perspective a similar pattern was recorded but with positive (0.47, P = 0.001) instead of negative correlation between the water depth and the total abundance of rotifers.

Within the rotifer community were identified the so called "rotifer complexes" – relatively compact groups of 2 and more rotifer genera presented with 2 or more species/ subspecies (according the classification of Kutikova [4]). This classification expresses not only the structural but also the functional features of the organisms. Thus, the forming and development of rotifer complexes follows the pattern: similar names – similar structure – similar functions – similar ecological niches. Sometimes into the rotifer complexes are

included Asplanchna species which are a regulating and self-regulating component of the balance of aquatic ecosystems, not only of the zooplankton community, performing some controlling functions in sufficiently high abundance [6].

The dynamics of the rotifer complexes could be likened to the movement of the pendulum. Thus, the highest position is recorded during the periods following the floodings when 2-6 genera with a maximal number of species/sub-species occurs (Fig. 5 and 6). The data of the different studies [7] suggest that in this period the most intensive processes occur within the aquatic ecosystem. After that with the time the pendulum goes down, i.e., the number of species and sub-species reduces and at the lowest point there are no rotifer complexes at all.

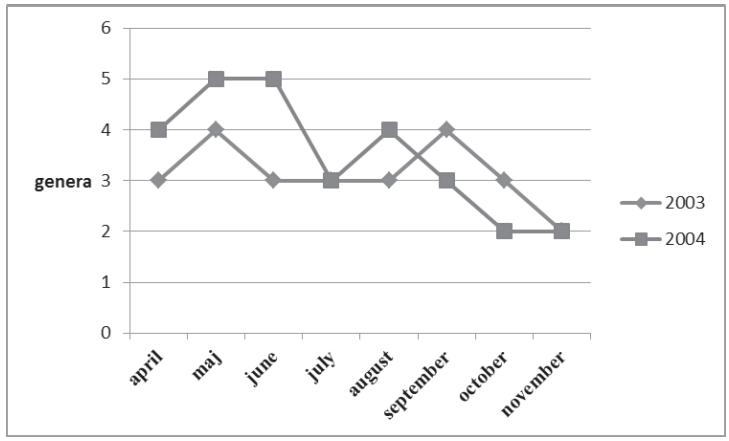

Fig. 5. Seasonal dynamics of the rotifer complexes according number of genera in 2003 and 2004

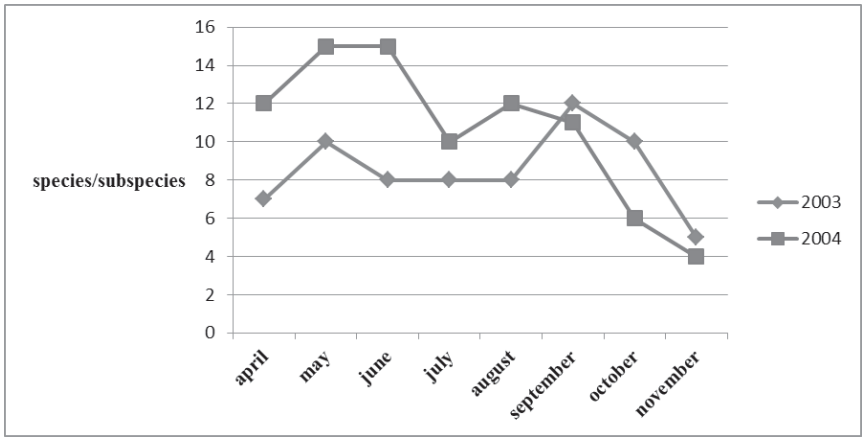

Fig. 6. Seasonal dynamics of the rotifer complexes according species/subspecies in 2003 and 2004

Two hypotheses could be putt forward to explain the occurring and dynamics of the rotifer complexes:

1. Rotifer complexes occur as a result of import of plankton species from the Danube by the flooding waters.

It impresses that in the rotifer complexes in the Danube water during the flooding periods the total number of species and subspecies varies between 7 and 11. With a smaller number of genera the number of species and sub-species is greater. Brachionus sp. predominates in the rotifer complexes in the Danube by the number of species (Table 1) what is an expected result taking in account the high ecological tolerance of this genus.

Table 1. Rotifer complexes in the Danube water flooding the Srebarna lake in summer (August 2004)

Date/site	South sluice – flooding water		
22.04.2004	Rotifer complex 6:2:2		
Genera	*Brachionus* spp.: *Keratella* spp.: *Notolca* spp.		
Species and/ or subspecies	*B. angularis bidens* *B. calyciflorus dorcas* *B. c. spinosus* *B. diversicornis diversicornis* *B. leydigii rotundus* *B. l. tridentatus*	*K. cochlearis hispida* *K. quadrata quadrata*	*N. squamula squamula* *N. aciminata extensa*

The similar situation is retained in time in the lake (Table 2, 3)

Table 2. Spring rotifer complex in the lake (flooding in winter)

Date/Site	Point 1			
11.05.2004	Rotifer complex 4: 3: 2: 2			
Genera	*Keratella* spp.: *Brachionus* spp.: *Asplanchna* spp.: *Filinia* spp.			
Species and/ or subspecies	*K. cochlearis cochlearis* *K. c. hispida* *K. c. tecta* *K. quadrata quadrata*	*B. diversicornis diversicornis* *B. d. homoceros* *B. quadridentatus quadridentatus*	*A. girodi* *A. priodonta*	*F. longiseta longiseta* *F. major*

Table 3. Autumn rotifer complex in the lake.

Date/Site	Point 1		
27.09.2004	Rotifer complex 4: 3: 3		
Genera	*Lecane* spp.: *Brachionus* spp.: *Polyarthra* spp.		
Species and/ or subspecies	*L. (s.str.) ludwigii* *L. (s. str.) luna luna* *L. (Monostyla) bula diabolica* *L. (M.) goniata*	*B. angularis bidens* *B. forficula minor* *B. quadridentatus zernovi*	*P. dolichoptera dolichoptera* *P. euryptera* *P. luminosa*

2. The dynamics of rotifer complexes follows those of the PEG-model [8]

After the reconnection of the lake with the Danube the succession of the lake ecosystem follow the patterns of the Flood Pulse Concept [2] which predetermines a dependence of the processes in the aquatic communities on the flooding regime. Probably the main factors driving the seasonal change in community composition are nutrient availability, water temperature and the increase in predation pressure by the YOY fish [9] as predicted by the PEG model even if this model was developed for dimictic lakes. Since the Srebarna Lake is a polimictic water body one can assume that the main driving factor instead of the seasonal mixing of water column is the flooding of the Danube.

The similarity of seasonal changes in the rotifer complexes to these in the zooplankton as a whole gives a reason to suppose that the drivers could be the same as in the PEG-model.

On the other hand, the comparison shows the same seasonal dynamics of rotifer complexes in years with spring (2003) and summer (2004) flooding (Fig. 5, 6) what suggests that the flooding is not the only factor determining the forming of these complexes.

Conclusion

In the rotifer community of the Srebarna Lake were recorded annual and seasonal changes associated with the fluctuations of the water level in the lake which is a function of the flooding regime of the Danube. The seasonal succession of rotifer community together with the zooplankton as a whole can be explained with the nutrient availability, water temperature and the intensity in predation pressure by the YOY fish in accordance with the patterns of the PEG model.

Rotifer complexes including 2 or more genera with several species and sub-species occur within the zooplankton community of the lake. The main trigger inducing formation of rotifer complexes is considered the flooding of the Danube water through the drift of adults and/or eggs of rotifers in the current. The seasonal changes of rotifer complexes as a response of the dynamics of the environmental parameters also follow the patterns of the PEG model.

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SHORT COMMUNICATIONS

Topic:
ECOSYSTEM RESEARCH, SERVICES AND ECOLOGICAL AGRICULTURE

MONITORING THE NITROGEN LEACHING FROM ARABLE FIELDS AND ITS ECOLOGICAL ASPECTS

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The purpose of this investigation is to study the nitrogen leaching from arable fields and evaluation of the best management practice.

Materials and Methods: The area - village Tsalapitsa is located in South Bulgaria (24°35'E; 42°14'N and 180 m above the sea-level), near the town of Plovdiv, Bulgaria. The region belongs to the transition subzone between the Moderate and Mediterranean continental climate zones. The region is characterized by mean annual precipitation of 814 mm, that exceeds the average rainfall for this region of 500-540 mm, and the mean annual air temperature is 12.2°C. The soil on which the experiment was set up is classified as Alluvial-Meadow (Fluvisol). It is characterised with slight acidity, low total nitrogen and humus contents, and low cation exchange capacity of the plough soil layer. Potato crops were growing during the experimental 2014 year. The potatoes are part of the long-term vegetable experiment for the period of over 30 years. On a background of phosphorous and potassium $P_{100}K_{100}$ (kg ha^{-1}), four N – fertilizer rates have been tested: fallow $T_0(N_0)$; $T_1(N_{60})$; $T_2(N_{100})$; $T_3(N_{140})$ kg ha^{-1}. The rates of nitrogen for optimum treatment is determined according to the N. Poushkarov Institute model for fertilizer recommendation [1]. The field plots were equipped with Ebermeir lysimeters [2] for water collection from soil depths of 50 and 100 cm. The nitrogen content was measured by „Spectroquant Pharo 100".

Results and Discussions: A characteristic feature of Fluvisols is their significant spatial heterogeneity and the great variety in the arrangement of the alluvial materials in the whole profile. These facts result into the variation of the quantities of the lysimeter water during the considered period. Lysimetric water strongly depends on the precipitation, its seasonal distribution, and the intensity of the rainfalls [3]. The highest drainage in the studied soil is observed when great rainfall and irrigation events coincide. Experimental results show that, lysmeter water for the year 2014 ranged from 27 l m^{-2} to 60 l m^{-2}. The nitrate nitrogen contents in the lysimetric water for all treatment variants are from 6.55 mg l^{-1} to 30.75 mg l^{-1}. The results show an increase of nitrate nitrogen in lysimetric water at maximum fertilization rate, and it was about three times over the maximum permissible contaminant level (MPCL – 11.3 mg l^{-1} NO_3^-N) for drinking water [4]. At the optimal fertilization variant T_2, it was found a slightly increase of the NO_3^-N content (20.53 mg l^{-1}) over the MPCL. Nitrogen leaching through the soil profile is strongly affected by fertilization rates. The analyses of data for nitrate nitrogen leaching during the potatoes growth shows that the exported quantity of fallow (control) in all cases is lower (0.0 and 0.5 kg ha^{-1} for depths of 0-50 cm and 0-100 cm, respectively) than in the fertilized variants. The obtained results show an increase in losses of nitrogen from fertilized variants, especially that of the highest fertilization rates T_3 (N_{140}), whose absolute value reaches 12.8 kg ha^{-1} for 0-50 cm soil layer, and 10.11 kg ha^{-1} for 0-100 cm soil layer. The results show that 11.8 kg ha^{-1} nitrogen is drained up from the 0-100 cm soil layer of the var. T_2 (N_{100}).

Conclusions: The long-term field data shows that the application of N rate 50% higher than the optimum was a precondition for the formation of lysimetric water with NO_3^-N content 2-3 times higher than the MPCL for drinking water. Formation of a considerable amount of residual mineral nitrogen and increase of nitrate nitrogen losses throughout the soil profile is possible in the case of treatment of Alluvial Meadow soil (Fluvisol) with maximum N fertilizer rate. The results from this study show that the use of optimum fertilizer rate in vegetable growing is the main factor for sustainable production without risk of agroecosystem pollution.

Keywords: fertilization, potato crop, leaching of nitrates, lysimetric water, agricultural practices

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REPORTS

Topic:
LANDSCAPE ECOLOGY

LANDSCAPE ECOLOGY AND BIODIVERSITY

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Abstract

Aim: The main objective of the paper is to reveal the relationship and interaction between hierarchical systems of biodiversity occurring in geographic space.

Materials and Methods: The international conventions and biodiversity directives, and their application in Bulgaria are analyzed. The hierarchical systems of the complex scientific category biodiversity in its genealogical and functional nature as genetic, species, syntaxonomic, ecological and landscape diversity

are discussed. The existing classification systems for landscapes differentiation in Bulgaria are compared and analyzed.

Results: It is assumed that the inventory of habitat diversity in our country is crucial for landscapes differentiation. The existing possibility of overlapping of boundaries between a landscape type or an elementary landscape, and a habitat type should be seen as the exception, not the rule. It was found that the problem of creating a unified European system for the classification of landscapes meeting the priorities of the European Landscape Convention is not yet solved and the conclusion is made that the development of a universal classification system of landscape looks increasingly hardly realizable.

Conclusion: The existing diversity of classification systems for differentiation of landscapes rather repels than attracts research interest in the field of landscapes and in that context the use of the European Classification System LANMAP2 has positive significance as a methodology for landscape studies in Bulgaria.

Keywords: Habitat type, Landscape differentiation, Directive 92/43/EEC, Landscape Convention

Introduction

The emergence of the term landscape dates back to the Middle Ages as a pre-scientific category and only in the early nineteenth century through studies of Humboldt [1] it came to be perceived as a scientific concept. The term habitat has been introduced in science almost 200 years ago by Augustin Pyramus de Candolle [2], who certainly did not presume what will be its relevance to the modern world. Approximately parallel incurred, both terms appear in the bosom of phytogeography but take a different path of development. Approximation of geographical and environmental research approaches led to the formulation of the concept of landscape ecology by Carl Troll [3], on the basis of which the new scientific direction develops. The division of Europe and the world in the 60s of the last century into two radically different geopolitical spheres of influence reflects on the development of science. In western democratic societies, research to a certain extent continue its natural course of development aimed at liquidating the consequences of the Second World War. In the eastern totalitarian sphere of influence, dominated by the Soviet Union, scientific studies are necessarily subordinated to competition with the West and strong differentiation of scientific fields in key scientific areas. In the west, Carl Troll [4] introduces as a synonym of landscape ecology the concept of geoecology and the research of plant communities is dominated by the phytosociological direction. In the Soviet Union and the Warsaw Pact landscape ecology is called landscape science, but the term geoecology is also perceived, and they developed through improved field research tools and the emergence of various classification systems for differentiation of landscapes. In terms of plant communities in Western Europe all syntaxons are inventoried and there is a search for a new approach in systematizing the diversity of communities that could more effectively respond to the needs of nature conservation posed by rapid economic development. Phytosociology in Eastern Europe has developed on the basis of the dominant approach and only in the last 20 years the determined effort for syntaxons inventory are noticed, for example in Bulgaria and even within the Russian Federation.

Regulative and theoretical background

International Conventions and Directives

The Convention on Biological Diversity was adopted in 1992 during the World Summit in Rio de Janeiro and has been ratified by Bulgaria in 1996. The most important tools for implementing the Convention in the EU are the Habitats Directive 43/92EEC [5] and the Birds Directive 2009/147EEC [6], as well as the created NATURA 2000 ecological network based on the two directives. Leading goal of the EU strategy on biodiversity is halting the loss of biodiversity and the degradation of ecosystem services in the EU by 2020 and, accordingly, their recovery as far as feasible, while stepping up the EU contribution to averting global biodiversity loss. Bulgaria as an EU member state strictly implements the strategy on biodiversity by 2020 and adopts the vision of EU biodiversity by 2050 according to which the EU biodiversity and the ecosystem services it provides – its natural capital – should be protected, valued and appropriately restored for biodiversity's intrinsic value and for their essential contribution to human well-being and economic prosperity, and so that catastrophic changes caused by the loss of biodiversity are avoided.

Through the European Landscape Convention, adopted in 2000 by the Council of Europe and ratified by Bulgaria in 2004, enforced on 01.03.2005, landscape ecology received recognition and opportunity for development. The lack of a uniform classification system in Bulgaria is considered in the European Landscape Convention and some authors [7] determine that the focus of the convention is the obligation of each state to identify the landscapes within its territory. The procedures for the identification and evaluation of landscapes will follow common standards, experience and methodology that will be agreed between the states at European level aiming at determining specific properties of natural complexes at the final stage [7]. European Landscape Convention is thoroughly analyzed as a prospect for the conservation of landscapes in Bulgaria by Konteva [8], who states in detail the problems associated with our country. The same author notes that the obligations of Bulgaria, adopted at the Third Ministerial Conference "Environment for Europe" held in October 1995 in Sofia, are not fulfilled, particularly the section on conservation of landscape diversity. This conference adopted a Pan-European strategy for biological and landscape diversity (PEBLDS) [9], whose 20-year period has expired, and the results show that in terms of landscape diversity as part of biodiversity very little has been done.

Hierarchical Systems of Biodiversity

All existing documents on biodiversity at the global, European and national level point out that the first cause of its loss is the loss of habitats. Whether habitats are natural habitats or habitats of the relevant species, or certain taxonomic rank of landscape, forming part of the geographical space, is a matter of research approach. The conventional schemes of the hierarchical structure of biodiversity assume that it occurs and is respectively studied at four levels - genetic diversity, species diversity, ecosystem diversity and landscape diversity:

a) Genetic diversity includes the variety of genetic information contained in all organisms. Genetic diversity exists within populations, between these populations and between species;

b) Species diversity includes the diversity of species;

c) Ecosystem diversity includes differences between the types of ecosystems and the diversity of habitats and ecological processes in any type of ecosystem;

d) Landscape diversity encompasses all geoecological aspects of the landscape and the functional nature of biodiversity, including various populations and communities of organisms, functionally tied to both the living and the abiotic nature components.

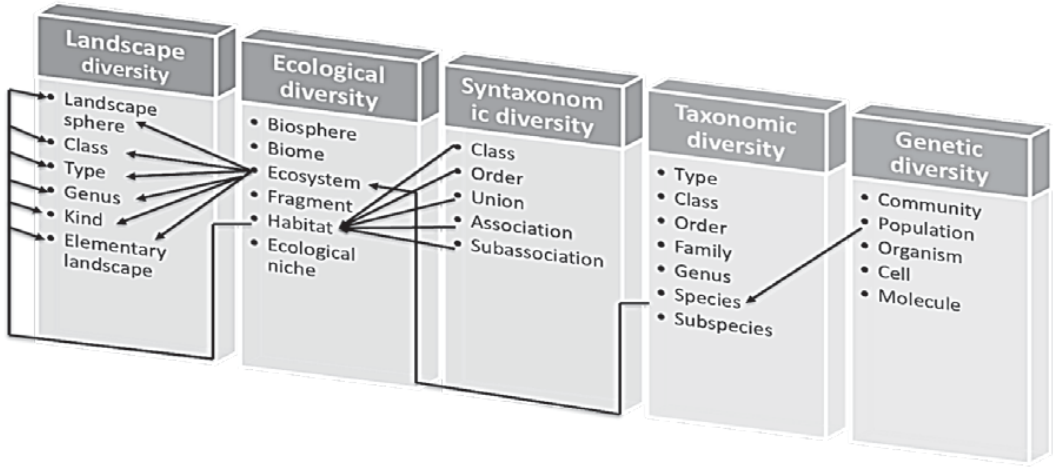

Fig. 1. Hierarchical Systems of Biodiversity [10]

The functional nature of biodiversity is expressed by the ecological and the landscape diversity. Their positioning in the spatial perception of biodiversity is one after another, as the most comprehensive and complex categories, incorporating the other systems of biodiversity. The diversity of communities (syntaxonomic diversity) as a hierarchical system is located in the center (Fig. 1), between the genetic and taxonomic diversity on the one hand, and on the other - the environmental and landscape diversity. Syntaxons as plant communities are carriers of genetic diversity, while being close to the floristic taxonomic diversity. Syntaxons participate in the construction of ecological and landscape diversity with active influence on the functional nature of biodiversity. Comparing and relationships between the hierarchical systems of biodiversity show a certain intermediate position of plant communities as carriers simultaneously of genealogic and functional biodiversity. In the ecosystems hierarchical structure of biodiversity habitat diversity has a key location (Fig. 1) as a carrier of relevant genetic diversity and preservation of natural functions of habitats.

The habitat types with their relatively discrete parameters and boundaries compared to the continual parameters and boundaries of ecosystems turn out the most crucial concept in biodiversity. Habitat diversity is directly related to syntaxonomic diversity and directly derived from it, and in turn some of the habitat types are comparable or similar to certain categories of landscapes. The connection of habitats with landscapes is shown

(Fig. 1) with arrows which illustrate that each habitat is associated with a certain category of landscape, and in certain situations at the level of kinds of landscape or elementary landscape they may coincide. Hierarchical categories of syntaxonomic diversity, specified in the geographic space, are involved both in determining the habitats, but can participate independently in determining the landscapes at a certain level. Syntaxonomic diversity is situated between the systems of ecological and taxonomic diversity as a core constituting and bonding system in the functional structure of biodiversity.

The contemporary significance of the scientific category biodiversity is associated with optimizing the EU set of sustainable development indicators regulated in the information system of the EU (Eurostat, 2016), where one of the ten themes is natural resources, and the first sub-theme is biodiversity. Preserving biodiversity in each country has particular value as part of the national wealth and as quality and quantity of ecosystem/landscape goods and services provided to the respective country, the EU and the rest of the world.

Classifications of Landscapes

In the differentiation of landscape sphere (Table 1), "regarded as biogenic focus of geographic space" [11], particularly important are the applied classification systems, distinguished by their hierarchy. The lack of a uniform classification system for differentiation of landscapes is the basis for discussions and intractable problem in the comparison of taxa from various classification systems. In Bulgaria there are several landscape classification systems, and in the presented four systems (Table 1) the taxonomic levels are almost identical but have different features. For the third basic taxonomic level, named group of landscapes or genus of landscapes, in the first three variants of classifications the diagnostic features are the mezzo-forms of relief, relief-forming processes and to some extent soil subtypes and varieties, as well as phytocoenoses. In the fourth landscape classification [12], the third basic taxonomic level is distinguished only by the potential (indigenous) vegetation. The fifth basic taxonomic level in all four variants is established as a taxonomic category kind of landscape with diagnostic criteria for a maximum degree of uniformity of natural conditions, homogeneous conditions of relief-forming and morphological features, while Popov [12] uses as a diagnostic criterion also the nature of land use. At the kind of landscape level it is likely, not as the norm, but rather as an exception, the kind of landscape to coincide with a certain category of habitat type. Even more likely this possibility may occur in the case of micro-landscape or any of the synonyms of this last taxonomic level as elementary landscape, landscape section, locality, facies or subkind of landscape.

Table 1. Comparison of classification schemes applied to analyze the landscape structure of Bulgaria (adapted after Borissova [11])

Petrov P. [22] Landscape map at 1:400 000	Velchev A. et al. [23] Landscape map at 1:500000	Petrov P. [24] Landscape structure	Popov A. [12]
Diagnostic criteria for the taxonomic levels of landscapes			
Class Macro geomorphological features	**Class** Relief and geological base	**Class** Mountain, plane, valley and inter-mountain flat landscapes	**Class** Morphographic types of relief **Subclass** – rock base
Type Typical zonal indicators - radiation index of dryness, biological cycling of air migrants, typomorphic element of water migration, type of soil and vegetation	**Type** Uniform hydro-climatic conditions, community migration of chemical elements	**Type** Zonal hydro-climatic parameters (soil type, vegetation type)	**Type** Climate (humidity index of Thornthwaite)
Subtype Indicators for altitude and latitude zoning, subtypes of soils and vegetation	**Subtype** Secondary zonal and bioclimatic features	**Subtype** Zonal hydro-climatic indicators (subtype of soil, subtype of vegetation)	**Subtype** Waters
Group Mezzo-relief, nature of rock substrate and modern deposits, soil types and phytocoenoses, extent of ploughing and deforestation	**Group** Type of relief (erosive, erosive-denudating, accumulative), subtype of soil and plant formations	**Group (Genus)** Mezzo-morpho-lithogenic indicators, modern sediments, soil varieties, plant associations, extent of ploughing and deforestation	**Group (Genus)** Potential (indigenous) vegetation Subgenus (Subgroup) Soils
Kind The greatest degree of homogeneity of environmental conditions, uniformity of the planned structure	**Kind** Homogeneous conditions of relief, rock and chemical base, soil and vegetation cover, microclimate	**Kind** Features of morphological structure	**Kind** Nature of land use
Micro-landscape **Elementary Landscape**	**Landscape section** **Locality** **Facies**		**Subkind** Degree of anthropogenization

Repeatedly the declared lack of commonly accepted classification system of landscapes in Bulgaria [7, 8, 13, 11, 14, 15] and the commitment of our country to the European Landscape Convention determine the demand for a convertible version of the classification of landscapes based on the European landscape ecological research - The European Landscape Character Initiative (ECLAI) [15, 17]. The classification system LANMAP 2, suggested by those authors, is implemented for the first time in Bulgaria by Borissova and Kotsev [18] in the Strandzha Nature Park. The existing criticism of the Pan-European classification system for landscapes differentiation LANMAP 2 for being difficult to apply to large-scale studies has led researchers [14, 15, 19] to implement the genetic approach (according to origin) for systematization and classification. This allows to take into account all possible factors of landscape differentiation (natural and anthropogenic) under the direct influence of the existing zonal and azonal natural and geographic regularities. The principle of "single spatial dimension" of the classification categories is strictly and

correctly used, well justified in the proposed by Popov [12] geoecological classification of Bulgaria.

Landscape units are defined by diagnostic criteria and specific level of generalization of indicators. The spatial generalization of data applied in their studies corresponds to the level of microstructure of landscapes (according to Popov [12]) or to the chorological level of analysis (according to Makhdoum [20]). The authors adhere to the principle of the geoecological informativeness of landscape classification by sticking to the criteria basis for differentiation of the hemeroby index as an indicator of anthropogenic transformation of landscape systems. New methodological decision in this adapted version of classification is the introduction of more precise information on modern vegetation through information about habitat types and their boundaries [21].

Similar adapted changes occur with other contemporary landscape classifications applied for the purposes of practical research. For example, in developing a management plan of Bulgarka Nature Park, Popov and Nedkov [25] apply the landscape typological classification using the taxons of the classification of Popov [12]. The development of the classification system is linked to compliance with the following principles: a) principle of complexity - the landscape appears as a definite interrelated combination of basic natural components; b) principle of relative uniformity - every landscape may hold different sized spaces that are not necessarily uniform in scope, i.e. the areas of a type or kind of landscape are most often disintegrated; c) principle of logical correctness - compliance with the basic logical principles for building a classification system; d) principle of a single spatial dimension of the classification categories; e) general feasibility - the classification has to be easily applicable for various practical purposes (visual-informational, conservation, administrative, etc.).

The accepted landscapes classification scheme [25] consists of two parts. The first concerns the natural landscapes, formed under the influence of natural processes and is the primary as regards the creation of landscape map, and the second reflects the impact of human activity on them. The second part of the landscapes classification system, called classification by degree of anthropogenic transformation, consists of two levels. The first sublevel takes into account the extent of the transformation of landscapes and the second sublevel implies dividing of landscapes according to the nature of anthropogenic transformation. In the second part of the classification system the authors emphasize the principle of practical feasibility in compliance with the included tasks in the European Landscape Convention, according to which each state undertakes to “identify landscapes on its territory, to analyze their characteristics, the forces and pressures transforming them, and to accept tools aimed at protecting, managing and planning the landscape”.

Discussion

The contemporary development of landscape ecology is driven by the increasing anthropogenization of landscapes worldwide. Pressure on natural landscapes causes an increased scientific interest in the practical aspects of landscape ecology and particularly to landscape planning. The development of landscape ecology is in direct connection with

all hierarchical systems of biodiversity. The most important cause of biodiversity loss is associated with the alteration or destruction of habitats of species and natural habitats, and the regulation of this process is related to the regional development and implementation of landscape planning as applied aspect of landscape ecology. The globalization of the modern world requires the application of an interdisciplinary approach to research, and the relationship of landscape ecology and biodiversity is a classic example of synergy between classical ecology and geoecology. Landscape planning as applied aspect of landscape ecology is included more often in an integrated approach to regional development and environmental protection. The successful and sustainable planning of a defined territory largely depends on the performance of landscape planning, which is always associated with the execution of landscape differentiation and the visualization of certain space on a landscape map. The landscape classifications used in Bulgaria which are analyzed in the paper illustrate the Bulgarian experience, while the successful implementation of environmental and regional policies in the EU is associated with the use of a unified system for landscape classification, in compliance with the recommendations of the European Landscape Convention. Regardless of the debatable character of the classification system Lanmap 2, defined as applicable only to large areas, the examples provided in the paper demonstrate that an adapted version of this classification proposed in the classification of Popov [12] is successfully applied to various municipalities in Bulgaria.

Conclusion

The examined relationships between landscape ecology and biodiversity illustrate the interdisciplinary nature of landscape ecology or at least the author's understanding coincides with this view. The landscape ecology development in Bulgaria needs to follow the European traditions in the development of this scientific trend, which are focused on the applied character of research using standardized scale for landscape differentiation. The analysis of the hierarchical systems of the biosphere and the landscape sphere, and conventions governing their implementation raises the need to formulate some conclusions of applied character:

1. The existing diversity of classification systems for differentiation of landscapes in Bulgaria is largely due to the influence of Russian landscape science dominating research in the country in the second half of last century;
2. The problem of creating a unified European system for the classification of landscapes that meet the priorities set in the European Landscape Convention is not yet solved;
3. The existing diversity of classification systems for differentiation of landscapes repels rather than attracts research interest in direction of landscape and the development of a universal landscape classification system looks increasingly difficult to realize;
4. The use of the European classification system LANMAP2 has positive significance as a methodology for landscape studies in Bulgaria;
5. Landscape diversity is undoubtedly part of the complex category of biodiversity and is a prerequisite for the realization of sustainable development as a concept, paradigm and/or management model.

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LANDSCAPE ASSESSMENT AND ANALYSIS OF MALA PLANINA BASED ON THE HEMEROBY INDEX

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Abstract

Aim: The main aim of the study is to analyze the importance of the hemeroby index in landscape assessment and monitoring of Mala Planina.

Materials and Methods: Investigating the significance of the hemeroby index as an indicator in landscape assessment and monitoring of mountain areas is crucial. Hemeroby levels are interpreted as degrees of anthropogenic transformation and are defined in accordance with the hemeroby criteria basis. The investigation

has theoretical and practical importance. Cameral and preparatory terrain research of the habitat types is done. The study is based on a landscape map and map of the habitats in Mala Planina.

Results: All possible factors for the landscape differentiation are considered. The presented map of the hemeroby points out the contrasts in Mala Planina.

Conclusion: The anthropogenic influence in Mala Planina is indisputable and it is proved by revealing the hemeroby index of the territory. The results can be used for the creation of a plan for the development of this mountainous area.

Keywords: hemeroby index, landscapes, habitats, NATURA 2000, Mala Planina

Introduction

Environmental indices are crucial to provide information about the state of nature. Currently we live in an anthropogenic environment, so measuring the level of hemeroby is a problem of major significance and that is why the hemeroby index was developed. The term hemeroby, which literary means "tamed life", was developed and was introduced in the 1950s by Jalas [1]. The studies of Brentrup et al. [2], Hill et al. [3] and Kiedrzyński et al. [4] are a proof that a lot of work, concerning hemeroby and the hemeroby index has been done in the European mainland in the recent years. Walz and Stein [5] outline a method to establish the indicators of hemeroby for the whole of Germany and present a hemeroby index that considers all hemeroby classes of a reference area as an example of the vital importance of the use of the hemeroby index today. The statistical office of the European Union (Eurostat) asserts that the hemeroby index measures the hemerobic state of an area. It is stated that the hemeroby index shows the magnitude of the deviation from the potential natural vegetation caused by human activities. It is affirmed that the degree of hemeroby increases with the increase of the human influence [6].

The identification of the hemeroby level of the landscapes and habitats in Mala Planina is a matter of present interest. The mountainous area is a part of the biogeographic region of the Balkans [7] and is in immediate proximity to the capital of Bulgaria, so there is no place for argument that the landscapes and habitats are under pressure. Luckily, the use of the hemeroby index is applicable to mountainous areas in Bulgaria. Borissova et al. [8] has already skillfully proofed it. The current research is based on it accordingly.

Materials and Methods

The anthropogenic activity is undoubtedly a major factor for the formation of the landscapes in Mala Planina and vegetation is one of the landscape components that is much altered. The mountain is a part of the Western Balkans and is situated to the west of the Iskar river gorge, to the north of Sofia valley and to the south of the mountains Ponor and Chepan.

Taking the aims of the system analysis into account, the hemeroby index is integrated in this research. Paracchini and Capitani, [9] adopt a scale, assessing the gradients of human influence (Table 1). The lowest values correspond with non-disturbed landscapes and habitats, while the highest ones belong to totally altered landscapes. The hemeroby index values in Mala Planina are presented on Table 2 and Table 3.

Table 1. Reference scale for hemeroby values

Source: Joint Research Centre, European Commission
(http://ec.europa.eu/eurostat/statistics-/index.php/File:Reference_scale_for_hemeroby_values.png)

Hemeroby value	Hemeroby level	Degree of naturalness	Example	Processes/Human impact
1	Ahemerobe	Natural	Bogs, tundra	No disturbance
2	Oligohemerobe	Close to natural	Forest with species typical for the site, semi-natural grasslands	Limited removal of wood, pastoralism, minor changes in matter circles, emissions through air and water
3	Mesohemerobe	Semi-natural	Forest with species atypical for the site, extensive grasslands	Clearing and occasional ploughing, extensive grazing, rare and small doses of fertiliser
4a	β-euhemerobe	Relatively far from natural	Annual crops associated with permanent crops (extensive), agro-forestry	Use of fertilisers and biocides melioration, ditch drainage
4b			Intensive grassland, extensive arable land,	
5a	α-euhemerobe	Far from natural	Intensive arable land (short rotations), intensive vineyards	Deep plowing, planting, major changes in matter circle, drainage, heavy use of fertilizers and biocides
5b			Cereal monocultures, rice fields and irrigated crops (intensive)	
6	Polyhemerobe	Strange to natural	City green, golf courses, pits	Strong changes in biocenosis, covering of the biotope with external material
7	Metahemerobe	Artificial	Streets, buildings	Sealed surface, biocenosis destroyed

Table 2. Diagnostic criteria for the landscape differentiation in Mala Planina

Initial data	Generalized classes	Symbol
Level 1 (Class): Diagnostic criterion – relief morphology		
Topographic map	Denudation level with karst	K
	Denudation level without karst	WK
	Slope	S
Level 2 (Subclass): Diagnostic criterion – geological foundation		
Clayey, sandy, organogenic, dolomite limestones; dolomites; marlstones	Carbonate sedimentary rocks	cr
Sandstones, argillites, conglomerates, breccias	Noncarbonate sedimentary rocks	ncr
Dacite, quartz-diorite porphyry, syenite, shonkinite, tinguaite-porphyry, quartzite, andesites, trachyandesites	Magmatic and metamorphic rocks	mg
Clays, sands and gravel	Unconsolidated sediments	u
Level 3 (Type) Diagnostic criterion – climate		
Thornthwaite moisture index (Im):		
20-40	Humid climate	H
0-20	Wet sub-humid climate	SH

Level 4 (Genus): Diagnostic criterion – vegetation		
Maps, provided by Sofia and Svoge Forestry	*Robinia pseudoacacia*	rp
	Quercus frainetto	qf
	Betula pendula	bp
	Fagus sylvatica	fs
	Pinus sylvestris	ps
	Carpinus betulus	cb
	Pseudotsuga menziesii	pm
	Quercus dalechampii	qd
	Carpinus orientalis	co
	Quercus pubescens	qp
	Populus sp.	p
	Quercus cerris	qc
	Quercus rubra	qr
	Pinus nigra	pn
	Pastures and meadows	m
Level 5 (Subgenus): Diagnostic criterion – soils		
	Eutric Fluvisols	1
	Dystric and Gleyic Colluvisols	2
	Chromic Luvisols	3
	Eutric Vertisols	4
	Eutric Cambisols	5
	Rendzic Leptosols	6
	Albic Luvisols	7
Level 6 (Kind): Diagnostic criterion – anthropogenic level (hemeroby)		
Beech forests	Ahemerobe/Oligohemerobe	AO
Mixed oak-hornbeam forests	Ahemerobe/Oligohemerobe	AO
Mixed deciduous forests	Oligohemerobe	O
Oak forests	Oligohemerobe	O
Shrubs and grassland vegetation	Oligohemerobe	O
Grassland communities	Oligohemerobe	O
Riverside forests	Oligohemerobe	O
Conifer forests	Mesohemerobe	M
Agricultural territories	α-euhemerobe	AU|
Vegetation comprised of introduced and invasive species	beta-euhemerobe	BU
Urbanized areas	Polyhemerobe/Metahemerobe	PM
Quarries	Metahemerobe	MT

Table 3. Vegetation as a diagnostic criterion for landscape differentiation in Mala Planina

Vegetation	Habitats
Shrubs and grassland vegetation	**40A0** *Sub-continental peri-pannonic scrub
	5130 *Juniperus communis* formations on heaths or calcareous grasslands
Beech forests	**9110** *Luzulo-Fagetum* beech forests
	9130 *Asperulo-Fagetum* beech forests
	9150 Medio-European limestone beech forests of the *Cephalanthero-Fagion*
Conifer forests	**91CA** Rhodopide and Balkan Range Scots pine forests
	9530 *Sub-Mediterranean pine forests with endemic black pines
Mixed oak-hornbeam forests	**9170** *Galio-Carpinetum* oak-hornbeam forests
Oak forests	**91H0** *Pannonian woods with *Quercus pubescens*
	91M0 Pannonian-Balkanic turkey oak-sessile oak forests
Grassland communities	**6510** Lowland hay meadows
	6410 *Molinia* meadows on calcareous, peaty or clayey-silt-laden soils *(molinion caeruleae)*
	6110 Rupicolous calcareous or basophilic grasslands of the *Alysso-Sedion albi*
	6210 Semi-natural dry grasslands and scrubland facies: on calcareous substrates (*Festuco-Brometalia*) (*important orchid rich sites)
	6240 *Sub-Pannonic steppic grasslands
Riverside forests	**91E0** *Alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior* (*Alno-padion, Alnion incanae, Salicion albae*)
Mixed deciduous forests	**9180** **Tilio-Acerion* forests of slopes, screes and ravines
Vegetation comprised of introduced and invasive species (*Quercus rubra, Robinia pseudoacacia*)	
Cultural vegetation	

A landscape map of Mala Planina has been developed as a continuation of a landscape research in the northeastern part of the mountain by Grigorov et al. [10]. The work follows the landscape character mapping and typologization by Borissova and Kotsev [11]. The applied methods consist of spatial analysis and mapping by the use of the software product ESRI ArcGIS 9.3. The process includes preliminary activities of data processing and vector layers development for every one of the diagnostic criteria of the classification system. The creation of landscape map by integrating the different layers is the culmination point.

Several vector layers have been created: a vector layer of the morphological relief types, based on ASTER GDEM morphometric parameters; a layer of georeferenced geological maps and vectorized lithological units according to the developed scheme for generalization; a vector layer of the climate types by vectorization of the map with the Thornthwaite moisture index; a vector layer of the vegetation based on the maps provided by Sofia Forestry and Svoge Forestry; a vector layer of the soils by generalization according to the developed scheme. The minimal area of the polygons is 5 ha.

The standard procedures for spatial analysis in GIS environment lead us to the creation of the landscape map, which is achieved by combining the vector layers. The integration of the diagnostic criteria is done in several steps. At first, the relief morphology layer and the layer with the geological information were combined and spatially generalized. Then the climatic vector layer was added and a generalization was done. The layers with the soil and vegetation components were combined with the rest of the layers and then the final generalization was done.

Results and Discussion

A hemeroby map, presented in Fig.1, unveils the specific hemeroby level of the areas in Mala Planina. Undoubtedly, most of the surface (67%) falls within the zone of the oligohemerobe level. Forest and grassland vegetation is typical for these sites as it is deeply connected with the altitude and climatic conditions. Although there is human impact, due to the accessibility of the mountain, we can affirm that this does not lead to the significant alteration of the vegetation cover.

The ahemerobe landscapes (5.5% of the territory) are situated in the northern part of Mala Planina. They consist of beech and mixed oak-hornbeam forests (some of which are among the oldest in the mountain) that are more difficult to get access to and thus the anthropogenic impact is reduced. These areas cover territories around the highest sections of the mountain, including Tseria Peak (1234 m), and are located comparatively away from villages, country houses and main road arteries. This statement can be enhanced by the fact the zones near a main road, cutting through the mountain and connecting Svidnya and Chibaovtsi, have different hemeroby level. The combination of these conditions along with factors that determine landscape differentiation such as soil type, lead to the high level of naturalness of this part of Mala Planina. However, we must stress on the fact that not all forests here belong to the ahemerobe level. There are zones dominated by *Fagus sylvatica, Carpinus betulus* and *Quercus* sp. that pertain to the oligohemerobe level.

The semi-natural (mesohemerobe) landscapes cover 9.6% of Mala Planina and are located near the villages. They consist of coniferous species (*Pinus sylvestris, Pinus nigra, Pseudotzuga menziessii*), atypical for the territory and thus are included in this level. Mala Planina is not one of the highest mountains in the Balkan range and the specific climatic conditions here prevent the natural formation of coniferous forests. Nevertheless, forest managers considered growing them and this plantations are a direct consequence of the anthropogenic influence, aiming to reduce erosion in the last decades.

The areas with vegetation comprised of introduced and invasive species (Q*uercus rubra* and *Robinia pseudoacacia*) fall within the beta-euhemerobe group. There are other types of invasive species (*Ailanthus altissima, Acer negundo, Amorpha fruticosa, Bidens frondosus* etc.), but the extent of the territories they cover can be neglected as they do not fall within the chosen minimal polygon area for this research. Beta-euhemerobe territories cover under 1% and are located in the eastern part of the mountain, mainly close to Novi Iskar and in the northern part near Iskrets and Svydnia.

The agricultural territories in Mala Planina (12% of the territory) belong to the alfa-euhemerobe level. The information about their exact location is derived from terrain observation, Landsat8 [12] of 2014 and CORINE Land Cover 2006 and 2012 [13]. The two CORINE databases were compared and it was unveiled that there is no difference between them when it comes to agricultural lands, so it can be concluded that there is no agronomic expansion.

There are agricultural areas in the northwestern and southern part of the mountain. These territories are situated near the villages and the cultivated crops are mainly for local consumption rather than for major trade supplies, nonetheless we undoubtedly observe deep plowing, planting and the use of fertilizers in some places. The agricultural areas in the eastern and the central part are mixed with urbanized areas or are among territories with other types of land use, therefore it is difficult to differentiate them in the CORINE Land Cover's 2.4.2 complex cultivation patterns.

We experienced a certain difficulty when it came to deciding which areas should fall into the polyhemerobe category. Mala Planina lacks of golf courses or vast green areas in the villages because they are quite small. Even so we concluded that the territories that can be included in the polyhemerobe level are a part of the surrounding areas of the villages. There is transition from metahemerobe to polyhemerobe areas from the center of the villages to their periphery because that is where the human influence is fading. The polyhemerobe landscapes cover under 1%.

The metahemerobe areas cover 4% of the landscapes in Mala Planina. Among the vastest metahemerobe landscapes are those situated near the two longest rivers in the research area – Iskar and Iskretska. The most populated areas, such as the town of Svoge, can be found there. An example, of which the land owners shouldn't be very proud of, is the large quarry, situated on the slopes northern of Dragovishtitsa and Balsha. This is a striking illustration of a metahemeroby. The quarry "Lyulyatsite" is developed for extracting and processing of dolomitic limestones. There are also other quarries near the villages of Tompsan, Gradets (stone quarry "Gradets" for extracting and primary processing of limestone), Vlado Trichkov and Chibaovtsi. Another metahemerobe area is the former poultry-raising Hybrid center Ranislavtsi situated in the center of the triangle Ponor, Drenovo, Dramsha.

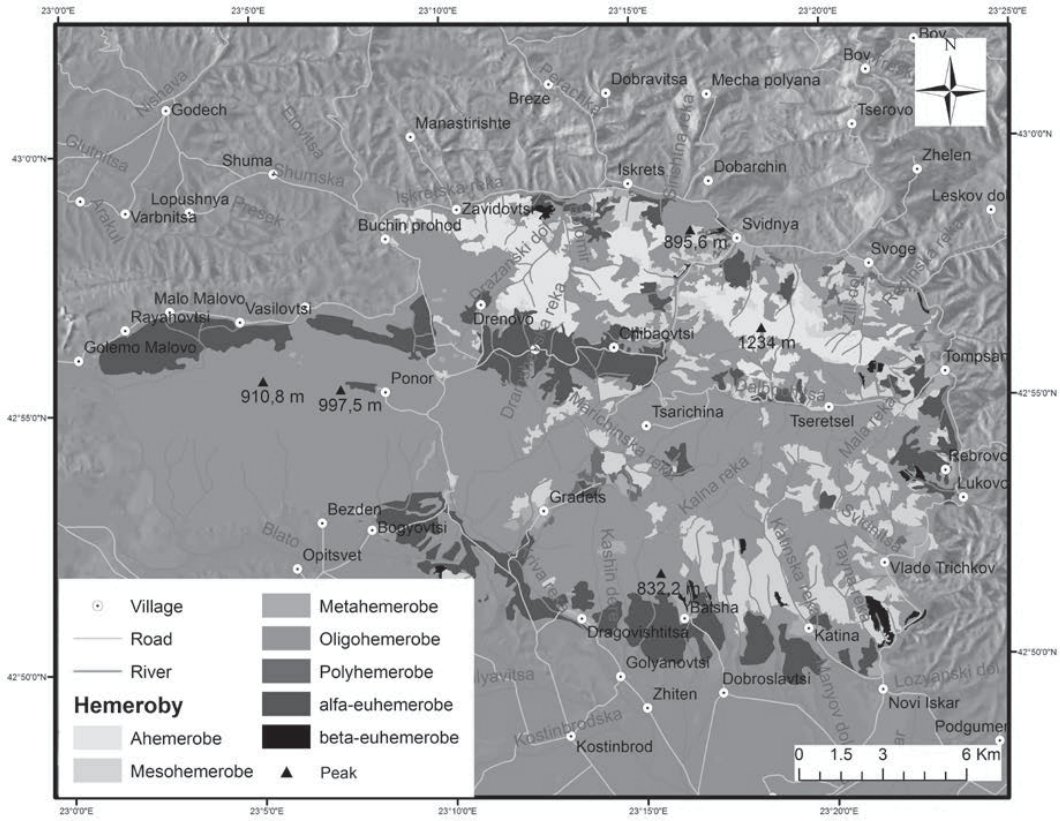

Fig. 1. Hemeroby map of Mala Planina (Borislav Grigorov & Assen Assenov)

Conclusion

The current research, concerning the landscape assessment by the use of the hemeroby index in Mala Planina was developed following the work of Borissova et al. [8] about landscape planning of mountainous areas. It is an attempt to understand in a deeper context the functioning of the landscapes in a mountain situated near the capital city. The specific hemeroby levels of the landscapes indicate that although it is easy to reach most parts of Mala Planina, most of its area falls within the low hemeroby value zone. Moreover, the western part including the ridge Kamuka that is a part of the NATURA 2000 protected zones: BG 0000322 Dragoman and BG 0001040 Zapadna Stara Planina and Predbalkan following the Habitats Directive and Rayanovtsi BG 0002001 according to the Birds Directive [14], is in a zone of low hemeroby value. Even so, the proximity of Mala Planina to Sofia should not be underestimated. The fact that landscapes with the highest hemeroby values are those around the most influenced areas by man, such as villages, mountain villas and quarries, have to raise the alarm and actions have to be taken under account in order to preserve the area.

Our final implication is that the presented work shows promising results and it can be used as an example for more comprehensive and elaborate future investigation of the landscapes in the western part of the Balkans.

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SPATIAL AND TEMPORAL ANALYSIS OF VEGETATION CANOPY AND THEIR RELATION WITH SLOPE PROCESSES IN ZEMEN GORGE (WEST BULGARIA)

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Abstract

Struma River forms 12 deeply incised valley sectors, one of which is the Zemen Gorge. Its steep slopes are shaped by various processes, closely related to presence or absence of vegetation and its type – forest or grass canopy.

Aim: Mapping and monitoring of vegetated and not vegetated areas are considered to be the aim of the presented paper.

Materials and Methods: Multispectral imageries from satellites Landsat 5 TM, 7 ETM+ and 8 OLI/TIRS from 1990 to 2015 are used to derive the Normalized Difference Vegetation Index (NDVI) in order to classify the land cover.

Results: Non-vegetated areas are extracted and represented as a percent of total area of interest. The results are compared with open source aerial imageries (Bing maps, Google maps) and field observations.

Conclusion: The presented results show a locally relevant information for land cover dynamics. They play important role for the stability of slope materials and landscape management.

Keywords: slope processes, vegetation, remote sensing, NDVI, GIS

Introduction

The Zemen Gorge is one of the Struma River's 12 gorges, located between Konjavska and Zemenska Mountains in the Kraishte Region, part of the transition area between the Balkanides belt and the Rhodopes Massif. Its length between the town of the Zemen and Razhdavitsa village is about 22 km. The study area (22.54 km^2) represents a part of the Stuma valley and Zemen Gorge (Fig. 1), enclosed between Zemen and Garbino village. With submeridional orientation, a maximal length of 13.16 km and a maximal width of 3.65 km, its perimeter is 21.32 km.

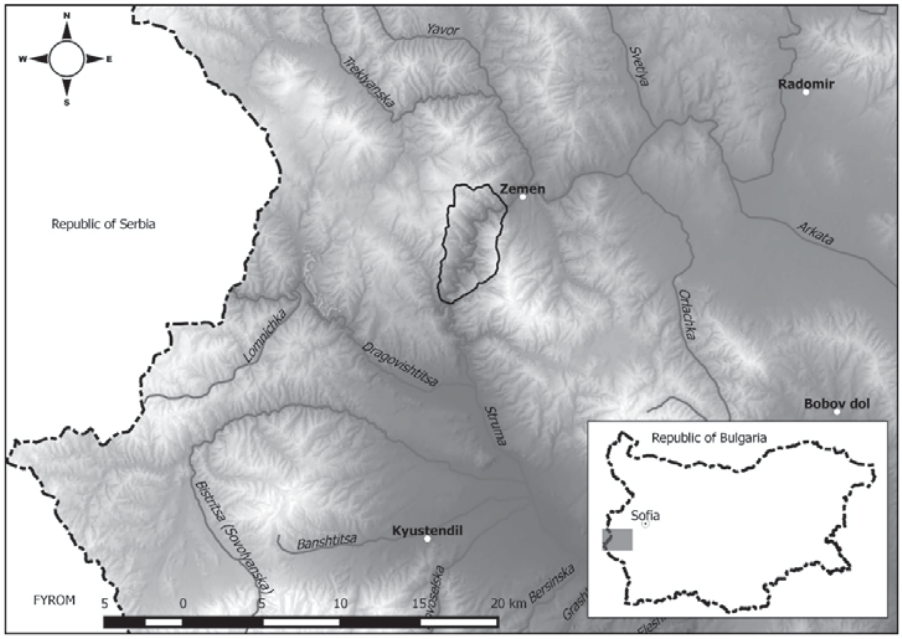

Fig. 1. Location of the study area (Petko Bozhkov)

The steep valley slopes, which in some places reach 70°, are shaped by various exogenic processes such as rock falls, rock slides, gully erosion, sheet erosion, soil creep, etc. The velocity, intensity, frequency and spatial distribution of slope processes are closely related to the presence or absence of vegetation and its type – forest or grass canopy. For instance soil creep occurs under vegetated slope often with forest canopy, whereas "sheet erosion occurs under conditions where the soil surface is insufficiently protected by vegetation cover" [1]. Gullies and rills are also associated with devegetation of the terrains. Plants and their root system anchor underlying soils and sediments and increase the stability of the slope. Therefore mapping and monitoring of vegetated and not vegetated areas over time is considered to be the aim of the presented paper. Three main tasks are set as necessary to be accomplished. The first one is to outline and measure the area of vegetation canopy. The second is to map out and measure the area of not vegetated areas and the third one is to assess the results.

Materials and Methods

Multispectral satellite images from the Landsat program are used to provide the spatial, spectral and temporal information for the presented paper. Different satellite imageries and derivative products are widely used in the environmental studies and are the foundation of remote sensing techniques and methods. Landsat is a long-term program, managed by the United States Geological Survey (USGS) with the cooperation of the National Aeronautics Space Administration (NASA), which acquires images of the Earth's surface since 1972 [2]. Each satellite scans the same area (also called scene) on the globe several times a month.

This allows monitoring and detection of surface (land cover) changes over a given span of time. Collected data, stored in the archives of USGS, is available free of charge and without restrictions at http://earthexplorer.usgs.gov/, http://glovis.usgs.gov/ or from a Landsat Look Viewer (http://landsatlook.usgs.gov/). In presented study a set of 5 images is used to detect the spatial and temporal changes of the vegetation canopy. Table 1 describes their main characteristics – identification (ID), acquisition date, coordinates (path and row) and cloud cover. All images from 1990 to 2015 have the same coordinate system (WGS84 zone 34N) and cell size providing data at a spatial resolution of 30 m.

Table 1. List of used Landsat images

ID	Date (dd-mm-yy)	Path x Row	Cloud cover (%)
LT51840311990192XXX03	11.07.1990	184x31	0.00
LE71840312000180EDC00	28.06.2000	184x31	0.14
LE71840312000228SGS00	15.08.2000	184x31	8.22
LT51840312010215MOR00	03.08.2010	184x31	8.57
LC81840312015213LGN00	01.08.2015	184x31	3.22

Landsat images have been applied for the quantification of global forest change from year 2000 to 2012 [3] and for assessment of natural forest expansion [4]. Vegetation indices, derived from different bands of satellite imageries, allow the detection and measurement of vegetation cover and its health status. They are widely used since the beginning of space observations of the Earth surface in the 1970s [5]. The Normalized Difference Vegetation Index (NDVI) is calculated from the set of Landsat images (Table 1). The index is the ratio between the near infrared and red bands:

$$NDVI = \frac{Near\ infrared - Red}{Near\ infrared + Red}$$

Values of NDVI vary between (+1) and (–1). Green vegetation absorbs most of the visible range of electromagnetic spectrum and reflects in the near infrared range, hence it has positive values of NDVI. On the other hand low chlorophyll level will result in different spectral characteristics of the vegetation and therefore – in low NDVI values. Negative values are associated with water bodies, bare soil, rock outcrops and man-made objects (like settlements, roads and quarries). Computations of the vegetation index are performed using a raster calculator tool in the open source GIS software – QGIS 2.12 Lyon. A separate raster layer with NDVI is created for each study year. Results are presented as a series of maps showing the spatial configuration of the vegetation cover. Field observations are made to validate or to disprove the results.

Results and Discussion

The NDVI values were calculated and presented as a series of maps showing the spatial extent of vegetation cover in the area of interest (Fig. 2). To improve the reading process values were classified in several intervals – bellow and equal to 0, from 0.0 to 0.2, from 0.2 to 0.3, from 0.3 to 0.4, from 0.4 to 0.5 and all other values, greater than 0.5. Areas with high and low index values are clearly visible. High values are associated with dense vegetation, whereas sparse vegetation and terrain with bare rocks and soil are related with low or negative NDVI. All maps the except map of year 2000th reveal similarity in the spatial configuration of vegetation. This can be explained with few things such as human activities (deforestation) and forest fire or could be related with climate and its characteristics. Human interference is not likely the reason because maps for 2010 and 2015 do not show same pattern and values of NDVI. A fire has same likelihood due to the same reason. Therefore the spatial extent of the vegetation in year 2000 is a consequence of the climate – possible drought, lack of rainfall and soil moisture will result in a change (decrease) of greenness and lowering of NDVI values. This hypothesis needs more data to be confirmed or disproved as a false statement. Landsat images from June and August 2000 were used to calculate the NDVI. However obtained results for year 2000 are similar and Fig. 2 show only the layer from June 2000.

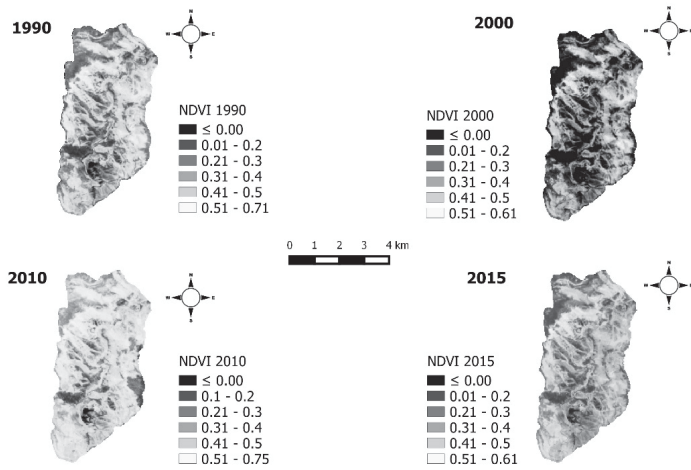

Fig. 2. Maps of NDVI values for each studied year (Petko Bozhkov)

Table 2. Quantitative analysis of the derived raster layers with NDVI values

Year	Maximum	Mean	Minimum	Standard Deviation
1990	0.7110	0.4495	0.0345	0.1318
2000	0.6125	0.2689	-0.3368	0.2009
2010	0.7500	0.5311	-0.0357	0.1264
2015	0.6127	0.4128	-0.0384	0.0998

The values of the NDVI vary in range of (-0.0345) in 1990 to (+0.7500) in 2015 (Table 2). The standard deviation for each raster layer is close to zero, which describes sampled data as close to the statistic mean. Only year 2000th is characterized with lower mean value and higher standard deviation than the other years. This difference results in the pattern of the raster layer, as seen in Fig. 2. Statistical maximum and minimum values describes raster layers as similar. However the arithmetic mean and standard deviation indicate different distribution of values in each data set. Fig. 3 shows relation between pixel count and pixel values in each NDVI layer. In year 2000th the count of low values (≤ 0.00) is larger than any other year.

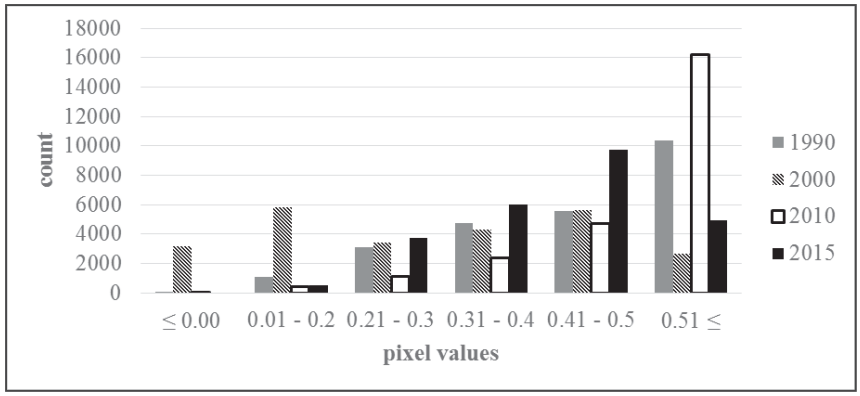

Fig. 3. Histogram displaying NDVI values for each studied year

Table 3. Total area (km²) and relative area (%) of vegetated and non-vegetated terrains (1990 – 2015)

Year	Class terrains	Area (km²)	Relative area (%)
1990	vegetated non-vegetated	1.92 20.58	8.51 91.49
2000	vegetated non-vegetated	9.58 12.93	42.56 57.44
2010	vegetated non-vegetated	0.81 21.69	3.59 96.41
2015	vegetated non-vegetated	1.82 20.68	8.09 91.91

Color composite images are created from the different bands of each Landsat image to ease the reading and verification of each NDVI layer. The produced "Natural color" (combination of red, green and blue bands) and "Pseudo natural color" (SWIR-1, near infrared and red band) composites facilitate the interpretations of each raster data set. In addition, aerial imageries from Bing maps and Google maps are used by swiping the NDVI raster datasets on top of them. Non-vegetated terrains are extracted from the NDVI layer for each year using an estimated threshold value of (+0.25) NDVI. Terrains with NDVI bellow 0.25 represent rocks outcrops, bare soils, talus slopes, screes, rockfalls and sparsely or non-vegetated areas. NDVI values above (+0.25) NDVI are associated with meadows, shrubs

and forest canopy. In this way NDVI-based simple land cover classification is performed for each layer. Binary raster layers are created, showing the spatial extent of each class. Non-vegetated areas in 2015 occupy 8.09 % of the study area (Table 3). Total and relative area of these class terrains vary in different years of observation. This is closely related with weather patterns and climate conditions as previously mentioned. Up to 96.41 % of the study area is covered with vegetation canopy.

Non-vegetated terrains for each year are extracted as polygons. The vector layer for year 2015 was displayed on top of a 3D elevation model. This approach allows further interpretation and determination of each polygon and its location on the slope surface. Non-vegetated areas encompass the crest of Zemenska Mountain and Konjavska Mountain, steepest valley slopes and many tributary gullies.

Several field observations are made to assess the results from remote sensing analysis. Key sites are established for monitoring of different slope processes. They represent given places where specific activities take place including taking a series of pictures. Since every key site has its own address (GPS coordinates and short topographic description) it is easy to relate all collected data form field work to a map. Coordinates of several pictures were plotted as points on top of the polygon layer with non-vegetated terrain for 2015 to determine if there any relation between real topographic situations and derived results from satellite image analysis. Pictures form different sites are used along with an aerial image of the "Skakavitsa" quarry (N 42° 26' 0.20", E 22° 42' 7.34"). All of them represent terrains with sparse vegetation or absence of vegetation canopy and fit to a certain polygon (Table. 4). Therefore the resulted layer is considered to contain accurate data.

Table 4. Pictures, taken during fieldwork and their geographic coordinates

 N 42° 28' 14.23", E 22° 43' 50.99" Key site № 1	 N 42° 28' 17.36", E 22° 43' 17.69" Key site № 2
 N 42° 27' 31.25", E 22° 42' 47.43" Key site № 3	 N 42° 26' 7.76", E 22° 41' 53.65"

Conclusion

Usage of satellite images and derived vegetation indexes NDVI allow recognition and mapping of vegetation canopy and extraction of rock outcrops and terrains with sparse vegetation canopy. Terrains with forest canopy and tree canopy are exposed to soil creep, while in areas with NDVI lower than 0.25 are shaped by sheet erosion, gully and rill incision, screes and rockfalls.

In the year 2000 NDVI values are lower than any other year of the study period (1990, 2010 and 2015). Perhaps that is related to the climate and its main elements. Further studies will eventually prove or discard such hypothesis.

Presented results facilitate the geomorphological mapping and allow detection of changes in the land cover over time. Important topographic information could be obtained using remote sensing. Spatial resolution is of great importance for the quality and applicability of derived data. Cell size of 30 m allows detection of changes in vegetation canopy. However smaller cells is required for more precise measurements and greater level of detail. Comparison between the images and the result layers from different years is possible by the implementation of descriptive statistics and cartography.

Satellite images provide necessary information for monitoring of slope processes, landscape, deforestation, vegetation health and canopy, ecoforcasting, etc.

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ASSESSMENT OF POTENTIAL ORGANIC POLLUTANTS IN LEACHATES FROM BULGARIAN LIGNITES

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Abstract

Aim: The aim of the study is characterization by wet chemical and spectral methods of leachates from Bulgarian lignites and their subsequent appraisal as potential organic pollutants in groundwaters.

Materials and Methods: In this study, lignites from the Thrace- and Sofia- coal basins, i.e. "Maritza-East" and "Stanjanci" mines, were subjected to water sequential extraction at 25^{o}C for 10 weeks. The process was tracked by pH and conductivity. The freeze-dried extracts (leachates) were characterized by yield, technical and elemental analysis. Leachates were characterized by a set of analytical techniques, i.e. X-ray photoelectron spectroscopy (XPS) and Infrared spectroscopy (IR).

Results: The chemical structural assignments of each component were done by curve-fitting method of the XPS, taking into account the binding energies reported for the C-, N- and S- functional groups. The main surface species include carbon atoms in aromatic and aliphatic structures in Maritza and Stanjanci leachates and their contribution is 48-53 atomic % from the total C 1s signal. In samples under consideration the most intensive N 1s signal is for pyrroles and amines at 400 eV (1-3at. %). The most abundant sulphur form is inorganic sulphates while the other ones are less than 1%. In this case the Maritza sample sulphatic sulphur content (13.6%) is almost seven times higher than the same for Stanjanci leachate. The FTIR spectra of the fractions show mainly the presence of oxygen-containing functional groups.

Conclusion: The analysis at the molecular level showed that the leachates contain organic compounds such as benzene carboxylic acids and their derivatives, short-chain aliphatic diacids, fatty acids and polyols.

Keywords: Lignite, Leaching, XPS, IR, Groundwater, Ecology

Introduction

Lignites are the main source for energy production in the power plants in Bulgaria. They created many environmental problems during mining, incineration and storage of waste products from mining and exploitation [1]. The main disadvantages of Bulgarian lignites are their low calorific value, high water content and harmful emissions during combustion. A possibility for gradual reduction of lignites energy use and finding alternative uses is the direct application of lignites in other areas of the economy, i.e. production of humic acids, adsorbents, soil conditioners, etc. At present, there is a little information, what and how much harmful to human health persistent organic compounds are leached into the soil by rainwater in artificial irrigation and during other irrigation activities. The contents of leached inorganic substances are under regulation [2] while organic impurities are somewhat neglected. In order to assess the potential impact of interactions between coal and water that might occur in soil systems during the agricultural application of lignite or in the waste water during sorption treatment, it is necessary to have more information on the composition of native lignite water-soluble fractions, water leachates [3].

The purpose of the present study is the characterization of leachates from Bulgarian lignites by wet chemical and spectral methods. On the base of the data an attempt for appraisal of potential organic pollutants in groundwaters will be done.

Materials and Methods

Lignites samples from Thrace- and Sofia-coal basins, i.e. "Maritza-East" and "Stanjanci" mines, were subjected to water sequential extraction at 25^{O}C for 10 weeks: Briefly, 10 g of grounded lignite samples and 150 ml of distilled water were placed into a 200 ml Erlenmeyer flask. The slurry was regularly agitated by magnetic stirrer. Each 7 days, the extract was separated from lignite by centrifugation for 10 min at 4000 rpm at 25^{O}C. Subsequently the supernatant was filtered to remove the finest particles eventually penetrated during manipulations. Likewise leachates were isolated. Their pH and conductivity were measured. According to the conductivity two portions were prepared: with high conductivity ("head" of the extraction curve) and with lower conductivity for the "tail" of the curve (Fig. 1). The leachates portions were freeze-dried. Products of leaching were light and voluminous. Due to the expected higher organic content for the "tail" leachates they have received special attention.

Leachates were analyzed and characterized by yield (wt. %), proximate, elemental analyses and by a set of analytical techniques, i.e. XPS and IR spectroscopy.

The XPS measurements were carried out in the analysis chamber of the electron spectrometer ESCALAB-MkII (VG Scientific) at a base pressure of $5x10^{-8}$ Pa. The spectra were excited with an Mg-K_{α} radiation at instrumental resolution of ≈ 0.9 eV. The base C 1s line had a binding energy of 284.6 +/- 0.1 eV and no charge effects were observed. The acquisition time for C 1s, N 1s and S 2p spectra were in the interval 30 ~ 5 min.

The IR spectra were recorded on dried spectrometry grade KBr pellets using a Bruker Tensor 27 Spectrophotometer in the range 4000–400 cm^{-1} with a 2 cm^{-1} resolution. On each sample 256 scans were performed.

Results

Bulk analysis: The water leachates obtained from the "Maritza-East" and "Stanjanci" lignites were yellow in color. After freeze-drying the first fraction was white in color unlike the others which were light brown to dark brown. The difference in color might be explained by the higher content of salts in the first fractions.

Table 1. Proximate (ad) and elemental analysis, in wt. %

Samples	Portions	Proximate analysis				Elemental analysis				
		W	Ash	VM	C_{fix}	C	H	N	S	O^{diff}
Maritza	"Head"	21.62	70.26	6.85	1.27	1.22	2.79	0.15	14.38	11.2
	"Tail"	15.93	35.93	43.44	4.7	10.91	3.10	0.39	6.76	42.91
Stanjanci	"Head"	19.92	63.09	11.1	5.89	7.11	2.80	0.27	9.08	17.65
	"Tail"	11.45	32.82	31.67	24.06	28.86	3.58	1.24	2.20	31.30

ad-air dried; W-moisture; VM—volatile matter; C_{fix}—fixed carbon; O^{diff}=100%-(∑(C+H+N+S)+Ash)

Fig. 1. clearly demonstrates that the conductivity of the leachate has sharply decreased from the fraction of the first week to the fraction of the second week and subsequently slightly decreased. Extracts of the first two weeks were gathered in "head" portions, and the rests in the "tails" (Fig.1). First leachates were slightly acidic in nature. The experimental results have indicated that first fractions were enriched in salts, 2000-4000 μS conductivity. Fractions containing organic compounds which were gradually liberated from the complex coal structure were collected in the "tail" portions. Proximate and elemental analyses of the freeze-dried samples are gathered in Table 1.

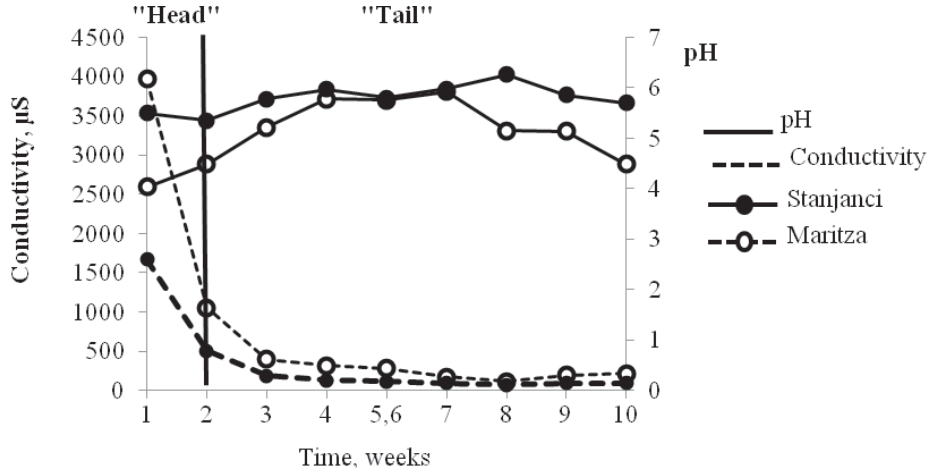

Fig. 1. Leachates curves tracked by conductivity and pH

The results of elemental analysis have confirmed the observation done on the base of conductivity:

– "head" portions were highly enriched in ash, 63-70% gradually reduced to 32-35% in "tail" portions;

– the tendency has mimicked VM (volatile matter);

– "tail" portions were partly enriched in C, H and O comparing to their counterparts of "head" portions;

– a sustainable decrease in S content for the "tail" fraction was depicted.

XPS analysis: X-ray photoelectron spectroscopy (XPS) was used to obtain qualitative information concerning the speciation of C, N and S species on the surface of the lignite water-extractable fraction of the leachates.

In order to provide a general picture of the distribution of several functional groups on the surface of the sample, curve-fitting method of the XPS spectra was applied. Chemical structural assignments of each component were made taking into account the binding energies reported for C, N and S functional groups [3-5]. The XPS data of the organic functional oxygen atoms are not presented because the general oxygen signal of the samples under study is due to predominantly of Si, Al, Ca, Mg, Na and other inorganic oxides. The XPS signal of the organic oxides is almost negligible compared to the signal from the inorganic oxides.

IR analysis: The FTIR spectra of the fractions have shown mainly the presence of oxygen-containing functional groups, though the appearance of gypsum and kaolinite made the interpretation of the FTIR spectra equivocal.

Discussion

XPS analysis:

Carbon species

As mentioned previously the XPS spectra were interpreted by using a peak fitting procedure. The XPS carbon 1s spectra obtained by a deconvolution procedure of the Maritza and Stanjanci fractions was presented in Fig. 2. For the two samples there were obviously four different structural groups of the carbon atoms with binding energies occurred at 284.6, 286.3, 288,4 and 292.3 eV, Table 3. They were assigned as follow:

– The 284.6 eV peak represents the contribution from the aromatic and aliphatic carbon (C-C, C=C, C-H).

– The carbon bound by a single bond to oxygen (i.e., C-O, C-OH, etc.) and to nitrogen or sulphur (C-N, C-S) will correspond to a signal at 286.3 eV.

– The 288.4 eV peak concerns mainly to carbon bound by three bonds to oxygen like carboxyl and ester functional group (O=C-O);

– Shake-up satellite peak at 292.3 eV is due to π-π transitions in aromatic rings [6].

The carbon atoms in aromatic and aliphatic structures are the main surface species in Maritza and Stanjanci leachates and their contribution is 48-53 atomic % from the total C 1s signal. Carbon atoms in carboxylic and ester groups are minor surface species 11-14 at.% (Table 2). The lowest C 1s signal has π-π satellite peak below 2 at. %.

Organic nitrogen containing species

Based on N 1s spectrum the organic nitrogen species mainly exist as pyrrolic nitrogen and amines, followed by quaternary and pyridinic nitrogen (Fig. 2). The peak at 398.4 eV can be assigned to N-pyridinic, while the peaks at 400 and 402.3 eV respectively to pyrroles, pyridones, secondary and tertiary amines, imides, protonated amines, N-quaternary atoms and oxidized nitrogen (N-O) [3, 7-11]. In our samples the most intensive N 1s signal is for

pyrroles and amines at 400 eV (1-3 at.%) (Table 2). Amino containing functional groups were also observed in the aqueous leachate from lignite [1].

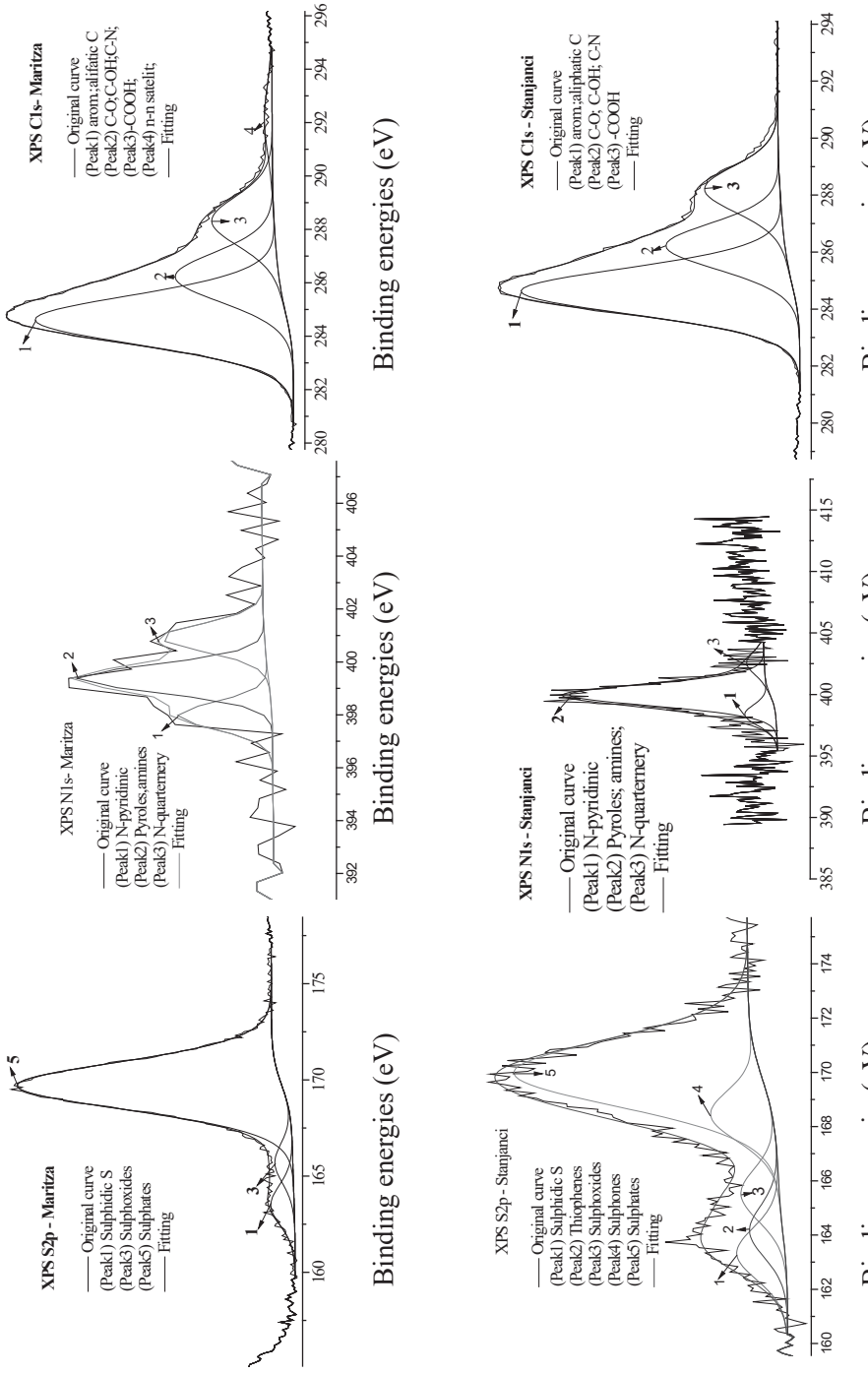

Fig. 2. XPS C 1s, N 1s and S 2p spectra of the Maritza and Stanjanci leachates

Organic sulphur containing species

The XPS signal of a sulphur single species was composed by two representing 2 $p_{3/2}$ and 2 $p_{1/2}$ components having a 2:1 relative intensity ratio and separated in energy by 1.2 eV. We performed peak synthesis for S 2p by mixed Gaussian and Lorentzian line shapes with full width at half maximum of 2.60 eV for each sulphur species. XPS spectra obtained by a deconvolution procedure are shown in Fig. 2. The assignments of the sulphur forms are based on references and our previous analyses [3, 4, 12, 13]. Their relative atomic concentrations in XPS spectra are determined using peak values fixed at 163.3, 164.1, 165.5, 168.4, and 169.1 eV of binding energies for inorganic (pyritic) and organic sulphidic, thiophenic, sulphoxidic, sulphonic and sulphatic sulphur forms, respectively. The main abundant sulphur form is for inorganic sulphates while the other ones are less than 1%. In this case the Maritza sample sulphatic sulphur content (13.6%) is almost seven times higher than the same for Stanjanci leachate. This is probably due to the higher sulphur content of initial Maritza-East lignite compared with Stanjanci lignites.

Table 2. Distribution of carbon, nitrogen and sulphur forms in leachates determined by XPS

Elemental peak	Functionality	Binding energies, (eV)	Atomic %	
			Maritza	Stanjanci
C 1s	Aromatic and aliphatic Single C-O, C-OH, C-N bond Carboxyl, ester π-π satellite	284.6 286.3 288.4 292.3	48.27 19.87 11.96 2.04	53.57 23.37 14.82 0.2
N 1s	N-pyridinic	398.4	0.59	0.52
	Pyrroles, pyridones, *sec./tert*. amines, imides	400	1.19	3.4
	N-quaternary, protonated amines, N-O	402.3	0.65	0.3
S 2p	Organic/inorganic sulphidic	163.3	1.03	0.37
	Thiophenes	164.1	0	0.26
	Sulphoxides	165.5	0.79	0.3
	Sulphones	168.4	0	0.47
	Inorganic sulphates	169.1	13.62	2.42

IR analysis: A comparison of the FTIR spectra for the wavenumber region 4000 to 400 cm^{-1} for the leachates of lignites samples from “Maritza-East” and “Stanjanci” mines is presented in Fig. 3. Gypsum was revealed by the bands at 3543, 671 and 603 cm^{-1} [14]. Kaolinite was related to bands at 534 and 470 cm^{-1} [15]. The FTIR spectra of the fractions show absorbance in the region of 4000-3000 cm^{-1} due to vibrations of OH groups (H-bonded).The sharp intense bands at 2920 and 2850 cm^{-1} can be assigned to stretching vibrations of $-CH_3$ and $-CH_2$ aliphatic groups and that at 1622 cm^{-1} correspond to aromatic C=C structure. Bending vibrations of phenols OH groups are observed at 1384 cm^{-1}. The vibrational frequencies of oxygen in C-O-R structure spread from 1143 to 1016 cm^{-1}.

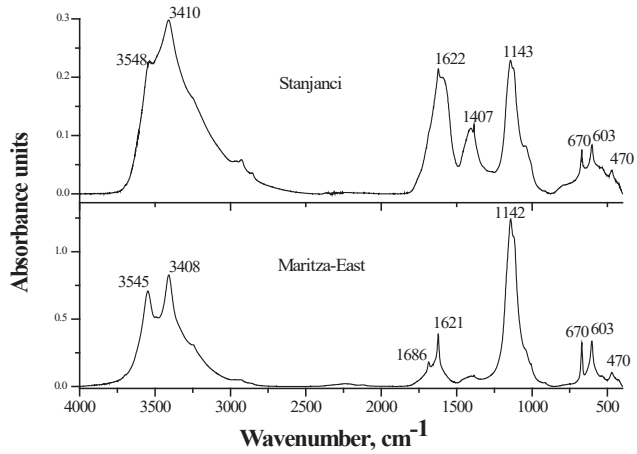

Fig. 3. IR spectra of leachates

The analysis at molecular level have shown that the fractions could contain compounds with –COOH groups such as benzene carboxylic acids and their derivatives, short-chain aliphatic diacids, fatty acids and polyols. Most of the identified molecules reflect clearly the presence of microbial remains in the lignite structure since microbial activity during coalification is well known.

Water-extractable fractions form compounds which may act as complexing agents and sources of nutrients for plants. On the other hand, the fractions might also take away nutrients from topsoils into bottom layers of soil or might pollute the groundwater. It seems that the identified compounds do not represent an acute toxic risk from an environmental viewpoint. However, nitrogen-containing compounds can raise concerns and further study is needed to focus on this. Applications of lignite as a soil conditioner will also call for a study on the penetration of compounds leached from lignite into plants and food.

Conclusions

The present study should be regarded as a first attempt to assess the mobile contaminants in lignites aqueous leachates. Although a very simplified separation procedure based on conductivity was applied some fractionation has been achieved. Portions relatively enriched in C, H, N and O have been isolated and characterized by spectral methods. Sulphur most likely was of pyrite type as it was concentrated in “head” portion, with high ash content, 60-70%.

The study should be developed by the appraisal of the influence of extractants, i.e. acidic leaching liquor (H_2SO_4), alkaline leaching solution (NaOH) and other experimental parameters (liquor: solid ratio, pH, t^OC, time, etc.) on the yields and compositions of leachates.

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REPORTS

Topic:
ECOLOGY AND EDUCATION

PROJECT-BASED LEARNING ON "ECOVILLAGES"

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Abstract

The objective is to increase the ecological knowledge of the students working on projects based on ecovillages. One of the main goals of this form of biological education is to activate the ecological self-consciousness in the process of learning. The study aims to show an innovative model for teaching. The theme is "Ecovillages", in which settings the students work on preliminary given algorithm and present their own developed projects after completion. Project-based learning gives the opportunity for individual expression for every student based on their knowledge and skills in the ecological area, and with this, a complete ecological self-consciousness.

Keywords: Ecovillage, Ecological self-consciousness, project-based learning

Introduction

Project-based learning (PBL) is a method of teaching and learning in which students acquire new knowledge and skills in the process of designing, planning and producing a certain educational product [1].

The structure of PBL is flexible and mobile in regard to organizing, duration and also in content. The educational content is not given in the beginning from the teacher so the students work on their own on the project in the process of its research, selection, structure and presentation. In this way they actively master the content in an actual context which makes their knowledge valuable and permanent. In this process the teacher is in the role of a partner and counselor, who guides the studying in a functional and meaningful aspect in the search of the answer to the questions "why?", "what?" and "how?" [2].

PBL gives the students an active role in creating a certain product. The impact of PBL is extremely valuable on the motivation, communicative skills and teamwork. In order for the student to realize the project they must plan, present their ideas, work in groups, discuss, review the authenticity of their own knowledge, make conclusions, hypothesis, create common strategies, etc. [3].

The purpose of this project is to develop and present a model of project-based learning on the topic "Ecovillages".

Materials and Methods

The study used methods of theoretical analysis and synthesis, pedagogical modeling.

In the process of PBL the roles of the students and teachers are exchanged. Students are entering the roles of people who actively solve problems [4]. They are responsible for their own learning and are motivated about the completion of something useful in which they are active participants. The teacher as a trainer manages the group's dynamics, supports the movement of the process forward, advises and evaluates [5].

The idea of this project is to motivate the students about an alternative way of thinking and lifestyle. The environment is the nature that surrounds us. Without it our existence would be impossible. If we want to live better we need to consider the needs of the nature and help with its conservation. We must stop the pollution of the environment and the depletion of its non-renewable resources. For that reason it will be satisfying if we create in children an ecological self-consciousness in their early childhood [6].

As humanity craves progression, there is an increase in the number of buildings being constructed on a global scale; this in turn leads to an increase in energy consumption to satisfy the development process which leads to more tons of greenhouse emissions can be released to accommodate the energy demand. Due to this there is increase in energy demand; the world has to face the fact that our resources will soon be depleted [7].

Nowadays people start thinking about changing their living habits towards being more socially, economically and ecologically responsible. One of the possible ways to do this is proposed by ecovillage movement. Ecovillage principles aim to combine social–cultural environment with a low–impact way of living. Choosing to live in the ecovillage is choosing an alternative way to the individualistic, materialistic and consumer-oriented lifestyle [8].

Ecovillage members are united by shared ecological, social or spiritual values. An ecovillage is often composed of people who have chosen an alternative to centralized power, water, and sewage systems. Many see the breakdown of traditional forms of community, wasteful consumerist lifestyles, the destruction of natural habitat, urban sprawl, factory farming, and over-reliance on fossil fuels, as trends that must be changed to avert ecological disaster.

Many designs, materials and techniques are available to create beautiful and ecologically sound homes. Examples of housing types include rammed earth, mud brick, straw bale, cordwood, yurts and super adobe or eco-domes.

People living in ecovillages are moving to renewable energy such as biogas.They are installing compost toilets and solar panels and also are using biodynamic agriculture techniuques (a method of organic farming) [9].

Results and Discussion

The project is given to students from 9th grade who are studying architecture and geodesy, after they have learned the section “Biosphere”. For the implementation the students are divided in groups and every group has an individual task. They will have precise time to accomplish the tasks. The project-based learning is realized in 3 stages [2].

1. First stage of preliminary study and planning:

The subject of the project is "Ecovillages". The stage begins by discussing the final goals in front of the class, which are:

-Increasing the student's interest in ecology, problems of environmental pollution;

-Developing social skills: teamwork, presenting in front of an audience;

-Forming a self-consciousness in protecting the environment and the wise use of the natural resources;

-Increasing the creativity of the students;

After that a schedule is created. The duration of the project will be two weeks. The dates in which tasks are going to be presented are also specified.

Planning activities:

The class is separated into three groups. Each one of them has a task.

First group: Finding the most suitable place for building an ecovillage;

Second group: Looking for the needed documents for building an ecovillage;

Third group: Searching for the resources which are needed for building an ecovillage;

A task for all the class will be to make a model of an ecohouse.

Students will have the freedom to use all available resources to find information.

2. Second stage of practical implementation:

According to the schedule teams gather together, select and structure the information.

The students will have the opportunity to consult with the teacher if they have any questions or difficulties.

3. Third stage of presentation, discussion, review and evaluate the results of the study:

Each group will present their finished products.

In the beginning of the class the students are motivated by recalling the final goals.

Each group has 10 minutes to present their project through a multimedia presentation.

After presenting the project there will be a discussion with the class. The activity of each group will be analyzed and evaluated separately to highlight their achievements and shortcomings.

Conclusion

To work on a certain project is not easy, but it is really satisfying and motivating for both sides taking part in the process of learning. When working in teams, students exchange experience and are more self-confident. After finishing the project they acquire many new qualities and skills like social skills, the ability to create a project and interest in environmental protection.

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ENVIRONMENTAL EDUCATION MODEL TITLED "ECOLOGICAL CORRIDORS - ROADS FOR ANIMALS"

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Abstract

Aim: This article presents a dynamic approach, called Project-based learning (PBL) to spread the idea of preserving the environment and minimizing the anthropogenic effect in wild animals’ natural habitats. Its goal is to draw attention of the young generation from Bulgarian high-schools to ecological problems.

Materials and Methods: Students play the active role in the educational process as they are required to conduct a research about the problem.

Results: Participants in the project are expected to create a product and present it in front of the class.

Conclusion: This approach is a way of improving the educational system and is reaching to create a feeling of responsibility to the environment in the student figure.

Keywords: Environmental Education, Ecological corridors, Project-based learning

Introduction

Project-based learning (PBL) is a teaching and learning method in which students gain new knowledge and skills in the process of designing, planning, and producing an educational project. This method ensures an active participation of students in creating a project into groups. PBL develops personal and collaborative skills that improve students' achievements in education [1]. This report - "Ecological Corridors - Roads for Animals" introduces the application of the method in Bulgarian schools with the target group of 9th grade students. The goals are as follows: formation of new, contemporary knowledge of the anthropogenic influence on animal habitats in Bulgaria and methods for dealing with the problem; heightening the interest of young people towards environmental issues; creating a mentality for conservation and responsible usage of natural resources; realization of interdisciplinary relations with physics, chemistry, geography and informational technology; development of social skills (thesis defense, teamwork). The main subject of this PBL is the importance of ecological corridors and ecoducts in general [2].

Ecoducts facilitate the transfer of organisms between two separated habitats so the wild animals could harmlessly cross over the obstacles made by man [3]. The most common obstacles are highways, crudely tearing nature and impeding animals from one side of the road to search food and new territory. Bridges, underpasses, overpasses, tunnels, viaducts, and fish ladders are just a part of the green installations which fit the definition of an ecoduct. The practice of building ecoducts isn't new. Because of the evident damages caused by people to the environment, the idea of preserving the biodiversity is getting more and more recognized. The first ecoduct was built in 1950 in France. Other countries such as the Netherlands, Switzerland, Germany, USA, Canada, and Japan soon followed suit. The number of ecoducts around the world is constantly increasing. Currently, the Netherlands is the primary example as they have built more than 600 tunnels. The longest ecoduct is also there - its 800-meter long, and encompasses a highway, a railway, and a golf course. In this way, for example, the population of the endangered European badger has increased greatly. Not only they managed to preserve animal population but also they decrease the number of killed animals and people in road accidents [4].

Materials and Methods

The key factors of the educational model, named "Ecological Corridors – Roads for Animals" are the motivation and purpose of conducting such a project, the used methods, and the procedure.

The objective motivation for this educational model is to develop secondary school students' practical skills and to raise their awareness of ecological problems due to the anthropogenic effects on the environment. During the project, participants will be able to practice and improve their team work skills as well as their personal commitment to the problem. Moreover, they will feel involved into the existing ways of protecting the wildlife on foundation of knowledge of the worldwide ecological issues.

In the case of this educational model, we aim to draw the attention of Bulgarian young generation in secondary school to the significance of preserving biodiversity of the environment. Students should be encouraged in an eco-friendly way of thinking in order to improve human mentality about the environment and our effect on it. People tend to neglect the ecological problems, but they exist and one of the purposes of the ecological education is to attract and motivate the young generation to deal with such problems. Making youngsters aware of the responsibility of human to preserve ecosystems, may affect their attitude to the nature.

The methods used in this particular PBL project are mainly working in groups, conducting a survey, presenting, and modeling [5]. Through these methods, students will develop essential cognitive abilities. The exact process of the project consists of 3 stages: fixed duration of the project, activity plan, and assessment criteria.

Duration of the project - The given time for this project may vary depending on the type of the product and how busy is the students' schedule. The assignment duration is usually two weeks, but in some cases it can be prolonged to one month. When every group takes a topic, then the teacher makes a schedule for consultations with each team during the implementation of the project, according to everyone's preferences.

Activity plan - In the beginning, the teacher separates the students into groups (this depends on the number of students), usually consisting of 4 participants in a team. Each group will receive a paper card with a place in Bulgaria that needs building an example of ecological corridor. For example, students can examine the Bulgarian highways that are crossing through reserved areas – highway *Trakia, Hemus* [6], and the new project for *Struma*. They are expected to gather information for the ecosystem and the endangered wildlife society living around the highways from scientific journals, encyclopedias, resources on the Internet, and even from their school books.

The activities of the project may include not only the help from biology teachers, but also the teachers from the geographic field. They can provide students with information about the characteristic features in the specific region, which they are investigating.

After each group has received a paper card, they are free to choose the type of project they will create. The expected results from them will be a report, accompanied by a model, presentation, poster, or collage.The necessary materials for all type of products may vary depending on participants' imagination and creativity but here are some examples that might be useful for them:

- *Model project:* materials that can be used here are plasticine or plastic clay for laying the soil as a foundation. Another idea for creating figures of animals and plants, typical for the given ecosystem, can be the usage of cardboard, fabrics, or even live objects from the vegetation (grass, plants, leaves, etc.).

- *Multimedia presentation project:* for this type they can download photos from the Internet, social networks, scientific books, and encyclopedias and include them in this type of project. The information can be presented in several ways, according to their computer competence. For example, they can prepare a presentation, a video or even an animation, based on the topic.

- *Poster project:* the enthusiastic ones can use paints, markers, and other art materials in order to express their idea of how the ecological bridge should be build, depending on the

characteristic of the particular ecosystem. Furthermore, it would be useful for them to create their idea on a painting program on a computer and after that just print out the product.

- *Collage project:* students may need different stickers, photos, application paper, which they can glue to a sheet of thick drawing paper (the size of that will be given beforehand). The collage must also consist of a section of text written with markers, paints, crayons, or pencils.

In addition, students will be given instructions to conduct a survey among a number of people from different ages. Each group will get a survey paper example and they will need to make copies in order to carry out the investigation. On basis of this survey, they have to estimate the results in how effective and useful people find the idea of ecological corridors in ways of ecosystems' conservation. The analyzed results can be used as introduction of their reports, saying if people are aware of environmental issues.

Survey paper example

Survey Paper	
This short paper will be in help for the students form the 9th grade in conducting a school project on the topic of *Ecological Corridors - Roads for Animals*. The survey aims to examine people's attitude toward the importance of ecological corridors, whose goal is to preserve the environment, preventing wild animals' isolation via specific save roads crossing above or under highways. The last question is open so feel free to express your opinion to the problem. Please, be honest and have in mind that this survey is anonymous and will be used only for educational purposes.	
1. How often you see killed animals on the roads?	□ Very often □ Rarely □ Never
2. In your opinion, how often you think is the process of extinction among wild animals?	□ Very often □ Rarely □ Never
3. In how many cases you think that human is responsible for damaging the environment?	□ Very often □ Rarely □ Never
4. Do you think that the government of every country should invest in such ecological corridors? Is this worth the effort?	
…… …… …… …… ……	

After analyzing the results from the survey and choosing a type of project, the students can arrange a consultation meeting with the teacher with the intention of helping develop their ideas and keep them motivated.

Assessment criteria - The assessment is on the basis of content, language and technical criteria. The general standards will be given in advance in the beginning of the project, so that students would be prepared what to expect in evaluating their products: logical evaluation of the problem, scientific knowledge in the field of ecology, presentational skills of the group, clear language and accurately used terminology. The criteria will be printed on sheets of paper and each group will be facilitated with them.

Presentation and results

The final steps in the project are inspection and assessment of the products created by students. For this purpose, it is necessary to arrange a meeting with all groups, when each

one will present their own product. For our project it is also essential to encourage creative thinking. For this reason, the most esthetical and original product will be valued greatly. The students' products may differ - multimedia presentation, model, poster, or a collage. Every project will be evaluated individually without comparing to the others as we aim to be extremely objective.

Discussion

In our opinion, Bulgarian educational system is mainly focused on students' theoretical achievements, but this innovative way of teaching can develop their practical and social skills. Project-based learning will give students an opportunity to actively participate in creating a product into groups. By doing this, students will be more likely to process the new educational information and be constantly motivated to reach goals. One more positive effect of PBL is the building of critical thinking that may be useful for their future realization into the adult world.

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REPORTS

Topic:
OTHER RELATED TOPICS

LIQUID CRYSTALS, BIODIVERSITY AND ADAPTATION MECHANISMS OF LIVING MATTER

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Abstract

The aim of the paper is, on the basis of the unique properties of liquid crystals (LC), to revue their role in accommodation mechanisms of living matter, as building blocks of biodiversity and in the origin of life on earth.

Materials and Methods. Liotropic, smectic and cholesteric LC are mainly in use. The methods of investigation include experiments with liotropic and native membranes, flexoelectric analysis, polarization microscope observations and others.

Results and Discussion. The results published up to date reveal that LC show properties of sensitivity and ability to interplay between mechanical, electrical and optical external influences. LC incorporated in living organisms act as proto-organs thus cause and facilitate the adaptive abilities of biological specimen.

Conclusion. LC are widely found as effectively functioning building blocks of the living organisms, facilitating their adaptation mechanisms and can be considered as predecessors of life on earth.

Keywords: Adaptation mechanisms, liquid crystals, origin of life.

Introduction

Liquid crystals possess some of the properties both of liquids (fluidity) and of crystalline state (ordering of molecules in some directions) and therefore are named also a mesophase. The thermotropic liquid crystals, one of the two big classes of liquid crystals is divided into three subclasses: smectics, nematics and cholesterics. The parameter that distinguishes between the thermotropic subclasses is the temperature. The thermotropic mesophase is studied for the purpose mainly of technical applications.

The second class are the lyotropic liquid crystals. They are obtained by solving of a substance into other one. The dissolvable substance is an object with polar heads which have electric charges and non-polar tails without charges. The polar heads are soluble in water and other polar solvents (hydrophilic parts), and the non-polar tails are soluble in oils (hydrophobic parts). The tails consist of one or several alkyl chains with different length. Examples are the water solutions of detergents, lipids of biological and synthetic origin,

proteins, nucleonic acids (DNA, RNA) cell membranes - native or laboratory modelled. Another example are chromonics, which are flat and rigid, have polar charges and are soluble in water. It is characteristic for lyotropic mesophase a tendency of spatial self-organization when placed in a solvent. They form spatially distinct three-dimensional objects, such as layers (open or closed), spheres, tubes and others.

The cholesteric mesophase as a representative of the thermotropic liquid crystals was discovered by the botanist F.Reinitzer in 1988 and studied further by the physicist O. Lehman. The lyotropic mesophase was first discovered by the german physician and scientist R.Vrchow in 1854 who observed a water solution of myelin.

Liquid crystals show high sensitivity to external stimuli such as light, mechanical and electrical influences and temperature which explains also their participation in living organisms as important building blocks - the liotropic liquid crystals [1]. Lehman studying the remarkable properties of liquid crystals showed a series of phenomena considered previously characteristic of living organisms and possessed by these crystals. In his book published in 1906 entitled “Liquid crystals and the theories of life” Lehman posed the problem to determine where actually “the life starts” and which actions are produced exclusively by “matter and force”. In 1920 Lehman extended considerably these results in his new book: “The science of liquid crystals and their connections to biology” Now, more than hundred years later the problem posed by Lehman is still unsolved.

Materials and Methods

As materials native membranes are used (cell membranes) as well as model membranes. An example is the BLM - bilayer (black) lipid membrane obtained by smearing of a lipid drop over a small aperture in a hydrophobic plate immersed in water electrolyte. Spontaneous thinning leads to formation of a lipid bilayer at the aperture which looks black due to destructive light interference from both interfaces. Material for investigation are cells, furthermore viruses, special test material from plants and animals.

The method of visual observation by polarization microscope using the birefringence of liquid crystal (a property displaying dependence of refractive index from polarization and direction of light) was used. The inner structure of the mesophase is possible to observe in polarized light. In 2014 in USA laboratories bacteria Flagella was first observed to exist alive in a nontoxic nematic phase of a liotropic liquid crystal. The nematic phase displays a long range constant direction of the rod-like crystalline molecules. Bacteria has 20 nM width and swims freely through the mesophase living after itself a wave of directional disturbance of ordered molecules. This can be observed in polarized light and serves to study properties of mesophase on a submicron scale.

The method of measurement of flexoelectric effect e.g. on BLM, or egg yolk lecithin membranes includes measurement of the corresponding electric current as a function of applied frequency or amplitude of applied deformations [2]. Similar method of measurement is used for native membranes, e.g. in case of muscle membrane [3]

A method to investigate flexoelectricity also for thermotropic liquid crystals with application of electric field on the liquid crystal and additional registration of light

transmission properties (the so called flexoelectric spectral analysis) is a tool to study various properties of these crystals [4, 5, 6]

Results

Due to the existence of electric dipole moments in liquid crystalline cell membrane, the cell can process electric fields of different magnitude. Normally they lie in the interval 1-200 mV and outside this interval the fields are considered to be very low or very high. The response of living cells to very weak electric fields up to the thermal noise limit was explored in [7]. Cell liquid crystalline properties enable electric fields to be recognized by the cell as transmembrane potentials in order to regulate cell reactions or to establish link with some other cells. For instance the transmembrane protein having molecular electric moment, interacts with direct currents by changing its form. Its interaction with alternating currents causes oscillations of the transmembrane protein between different protein conformations.

There exists a special organization of the liquid crystalline lipid bilayer of the cell membrane to accomplish biocommunication [8] i.e. transfer of energy, matter and information between internal organelles of the cell and the environment. Namely, the membrane has special receptors as hormones, peptides, proteins to bind particles and ion channels regulating cation and anion fluxes. Theoretical models show that electric fields can transfer energy to cause enzyme reactions and reversely, reaction process can generate electric fields.

Another way cell membranes to transmit electric signals is based on flexoelectricity [1]. For layered liotropic crystals and biomembranes the deformation of the molecular orientation leads to a splay which leads to electric polarization. The source of polarization comes mainly from the charges that are contained in the polar heads of the molecules.

The role of flexoelectricity in biology is confirmed also by the observation that bio membranes are usually strongly curved. As examples one can refer to the edges of retinal rod outer segments and the inner mitochondrial membranes in case of energized mitochondria. Further examples are the borders of intestinal epithelial cells, by virus budding (i.e. in stage of development), by organelle and cell morphogenesis. Frequently this membrane curvature is dynamic as in the contact area between contacting cells. Experiments with erythrocytes (red blood cells) show dependence of their membrane fluctuations on temperature, solutes etc. as well as dependence of light transmittance intensity on the fluctuations (Flicker phenomenon). By erythrocytes the membrane fluctuations can exceed 400 times the thickness of the membrane. Furthermore the peripheral proteins imbedded into the cell membrane matrix are capable to produce such curvature by their contraction.

Conversely an electric field can produce appearance of curvature on a model or native membrane (converse flexoelectric effect) [9]. Consequently, deformations of the cell liquid crystal membrane and appearance of electric field are mutually induced and contribute to the communication of the cell with the surrounding. This process is enhanced by the presence of proteins embedded in the membrane due to their rather large electric moment.

Intermembrane contacts are facsilitated by the existence of flexoelectric force as well as by the existence of a mosaic distribution of electric charges on the liquid crystalline

membrane. Both electric forces help to overcome the hydration barrier of water between cells. Flexoelectric effect facilitates the ion transport between the inner and outer cell spaces. This concerns the passive (along the transmembrane concentration gradient) transport and active (against the gradient). E.g. flexoelectric effect contributes to opening the ion channels in native membranes or pores in model membranes. The flexoelectric effect of muscle membrane of locust Schistocerca gregaria was investigated in [3]

There are differentiated organelles of the cell such as retinal photoreceptors of the eye and chloroplasts for photosynthesis build of liquid crystals. Some native membranes e.g. the retinal rods and discs are photosensitive. Such membranes have three degrees of freedom to interact: optical, mechanical and electrical degrees of interaction possibilities. Hence they are able to display electro- mechanical – optical interconversion of energy. So far experiments have been performed with model photosensitive membranes e.g. BLM membrane made photosensitive. Photosensitivity of a model membrane is achieved by decorating it with nanoparticles of CdS which are highly sensitive to light and produce electric charges upon illumination. Using this property of model nanomembranes the photoinduced flexoelectricity is investigated [1]. Photoinduced pore formation on model membranes and photoinduced shape changes of erythrocites was investigated e.g. in [10].

The idea behind modern cryobiology is using special thermal regime and crioprotectors to preserve the liquid crystal order of the living sell membrane avoiding its crystallization [11].

Liquid crystals are found not only in cell membranes but e.g. in the bile secreted from the liver containing (among others) also lecithin and cholesterol. By the already mentioned process of liquid crystal self assembly, they form oval vesicles and micelles in the bile. If the normal equilibrium between the vesicles and micelles is changed then cholesterol crystals (gallstones) may arise in bile duct requiring surgical intervention. In human blood the cholesterol appears as small spheres with dimensions between 1 micron (10^{-6} M) and 0.3 millimeter. When attached to the blood vessels they do harm to the vessel walls destroying the nearby cells not because of the toxic chemical properties, but due to the liquid crystalline nature.

The virus of tobacco mosaic when observed in polarized light displays the property of birefringrnce. A microphotograph of the virus shows a layered structure as by smectic liquid crystals. The virus is a tenacious enemy of the plant's leaves- it endures unlimited time at temperature 40^{O}C and 30 minutes at temperature 50^{O}C without water (i.e. on a dry leaf).

The myelin cover of nerve fibres is a liotropic liquid crystal forming a structure of layers wrapping around the axon. It plays the role of insulator of the nerve. The myelin layers consist of lipid and protein molecules. An analysis in polarized light shows that the lipid molecules are situated in a perpendicular direction to the nerve axis while the protein molecules are oriented parallel to the nerve axis.

The brain is a complicated liquid crystal structure. The grey brain substance consists mainly of a liotropic state of matter. Liquid crystals play the role mainly as insulators in the white brain substance and by the nerve fibres.

The proteins myosin and collagen enter respectively the muscle tissue and the sinews and bones.

Discussion

The liquid crystal state in contradistinction to solid state is much more suitable to enter living organisms in particular as an ingredient of cell membranes. The reason for this is the extreme sensitivity of liquid crystals to external stimuli as light, temperature, mechanical and electrical action. In this way liquid crystals incorporated into living organisms can serve as protoorgans which facilitate the adaptation mechanisms. Not less important property of liquid crystalline state is its ability to restore the initial condition after removal of external forces. An example is the elasticity of native membranes (property used in particular by muscles functioning).

Liquid crystals are supposed to play essential role at the times (as conjectured about 4 bilion years ago) when life arose on Earth. Independently of whether life started as abiogenesis (i.e. from dead matter) or was restored in some form of previously existing life, the property of spatial self-organisation of liquid crystals should have been of \importance for the process. Lipid layers in waters of pre-biological times due to hydrophobic effect of their alkyl tails tend to form some closed spatial forms convenient to protect some pre-life processes inside their volume. It was claimed [12] that remnants of primitive organisms with the structure of liquid crystals have been found in the earth layers of ancient geology times.

The ideas of one of the first professors at Sofia University - P. Bahmetiev - have a lasting influence on modern cryobiology. Inspired by the liquid crystal research of his time he wrote an article on “Biological analogs of liquid crystals”. There he first pointed out in 1906 that anabiosis – discovered by him as a reversible condition of living organisms between life and death at low temperatures - is an analogy between the transition from liquid crystal to crystal state. Anabiosis helps organisms to survive at extreme conditions of low temperatures.

One of the founders of quantum physics, E. Schroedinger in 1943 in his book “What is life? The physical aspects of living cell” was more definitive than Lehman in posing the problem “Does life is based on the laws of physics?” In general life is certainly based on some complicated biological mechanisms such as accommodation, reproduction, and others. However, in the spirit of science to analyze phenomena by dividing them into parts, such a biological mechanism in its very small parts is expected to be ruled by physical forces executed on the corresponding material objects. A good example is the biocommunication between cells discussed in third section. The experience of biophysicists shows that more and more purely biological phenomena are based (in the above sense) on the physics laws. Nevertheless the problem posed by Schroedinger and Lehman is still open.

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HEALTH AND ENVIRONMENTAL ASPECTS OF SEWAGE SLUDGE UTILIZATION

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Abstract

Aim: Application of sewage sludge from urban wastewater treatment plants in the agriculture is the preferred treatment method according to EU requirements. This procedure for Bulgaria has been defined in special Ordinance since 2004. Under the Ordinance, an essential requirement to fulfill before sludge utilization is to ensure protection to human health. The aim of this study is to investigate the microbial contamination of sewage sludge, followed by statistical generalization and interpretation of the environmental health risk.

Materials and Methods: This study presents the results from microbiological analyses of 288 sludge samples from 43 wastewater treatment plants in Bulgaria, investigated in NCPHA within the period 2006-2015.

Results: While in most cases the sewage sludge samples appeared to be clean from harmful chemicals, the bacteria presented in some of the samples indicate the potential health risk of pathogenic contamination. The indicative quantifications of *E. coli* and *C. perfingens* prove their persistence in some samples.

Conclusion: The results of this study prove the need for further introduction of new and more efficient methods for final sewage sludge treatment and microbial decontamination.

Keywords: sewage sludge, wastewater treatment, environmental health

Introduction

Finding environmentally acceptable, health responsible, and economically feasible ways of utilizing municipal sewage sludge has received much attention in the last decades both from research and regulatory institutions, as well as the general public. According to EU requirements, application of sewage sludge from urban wastewater treatment plants (WWTP) in agriculture is their preferred treatment method [1]. This option has the potential to cease the broadly distributed in Bulgaria landfilling of sludge, or stockpiling it in the backyards of the WWTP. The procedure for Bulgaria is defined in "Ordinance on the order and the way of recovery of sludge from waste water treatment through its use in the agriculture (adopted with CM Decree 339/14.12.2004, SG112/23.12.2004)" [2].

Under the Ordinance, an essential requirement to fulfill before sludge utilization is to ensure protection to human health. Account must be taken of the nutrient needs of the plants, without impairing neither the quality of the soil nor that of surface waters and groundwaters, thus indirectly impairing human health.

The agricultural utilization of sewage sludge has to be regulated carefully, as sludge contains microorganisms that could pose a health hazard to humans [3]. The types of organisms present in the sludge are determined by the microbiological quality of the wastewater, from which the sludge is generated. These organisms include bacteria, viruses, protozoa and helminthes [4]. Three groups of bacteria are included, *Escherichia coli*, *Clostridium perfringens* and *Salmonella* spp., as sanitary-bacteriological indicators in the Bulgarian "Ordinance on the order and the way of recovery of sludge from waste water treatment through its use in the agriculture", to limit the microbiological health risk in connection to land use of sludge.

Material and Methods

This study presents the results from sanitary-bacteriological analyses of 288 sludge samples from 43 WWTP in Bulgaria, investigated in NCPHA within the period 2006-2015. Details of the investigation are discussed for sanitary-microbial contamination, followed by statistical generalization and interpretation of the environmental health risk.

The spatial distribution of the urban WWTP, from which the sewage sludge samples were investigated in NCPHA is presented in Fig. 1. The number of analyzed representative sewage sludge samples per year for the period 2006-2015 is shown in Fig. 2.

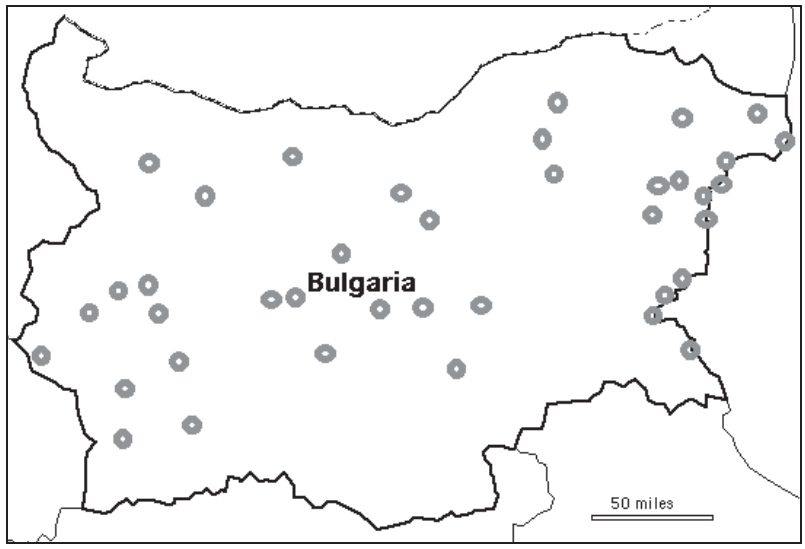

Fig. 1. Spatial distribution of the urban WWTP, from which sewage sludge samples were investigated in NCPHA for sanitary-bacteriological indices within the period 2006-2015 (*Outline map source: http://www.enchantedlearning.com*)

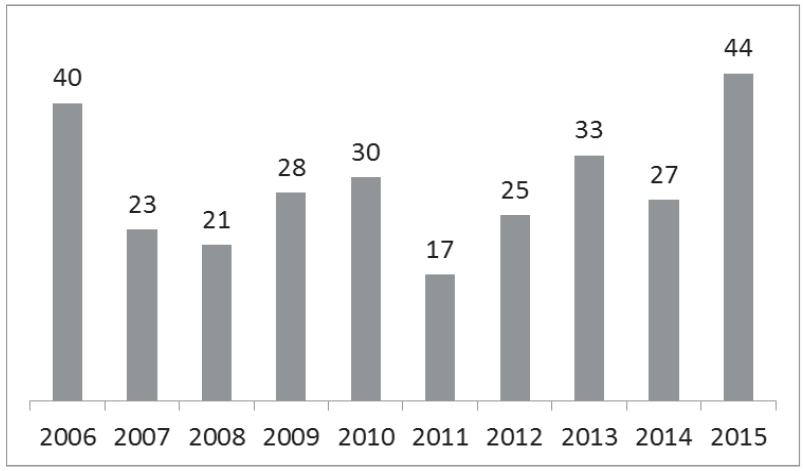

Fig. 2. Number of analyzed representative sewage sludge samples in NCPHA per year for the period 2006-2015

For each sewage sludge sample, a sampling protocol was provided, followed by a common laboratory protocol with the estimated analytical results. The determination of the sanitary-bacteriological indices involved a wide spectrum of accredited protocols. The presence of *E. coli*, *C. perfringens* and *Salmonella* spp. is determined through the adopted in NCPHA "Interlaboratory Method 02.01.: Soil, Biowaste and Sewage Sludge. Determination of Microbiological Indicators" [5].

Results and Discussion

Table 1 presents the results from the bacteriological investigation for presence of sanitary-indicator microorganisms in the sewage sludge. The data obtained reveal the main characteristics of the titer variables and their distributions, compared to the limit permissible content for agricultural utilization, stated in "Ordinance on the order and the way of recovery of sludge from waste water treatment through its use in the agriculture (adopted with CM Decree 339/14.12.2004, SG112/23.12.2004)", which determines the level of environmental health risk.

Table 1. Results from the bacteriological investigation for presence of sanitary-indicator microorganisms in the sewage sludge

N - number of analyzed sewage sludge samples; min – minimal analyzed titer value; max – maximal analyzed titer value; LPC - limit permissible content in sewage sludge for agricultural utilization

Statistical Index	*Escherichia coli*	*Clostridium perfringens*	*Salmonella* spp.
N	257	288	288
min titer.g^{-1}	0.0000001	0.000001	-
max titer.g^{-1}	>1.0	>1.0	-
Share of samples with titer >1	61.9%	22.2%	-
Share of samples with titer = 1	1.2%	5.2%	-
Share of samples with titer from 0.9 to 0.01	10.9%	21.2%	-
Share of samples with titer below 0.009	26.0%	51.4%	-
Share of samples with absence of *Salmonella* spp. (in 20 g)	-	-	100%
LPC for agricultural utilization	>1.0 titer.g^{-1}	>1.0 titer.g^{-1}	not to be isolated in 20 g

Concerning the sludge content in titer.g^{-1} for *E. coli* and *C. perfringens*, the results in Table 1 are presented correspondingly to the criteria for determining the sanitary condition of soils (in titer) according to a national reference document [5]. Qualitative scales are used for the presence of *E. coli* and *C. perfringens* - in three rank groups as follows:

1) samples meeting the requirements for agricultural utilization (titer.g^{-1} > 1.0 for *E. coli* and *C. perfringens*);

2) samples corresponding to slightly contaminated sewage sludge (titer.g^{-1} from 1.0 to 0.01 for *E. coli* and *C. perfringens*);

3) samples corresponding to severely contaminated sewage sludge (titer.g^{-1} ≤ 0.009 for *E. coli* and *C. perfringens*).

Salmonella spp. is not isolated in all 288 investigated sludge samples, which is a positive result from sanitary-bacteriological point of view. *E. coli* and *C. perfringens* are met in different proportions within the rank groups.

Table 2 displays the summarized data about the percentage of sludge samples, which have fulfilled the sanitary requirements for limited presence of *E. coli* and *C. perfringens*, when the sludge is utilized in agriculture. While with *E. coli*, still 61.9% of the sludge samples stand within the limit, the presence of *C. perfringens* is beyond the allowed limit in 77.8% of the sludge samples, which practically hinders the direct agricultural utilization of a big share of the sewage sludge from WWTP, generated in Bulgaria.

Table 2. Comparison of the presence of *E. coli* and *C. perfringens* in the sludge samples with the national requirement for agricultural utilization

Sanitary-bacteriological index	Total number of samples N	Percentage of samples with:		Requirement for agricultural utilization (titer.g^{-1})
		titer.g^{-1} > 1.0	titer.g^{-1} ≤ 1.0	
E.coli	257 (100%)	61.9%	38.1%	> 1.0
C. perfringens	288 (100%)	22.2%	77.8%	> 1.0

Only 21.5% of all investigated sewage sludge samples fulfill the sanitary-bacteriological requirements for direct utilization in agriculture, when taking in account both *E. coli* and *C. perfringens* upper limits for their presence in the sludge (samples with titer.g^{-1} above 1.0 for both *E. coli* and *C. perfringens*, Table 3).

Table 3. Combined comparison of *E. coli* and *C. perfringens* presence in the sludge samples with the national requirement for agricultural utilization

Sanitary-bacteriological index	Total number of samples N (%)	Percentage of samples with:		Requirement for agricultural utilization (titer.g^{-1})
		titer.g^{-1} > 1.0 for both *E. coli* and *C. perfringens*	titer.g^{-1} ≤ 1.0 for either *E. coli* or *C. perfringens*	
E. coli and *C. perfringens*	257 and 288 (100%)	21.5%	78.5%	> 1.0 for both bacteria

Conclusions

1. The sanitary-microbiological indicators are with major hygienic significance in order to assure health and safety both for the general population and workers, when applying sewage sludge in agriculture.

2. Sanitary-indicator microorganisms persist in 78.5% of all analyzed sewage sludge samples in quantities, exceeding the permitted values for agricultural utilization of the sludge.

3. The results from the microbiological analyses prove that the currently applied methods for stabilization of sewage sludge prior to agricultural use, experience difficulties in providing sufficient sanitization and minimizing the risk of pathogenic bacterial presence. Further technological treatments are needed, in order to improve the sanitary quality of the sewage sludge.

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