# 11TH "SEMINAR OF ECOLOGY – 2018"

with international participation

**Section „Biology“ – Union of Scientists in Bulgaria**
**Institute of Biodiversity and Ecosystem Research –**
**Bulgarian Academy of Sciences**

# 11th „Seminar of Ecology – 2018“, with international participation Proceedings

**26-27 April**
**Sofia, Bulgaria**

Този сборник съдържа доклади, изнесени на 11-ти „Семинар по Екология – 2018“, с международно участие, проведен на 26-27 април 2018 г. в Институт по биоразнообразие и екосистемни изследвания – Българска академия на науките, 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 – Българска академия на науките, гр. София, България, с любезната финансова подкрепа на фирмите БУЛГАП ЕООД; Л.К.Б – България ЕООД и Издателство Пенсофт ЕООД. Публикуваните в Сборник 11-ти „Семинар по Екология – 2018” материали са рецензирани и редактирани.

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От редакторите

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The editors

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**CONTENTS:**

**SHORT COMMUNICATIONS**

**Topic:**
**ECOSYSTEM RESEARCH, SERVICES AND ECOLOGICAL AGRICULTURE**

**REPORTS**



**SHORT COMMUNICATIONS**



**Topic:**
**LANDSCAPE ECOLOGY**

**REPORTS**



**Topic:**
**ECOLOGY AND EDUCATION**

**REPORTS**

# БЪЛГАРСКА АКАДЕМИЯ НА НАУКИТЕ

**До**
**Организационния комитет**
**на "Семинар по екология - 2018"**

**Уважаема проф. ЧАНКОВА**
**Уважаема доц. ГАНЕВА**
**Уважаеми участници, скъпи гости, дами и господа,**

**За мен е удоволствие да присъствам на този научен форум посветен на екологията, който стана традиция за ИБЕИ и СУБ. Екологията се превърна в много важен сектор на научното познание за изследване на околната среда и човешкото влияние Изменението на климата и глобалните процеси в земната биосфера, демографските промени, социалните и икономически фактори са може би най-голямото предизвикателство пред съвременната научна общност. Замърсяването на въздуха, деградацията на почвата, опустиняването и обезлесяването очертават необходимостта от търсене и прилагане на адекватни подходи за преодоляване негативните последици от човешката дейност и за постигане на благоприятна и устойчива околна среда.**

**Семинарът по екология дава възможност за изява както на видни учени, така и на млади участници – докторанти и студенти, да обменят опит и знания в различни области на екологията и биологията. За изминалите години, той се утвърди като трибуна 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. С всяка година отбелязваме и увеличен интерес на международната научна общност.**

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

**С уважение,**

**София**
**26.04.2018 г.**

**проф. дбн Димитър Иванов,**
**Научен секретар, БАН**

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ДО
УЧАСТНИЦИТЕ В XI-ИЯ НАУЧЕН
„СЕМИНАР ПО ЕКОЛОГИЯ - 2018"
С МЕЖДУНАРОДНО УЧАСТИЕ

УВАЖАЕМИ ГОСПОЖИ И ГОСПОДА,

УВАЖАЕМИ КОЛЕГИ,

С искрено удоволствие се отзовавам на поканата от името на Съюза на учените в България да поздравя участниците в станалия вече традиционен ежегоден Семинар по екология с международно участие.

Залегналата в семинара актуална проблематика предполага за поредна година участие на широк кръг от специалисти, млади учени, докторанти и студенти.

Основните проблеми на околната среда у нас са свързани със замърсяването на водите, въздуха, почвите и изхвърлянето на големи количества промишлени и битови отпадъци. Проблемите са много сериозни, имат интердисциплинарен характер и изискват бързи и адекватни решения.

Важен проблем за нас е и ниската екологична култура на нашето общество и слабото сътрудничество по въпросите на екологията между научната общност, обществените организации и управляващите държавни органи.

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Нашите действия в тази насока се изразяват чрез приетите форми на дейност, а именно: провеждане на тематично насочени конференции, симпозиуми, семинари, лектории, дискусии и др. и даване широка гласност на възникналите проблеми и на възможностите за тяхното решаване. Чрез компетентност, добре обосновани становища и постоянство можем да убеждаваме и налагаме своите предложения.

Нека да има повече такива изяви и форуми, а добрите идеи и творчески инициативи да не секват!

На всички участници в семинара пожелавам успешна и ползотворна работа!

София, 26 април 2018 г.

проф. д.б.н. Диана Петкова,
председател на СУБ

Добре дошли уважаеми колеги, участници и гости на 11-тия пореден „Семинар по Екология – 2018" с международно участие!

Известно е, че човечеството навлезе в 21-ви век с много екологични проблеми, произтичащи от задачите на високо индустриалното общество.

Благородната цел да се повиши жизнения стандарт на хората доведе до засилена интензификация на различни отрасли на промишлеността и все по-широка химизация на бита и селското стопанство.

Въпреки обстоятелството, че индустриализацията и химизацията осигуряват многобройни предимства, няма съмнение, че те замърсяват основните жизненоважни матрици в природата – въздух, почви, води, утайки и водят до негативни последици за природата и здравето на човека - нарушаване на климатичното равновесие в резултат от освобождаването в атосферата на вредни емисии; замърсяване на повърхностните, подпочвените води и световния океан и силно влошаване качеството на питейните води; намаляване и замърсяване на обработваемите земеделски площи; генетични промени на клетъчно и организмово ниво; обедняване на биологичното разнообразие; влошаване на жизнената среда в градовете, което води до нежелателни последствия за здравето на хората.

С всеки изминат ден обемът на антропогенното въздействие се разширява, то става все по - многообразно и опасно.

Според Chemical Abstract (http://www.cas.org) повече от 25 милиона химични вещества са регистрирани до момента, като ежегодно в околната среда постъпват повече от 10 000 синтетични химични вещества.

Как биотата реагира на тези въздействия?

Известен научен факт е, че в процеса на еволюцията живите организми са развили различни механизми на адаптация, за неутрализиране и справяне с нарастващото количество вредни за тяхното нормално функциониране фактори.....

А как реагират хората и какви мерки трябва да се предприемат?

Може би частични отговори на тези въпроси ще дадем и потърсим на настоящия форум.....

Желая успех на всички участници!

26.04.2018 Проф. Стефка Чанкова

# REPORTS

## Topic:
## BIODIVERSITY AND CONSERVATION BIOLOGY

# RED DEER (*CERVUS ELAPHUS* L.) POPULATION DENSITY IN A HUNTING AREA IN THE CENTRAL BALKAN MOUNTAINS (BULGARIA) REVEALED BY CAMERA TRAPS

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**Abstract**

**Aim:** The aim of this study is to apply the random encounter model (REM) for estimation of the population density of red deer in a mountainous hunting area based on camera trap data.

**Materials and Methods:** 38 camera traps were set up in the State Hinting Enterprise "Rositsa", on the Northern slopes of the Central Balkan Mountains, Bulgaria in July 2017. A total of 702 camera trap days were accumulated.

**Results:** The results indicate a population density of 4.72 ind./km$^2$, lower than the reported by the official census, but above the average for red deer populations in other parts of Europe. The sex ratio in the population is 1 male: 6.4 females.

**Conclusion:** The applied method of estimating population density based on camera trap data is cost-efficient, requires limited field effort and provides scientifically sound results. The observed high population density is due to the measures taken in the Hunting Enterprise. The disturbed sex ratio is caused by the current hunting practices (i.e. hunting mainly male individuals), which if not altered will lead to further development of this trend.

**Keywords:** red deer, camera traps, random encounter model

## Introduction

Monitoring mammal populations is a key aspect of their management and conservation, often required by legislation and used to determine hunting quotas for game species. All of this determines the need for exact and reliable data, based on scientifically sound approaches. Animal abundance estimates also enhance understanding of various

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ecological processes, such as predator-prey relationships, competition, expansion, and disease transmission [1].

The red deer (*Cervus elaphus* Linnaeus, 1758) is a popular game species throughout Europe. In Bulgaria, red deer hunting is primarily aimed at male individuals. The hunting quotas are skewed towards more males (for 2017 the planned harvest ratio was 1.3 males: 1 female) and are fulfilled almost completely only for adult males. To facilitate the successful management of the species, reliable monitoring of the population abundance and density is needed. However, the currently utilized method by the hunting and forestry units – the spring census, is not based on a standardized and rigorous approach [2].

A number of direct and indirect methods for estimating ungulate population size exist. Direct approaches include counting the detected individuals via drive, foot or aerial counts. Indirect counts are based on signs left by the animal's activity, such as dung, tracks or signs of feeding [3]. However, most of these methods are very labor-intensive and/or costly.

Camera trapping has been used extensively in recent years for various research purposes throughout the globe. Despite the initial investment of funds, their maintenance and operation is cost-efficient and does not require considerable field effort. The recently developed random encounter model (REM) utilizes camera trap data to estimate population density with accurate results [4]. The REM has been used to study red deer in Iran [5] and Sweden [3], where the authors conclude that it is reliable and precise and can be used to substitute other monitoring approaches. The method is also widely applied to other ungulate species in many parts of the world. It has recently been tested in Bulgaria: on roe deer (*Capreolus capreolus* L.) in the Western Rhodope Mts., where the results correlate with those obtained with classical approaches – snow tracking and direct observations [2], and on roe deer and wild boar (*Sus scrofa* L.) populations in Osogovo Mt. in relation to grey wolf (*Canis lupus* L.) predation and pack size [6].

In this paper we present the first application of the random encounter model to a red deer population in Bulgaria – specifically, the population in a hunting area in the Central Balkan Mountains. Furthermore, we present analysis regarding the sex ratio in the population and comment on the factors that shape these two parameters.

## Material and Methods

*Study area*

The study was based on the territory of the State hunting enterprise (SHE) "Rositsa" – Lagat, located on the Northern slopes of the Central Balkan Mountains, Bulgaria. Its area is 250 km$^2$, covered almost entirely by forests (98%), of which 82% deciduous and 18% coniferous. The most widely distributed species here are the European beech (*Fagus sylvatica* L.), Scots pine (*Pinus sylvestris* L.) and common hornbeam (*Carpinus betulus* L.). The altitude in the enterprise ranges between 250 and 1430 m.a.s.l. SHE "Rositsa" borders two other state hunting enterprises ("Rusalka" and "Mazalat") and the Central Balkan National Park. It is a popular hunting area for red deer (*Cervus elaphus* L.), roe deer (*Capreolus capreolus* L.), wild boar (*Sus scrofa* L.), fallow deer (*Dama dama* L.) and grey wolf (*Canis*

*lupus* L.). Supplementary feeding is provided regularly on the territory of the enterprise during the winter months, and measures are taken to minimize poaching.

*Camera trap data and analysis*

Between July 10th and August 4th, 2017 38 camera traps (Bestguarder DTC-880V) were set up in the field, located at an approximate distance of 1 km from each other. The camera traps were programmed to take 3 consecutive pictures (5 seconds apart) and a 10-sec video upon triggering, with an interval of 1 min between consecutive triggerings. The resulting photos and videos were analyzed through CameraBase 1.6 [7], adapted and translated in Bulgarian (Zlatanova, unpublished). Photos showing a prolonged stay of an individual in front of the camera trap were considered one independent event (also referred to as independent registration). The data were analyzed with the random encounter model (REM) for estimating animal density using camera traps without the need for individual recognition [4], based on modeling the rate of the contacts between the animals and the camera trap. It includes characteristics of the species: mobility (average daily distance travelled, taken from published telemetry studies) and the average number of individuals in a group (taken from the camera trap registrations); and the used models of camera traps: angle and radius of the detection zone of the camera trap (taken from the model specifications).

This approach was applied to the data in two ways:

1. Estimation of the total population density based on the pooled data of all red deer registrations. For this estimation, a correction of the REM formula was used [4], taking into account the groups red deer form – the estimated density derived from the number of independent registrations (in essence, the density of the observed *groups*) was multiplied by the mean number of individuals observed in a group;

2. Separate estimation of the population density of males and females (> 1-year-old). This was done to measure the sex ratio in the population. Each recorded individual in an independent registration was counted separately and assigned to one of the 2 categories. Thus, the number of observed males and females represent the density of their sex, without the need for corrections related to group size. Unidentified individuals were excluded from this estimation.

**Results**

A total of 702 camera trap days were accumulated, with 508 independent registrations of red deer during this study (Fig. 1, Table 1). Taking into account these results, the mean mobility of the species [8] and the mean number of observed individuals in a registration (ranging from 1 to 8, mean = 1.52 ind./group), a total population density of **4.72 ind./km²** was estimated. The estimated population density of males, females and juveniles/subadults is presented in Table 2 (excluding the unidentified individuals). The male to female ratio is 1:6.42.

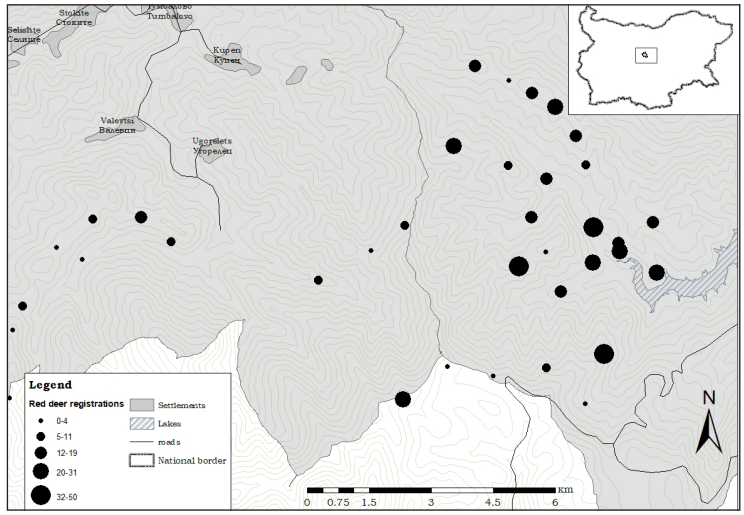


**Fig. 1. Map of the study area and the camera trap locations with number of independent red deer registrations**

**Table 1. Number of registered red deer individuals by sex, age, and habitat**

| individuals/habitat | coniferous forest | open habitat/ meadow | scattered vegetation | mixed forest | deciduous forest | Total |
|---|---|---|---|---|---|---|
| **Female** | 15 | 109 | 8 | 2 | 30 | **164** |
| **unidentified** | 12 | 101 | 10 | 1 | 21 | **145** |
| **1 female+juv./SA** | 15 | 60 | 5 | 3 | 8 | **91** |
| **Male** | | 22 | 1 | 2 | 8 | **33** |
| **juv./SA** | | 18 | 1 | | 10 | **29** |
| **2 females+juv./SA** | 3 | 9 | | | 6 | **18** |
| **larger group** | | 11 | 1 | | 3 | **15** |
| **male+female** | | 11 | 1 | | 1 | **13** |
| **Total** | **45** | **341** | **27** | **8** | **87** | **508** |

***juv. = juveniles, SA = subadults**

**Table 2. Number of registrations and estimated densities, estimated separately for males, females and juveniles/subadults**

| Sex/age | Number of registrations | Density (ind./km$^2$) |
|---|---|---|
| **males** | 62 | 0.38 |
| **females** | 398 | 2.43 |
| **juv./SA** | 179 | 1.09 |

## Discussion

*Accuracy of the method and other considerations*

The REM is based on a number of parameters, which need to be accurately estimated to obtain precise results. Camera trap characteristics taken from the model's specifications might be inaccurate, so additional field tests may be beneficial. Regarding the mobility of the studied species, telemetry data from the same region and season would provide the best estimate. However, such data is not always available, which brings bias in the results. There have been recent advances in determining speed of movement from camera trap data which might solve this problem [3]. Furthermore, the mobility of individuals from different age and sex classes might differ, although there is no indication of this in the literature [9]. Additionally, for group-living species, the number of individuals per groups should be independently assessed, which would be most successfully achieved through direct observations. Despite this, the random encounter model is relatively easy to apply and can be widely utilized for monitoring ungulate game populations.

*Population density*

Following a review publication [10] which defines three density classes, high (>5 red deer/km$^2$), medium (2–5 red deer/km$^2$) and low (< 2 red deer/km$^2$), our study population falls within the medium category (4.72 ind./km$^2$). According to the normative act regulating the inventorying and planning of forest territories in Bulgaria (Regulation 18/7.11.2015), the recommended population density for habitats of quality II (such as our study area) is 1.5-1.9 red deer/km$^2$. The official census reports a high population density of 6.64 ind./km$^2$ for the focal area and 4.55 ind./km$^2$ for the whole hunting enterprise (including the unsuitable habitats for red deer). These results are based on spring census counts and thus likely not accurate [11]. The staff of the State Hunting Enterprises in Bulgaria is required by the law to perform observations in their designated areas throughout the whole year and additionally during the breeding periods for red deer. However, this method often provides questionable results, since counting all individuals in a population is not possible due to the existence of „shy" animals that cannot be observed. Furthermore, some individuals may be counted multiple times by different observers which also brings bias in the estimates.

An older study (from 1981) for the area estimates densities of 2.0 ind./km$^2$ in summer and 3.5 ind./km$^2$ for winter [12]. There are no published studies on the topic since then, but most likely the population dynamics in the enterprise follow the trends for the whole country – increased population density in the 80s, followed by a sharp decrease around 2000 and subsequent recovery [13].

Red deer densities reported in other parts of Europe vary widely – from 0.08 ind./km$^2$ in Norway [11] to 42.6 ind./km$^2$ in Scotland [14], however, most authors report values between 0.5 and 7.0 ind./km$^2$. Habitat and climate, as well as the influence of predators, competitors, hunting and management regimes contribute heavily to these differences. For Bulgaria, only a limited number of studies on this topic exist: in State Hunting Enterprise "Palamara" [15] the reported density is 10 ind./km$^2$, a density which is described as the maximum capacity for breeding animals for trophy hunting.

In managing game populations, the aim is often to maximize density in order to enhance hunting opportunities. However, it is important to take into account that populations exceeding the carrying capacity of the habitat may have detrimental effects on other species or the habitat itself. Studies indicate that red deer density above 5 ind./km$^2$ can cause a negative impact on forestry. With increased density, the body mass of large herbivores typically declines, which affects individual performance traits like age of first reproduction and juvenile survival [17]. Population density below this threshold leads to an increase in the relative abundance of calves and the proportion of females (including 2-year-olds) participating in reproduction [16]. Thus, the value estimated for our study area (4.72 ind./km$^2$) falls within the optimal range.

The rich natural food base enhanced by management practices, the complete elimination of poaching on the enterprise's territory and the supplementary feeding during winter contribute to the wellbeing of the red deer population, which is one of the most abundant in the country. An additional advantage is the fact that SHE "Rosica" – Lagat is a part of a large complex of territories, including a National Park, surrounded by large game hunting enterprises, which ensure a vast non-fragmented habitat for the red deer. An interesting aspect for further investigation is the intra-specific relationship between the red deer and the roe deer (*Capreolus capreolus* L.). This smaller and subordinate ungulate is relatively less abundant in the enterprise compared to other parts of the country, most likely due to the competitive pressure of the larger and dominant red deer. The behavioral specifics of this coexistence are still largely understudied [18].

*Sex ratio*

According to the published literature, a typical sex ratio for red deer populations not subject to predation or culling is biased towards females, most frequently between 1.5-2 females per male [19]. Globally, a limited number of studies explicitly state the established ratios in the focal populations. However, data from Lithuania indicate male-female ratios between 1:3.3 and 1:1.4 [16]. An older study (from 1981) for the SHE "Rositsa"-Lagat estimates a sex ratio of 1 male: 2.6 females [12]. The author suggests that the optimal ratio for the breeding of well-developed males would be skewed towards more males (1.1-1.3 males: 1 female) and comments on the disturbed ratio his results indicate.

The observed results from our study (1 male: 6.42 females) are much higher, which is most likely due to the current hunting strategy, aiming predominantly at male individuals. Despite the reported from the Bulgarian Executive Forest Agency census data for the country that indicate a sex ratio of 1 male: 1.3 females, 2.17 times more males than females were harvested in 2017. A study in Norway found that the sex ratio increases nearly fourfold from a non-harvested to harvested population (from 1.01 female to 3.9 female per male respectively) [20]. This is also supported by the works of Bulgarian authors [21–23].

A heavily biased ratio like this may cause a number of direct or indirect changes in the population's demography and reproduction such as increase of the proportion of resident males that hold harems during the mating season; decline of the mean age of harem-holding males; reduction in the frequency of fights between stags; decrease in the productivity of the population through a decrease in pregnancy rates and delays in parturition, all of which should be taken into account when planning sustainable hunting

strategies [20, 24, 25]. A study on highland red deer in Scotland concludes that in places where females are subject to lower culling rates than males (such as our study hunting enterprise), increasing the proportion of culled females can lead to significant increase in the number of mature males in the population, a direct result of the improved male survival. A review study on the official census data for the ungulates in Bulgaria [26] also lists the disturbed sex ratio as one of the main threats to their populations.

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# NEW DATA ON MORPHOLOGY AND TAXONOMY OF THE FISHES FROM GENUS *ALBURNOIDES* (ACTINOPTERYGII, CYPRINIDAE) IN SOUTH-EASTERN BALKANS (AEGEAN AND BLACK SEA BASINS)

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**Abstract**

It has been considered for a long time that genus *Alburnoides* in Europe is represented by only one species – *Alburnoides bipunctatus*. Recent molecular study gives new light about the taxonomy of the genus and reveals 17 different Eurasian lineages. Four of them inhabit the studied territory and need detailed morphological description.

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**Aims:** The aim is to study the morphology of the main *Alburnoides* populations in the rivers flowing into Aegean and Black Seas and situated on the territory of the South-Eastern Balkans.

**Material and Methods:** A total of 182 specimens from eleven localities are examined. The localities are situated in seven different river basins on the territory of three countries – Greece, Bulgaria and Turkey. A total of 22 morphometric and 5 meristic characters are used in the study.

**Results:** The study demonstrates differences in the morphology of the four different molecular lineages and describes them well as a distinct species.

**Conclusion:** The pattern of morphological variation well corresponds to the published molecular lineages and supports the taxonomic validity of the species *A. tzanevi, A. strymonicus*, *A. thessalicus* and *A. economoui*.

**Keywords**: *Alburnoides,* morphology, taxonomy, Balkan Peninsula

## Introduction

The spurlins from genus *Alburnoides* Jeitteles, 1861 are small fish, which inhabit mainly the middle courses of fast flowing rivers, rich in oxygen. They are widespread in the temperate Eurasian rivers and some lakes, part of the basins of North, Black and Sea of Azov as well as in Caspian Sea and the middle course of the Volga River, south to Kura drainage. They occur also in Amu Darya drainage, part of Aral Sea basin, as well as in the basins of Euphrates and Tigris. In the Mediterranean the distribution covers Rhone and many smaller rivers from France eastward to Genova (Italy). Spurlins could be found also in the Drin River, Skadar, Prespa and Ohrid Lakes and some other rivers in Albania, as well as rivers in the Eastern part of the Balkan Peninsula and the Western Anatolia, flowing into the Aegean and Marmara Seas [1, 2, 3, 4].

It has been considered for long time that the genus *Alburnoides* in Europe and parts of Asia is represented by only one species – *Alburnoides bipunctatus*. With the advance of genetic research and modern statistical interpretation of the data, a lot of new taxa inside of the genus have been described for the last 10 years and nowadays more than 30 species are included in *Alburnoides* [5].

In the surveyed area the spurlin has been reported for the first time (as *Alburnus bipunctatus*) from the Treklyanska River, a tributary of Struma [6], as well as in the Vardar River [7]. Later Chichkoff found spurlins in the Rezovska River and described them as a new subspecies – *A. b. tzanevi* [8]. The same author conducted the first detailed morphological description of spurlins in the area and described another subspecies – *A. b. strymonicus*, from the Mesta and Struma Rivers [9]. He presumes that the subspecies *A. b. strymonicus* should be the one distributed in the Vardar River.

According to Drensky the subspecies *A. b. tzanevi* is a junior synonym of the subspecies *A. b. rossicus,* but he does not provide any evidence to prove it [10].

Berg claims that the spurlins from the Struma and Vardar Rivers are identical and they should belong to one of the already described subspecies *A. b. fasciatus* or *A. b. smyrnaeus* [1]. According to him the spurlins from the Rezovska River belong to the infrasubspecific taxa *A. b. fasciatus* natio *tzanevi*.

Stephanidis described the taxa *Alburnoides bipunctatus* var. *thessalicus* from the rivers Pinios and Sperchios [11].

According to Drensky there are three subspecies of spurlins in Bulgaria: *A. b. bipunctatus* – in the upper courses of the rivers part of Danube River basin, including Ogosta, Iskar, Vit, Osam and Yantra; *A. b. fasciatus* – in the rivers, which are flowing directly into Black Sea, i. a. Kamchia, Veleka and Rezovska and *A. b. strymonicus* – in Struma and Mesta Rivers [12].

An overall revision of the genus *Alburnoides* in Bulgaria has been made by Marinov [13]. Using statistical processing of the data from the morphological survey he stidied the spurlins from all three river catchments in the country – Danube River, Black Sea and Aegean Sea. He made the following conclusions:

- The subspecies *A. b. tzanevi* and *A. b. strymonicus* are morphologically identical with the nominant subspecies;
- There is no evidence that *A. b. tzanevi* is a junior synonym of *A. b. fasciatus*;
- *A. b. fasciatus* does not occur in Bulgaria;
- The only subspecies, distributed in all rivers of the country is *A. b. bipunctatus.*

Wherefore, after Marinov's revision only the subspecies *A. b. bipunctatus* exists in the Bulgarian ichthyological literature.

The recent study by Stierandová et al. [14] gives new light about the taxonomy and distribution of spurlins in Europe. It is based on detailed comparative sequencing analysis of mitochondrial and nuclear markers of all European spurlins. Molecular analysis revealed 17 different Eurasian lineages divided into two main clades, termed the Ponto-Caspian and European in accordance with the lineages distribution. In total, the European clade is represented by 10 monophyletic lineages and populations which are defined as the *Alburnoides prespensis* complex. The paper confirmed that the species richness in the genus *Alburnoides* in Europe is much underestimated. According to the authors the genetic analysis support the validity of 11 morphologically accepted species and four more phylogenetic lineages, which required descriptions as separate species. It is interesting that the distribution of the nominotypical species *A. bipunctatus* sensu stricto is redefined and it covers only a small part of Western Europe. Thus, this species does not occur in the surveyed area, but as a part of the European clade, another four species should be considered for the South-eastern Balkans.

*Alburnoides tzanevi* is the only lineage, which represented the so called South Bulgarian group. There is a strong genetic support for the validity of this species, and it stays well separated in the phylogenetic tree of the genus [see in 14].

There are a total of three distinct lineages in the area, which belong to the so called Aegean group.

*Alburnoides strymonicus* inhabits the rivers Struma and Mesta in Bulgaria and Greece and all genetic data, demonstrated by Stierandová et al. establish its status as a separate species [14]. Another lineage combines individuals from the Aliakmon, Pinios and Vardar river drainages in Greece and FYROM. This lineage is characterized by great haplotype richness and high genetic divergence from the other *Alburnoides* lineages. The last lineage is from the Sperchios River and achieves the highest genetic divergence from all other lineages in the European clade [14]. Its independent species status is confirmed also by a recent barcoding study [15]. These last two lineages are designated by Stierandová et al. as *Alburnoides* sp. 3 and *Alburnoides* sp. 4, respectively, because the name *Alburnoides*

*bipunctatus v. thessalicus* given to the newly described taxa by Stephanidis refers to both populations in Pinios and Sperchios rivers and overlap the distribution of the two species. Later this taxonomic problem was solved by Barbieri et al. as they described the population from Sperchios River as a new species – *A. economoui* and the name *A. thessalicus* remained restricted to the populations from the Pinios, Aliakmon and Vardar rivers [16].

The aim of the paper is to study the morphology of the different *Alburnoides* populations in the rivers flowing into the Aegean and Black Seas and situated on the territory of the South-eastern Balkans.

**Material and Methods**

The main part of the material for the present study has been collected in the period 2010 – 2017 by electrofishing, following the CEN standard (EUROPEAN STANDARD – WATER QUALITY – SAMPLING OF FISH WITH ELECTRICITY/2003) with backpack electrofisher Hans Grassl IG 200-2. The rest of the fishes used for this study are part of the ichthyological collections of the National Museum of Natural History in Sofia, the National Museum in Prague and Naturhistorisches Museum in Vienna.

A total of 182 specimens from 11 localities are examined. The localities are situated in seven different river basins on the territory of three countries as follows: *A. tzanevi* (Veleka and Rezovska rivers in Bulgaria and the Istrancha River in Turkey), *A. strymonicus* (the Struma River in Bulgaria), *A. thessalicus* (Vardar and Aliakmon rivers in Greece) and *A. economoui* (Sperchios River in Greece) (Fig. 1). Fish were fixed in 4% formalin and preserved in 70% ethanol.


**Fig. 1. Sampling localities: 1. Veleka River, near Kachula district (Bulgaria); 2. Mladezhka River, near Mladezhko (Bulgaria); 3. Rezovska River, near Valchanov most (Bulgaria); 4. Istrancha River, near Karamandere (Turkey); 5. Eleshnitsa River, near Drumohar (Bulgaria); 6. Eleshnitsa River, near Vaksevo (Bulgaria); 7. Eleshnitsa River, near Chetirtsi (Bulgaria); 8. Vardar River, between Axiopolis and Fanos (Greece); 9. Vardar River, near Axiopolis (Greece); 10. Aliakmon River, near Elevterochori (Greece); 11. Sperchios River, near Lamia (Greece)**

We have used 22 morphometric and 5 meristic characters for the analysis. Measurements of the morphometric characters were made point to point, by electronic calipers, to the nearest of 0.1 mm. Body length [Bl] is measured from the tip of the snout to the end of the last scale on the caudal peduncle. Head length [hc] and interorbital width [io] were measured to their bony margins. The following abbreviations for the different morphometric characters are used in the text – head length [lc], length of the caudal peduncle [lpc], body depth [H], depth of the caudal peduncle [h], pre-dorsal length [pD], post-dorsal length [poD], pre-pelvic length [pV], pre-anal length [pA], pectoral-pelvic fin origin [P-V], pelvic-anal fin origin [V-A], length of dorsal fin base [lD], depth of dorsal fin [hD], length of the anal fin base [lA], depth of the anal fin [hA], length of the pectoral fin [lP], length of pelvic fin [V], snouth length [prO], eye diameter [do], post-ocular length [poO]. The lateral line scales count includes all pierced scales, from the first one behind the supracleithrum to the posteriormost one. The total number of the lateral line scales is provided in the study. The number of dorsal and anal fin branched rays is counted in a way that the last two branched rays, articulated on a single pterygiophore, are noted as one. The character states of the ventral keel scale cover were estimated as a percents of the keel covered by scales. The number of the vertebrae is examined from radiographs.

All 22 morphometric characters were analyzed using Discriminant analysis (DA). Their number had been decreased down to 9 with „*Forward stepwise*" procedure with *Willks' Lambda* and *F-test* criteria. Only the most informative characters among them are used for the further analysis. These are the characters, which do not correlate with others.

Statistical analyses were performed using Microsoft Excel and Statistica for Windows 7.0 (Stat-Soft, Inc., Tulsa, USA, 1993).

**Results and Discussion**

The comparative morphology study is based on 22 morphometric characters, which are represented as an appendix at the end of the paper, but not all of them are used in the multivariate analysis.

The discriminant function analysis run after the Forward stepwise procedure shows the most informative morphometric characters which best contribute to the discrimination of the analyzed groups on the two canonical axes (Table 1).

**Table 1. Discriminant Function Analysis of the observed characters of four species in genus *Alburnoides***

| N=182 | Discriminant Function Analysis Summary (procenti)<br>Step 12, N of vars in model: 10; Grouping: lineage (4 grps)<br>Wilks' Lambda: ,04751 approx. F (30,496)=30,195 p<0,0000 | | | | | |
|---|---|---|---|---|---|---|
| | Wilks' Lambda | Partial Lambda | F-remove (3,169) | p-level | Toler. | 1-Toler. (R-Sqr.) |
| hc | 0,069757 | 0,681065 | 26,38027 | 0,000000 | 0,857836 | 0,142164 |
| do | 0,083144 | 0,571405 | 42,25408 | 0,000000 | 0,812164 | 0,187836 |
| io | 0,053145 | 0,893942 | 6,68342 | 0,000273 | 0,740848 | 0,259152 |
| prO | 0,056218 | 0,845086 | 10,32654 | 0,000003 | 0,779322 | 0,220678 |
| pA | 0,059444 | 0,799219 | 14,15211 | 0,000000 | 0,721957 | 0,278043 |
| h | 0,055771 | 0,851860 | 9,79649 | 0,000005 | 0,892085 | 0,107915 |
| lA | 0,054923 | 0,865011 | 8,79105 | 0,000019 | 0,562972 | 0,437028 |
| lV | 0,055265 | 0,859652 | 9,19705 | 0,000011 | 0,769696 | 0,230304 |
| lD | 0,056459 | 0,841475 | 10,61263 | 0,000002 | 0,616655 | 0,383345 |
| P-V | 0,053542 | 0,887322 | 7,15358 | 0,000150 | 0,754634 | 0,245366 |

The obtained results of the DA, based on the morhometric characters showed that three of the species, included in the analysis and inhabiting the Black Sea and Aegean Sea basins of the South-eastern Balkans, are well defined morphologically (Fig. 2). These are *A. strymonicus, A. thessalicus* and *A. tzanevi*. The specimens from *A. economoui* are somehow spread between the first two species and do not form well a defined cloud on the scatter plot. Although, it possesses the highest interspecific genetic divergence compared with the other species [16], according to the studied plastic characters it doesn't show obvious difference. One of the reasons for that could be the small number of studied individuals (n=9), which doesn't fit the sample size requirements of DA – each sample should possess at least two more entities than variables.

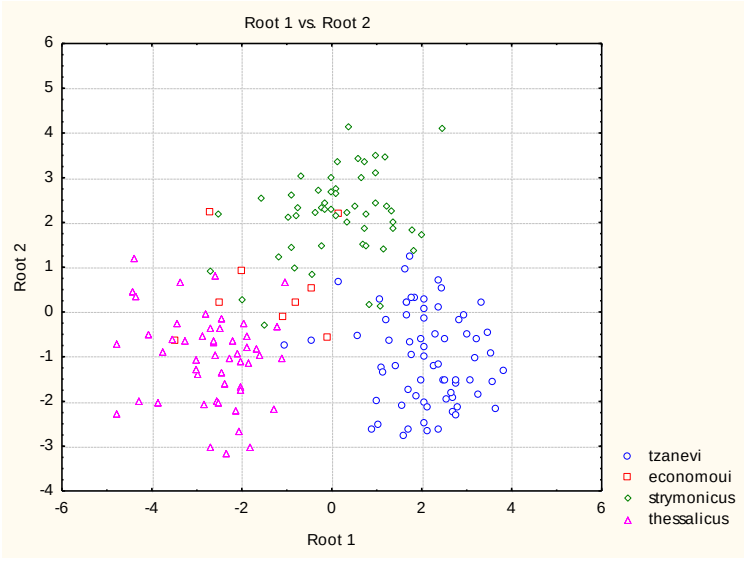


**Fig. 2. Canonical analysis chart of the surveyed characters of the four *Alburnoides* species**

According to the performed analysis the species *A. strymonicus, A. thessalicus* and *A. tzanevi* are well separated. The scatter plot of scores of the individual specimens in the projection of the first two discriminant axes showed that there is almost no overlap between the different groups (Fig. 2). The results confirmed the separation of the surveyed lineages shown by the genetic data [14], which are also well geographically isolated. Although, *A. economoui* is not well separated on the plot, this species is well distinguishable according to the studied meristic characters (see below). It also inhabits the distinctively well isolated region – the Sperchios River in Greece.

The total number of lateral line scales is shown in Table 2. The variations in this character are very high and they are widely overlapping in the different species. The highest variation is observed in the species *A. strymonicus* while highest number of lateral line scales is counted in *A. tzanevi* and *A. strymonicus*, with mean values of 50.03 and 50.72, respectively. The lowest number is found in the species *A. economoui* and *A. thessalicus* (47.89 and 48.49, respectively).

**Table 2. Number of lateral line scales in the surveyed four *Alburnoides* species**

| lateral line scales | 44 | 45 | 46 | 47 | 48 | 49 | 50 | 51 | 52 | 53 | 54 | 55 | 56 | 57 | 58 | n | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *A. tzanevi* | 2 | | 1 | | 6 | 17 | 16 | 13 | 8 | 5 | 1 | | | | | **69** | **50.03** |
| *A. strymonicus* | | | 2 | 5 | 4 | 7 | 11 | 7 | 5 | 4 | 3 | | 2 | 1 | 2 | **53** | **50.72** |
| *A. thessalicus* | | | 2 | 13 | 13 | 11 | 6 | 5 | 1 | | | | | | | **51** | **48.49** |
| *A. economoui* | | | 2 | 2 | 2 | 1 | 2 | | | | | | | | | **9** | **47.89** |

The data about the ventral keel scale cover is presented in Table 3. According to our data, in two of the species the ventral keel is less covered by scales. These are *A. strymonicus*, in which the keel varies between completely naked to half covered by scales (mean 30.70%) and *A. economoui*, in which about half of the keel is covered – between 12.5-66.7% (mean 45.71). In *A. tzanevi* and *A. thessalicus*, the keel is always more than half covered by scales – variation between 50.0-88.9% and 50.0-90.0% respectively and mean values of 67.97% and 73.78%, respectively.

**Table 3. Ventral keel covered by scales (in %) in the surveyed four *Alburnoides* species**

| Keel V-A | limites | mean – st. error | n | st. dev. | coef. var. |
|---|---|---|---|---|---|
| *A. tzanevi* | 50.0 – 88.9 | 67.97 – 1.0378 | 69 | 8.6203 | 74.3098 |
| *A. strymonicus* | 0 – 66.7 | 30.70 – 2.7846 | 53 | 20.2723 | 410.9650 |
| *A. thessalicus* | 50.0 – 90.0 | 73.38 – 1.0832 | 51 | 7.7356 | 59.8389 |
| *A. economoui* | 12.5 – 66.7 | 45.71 – 6.6048 | 9 | 19.8144 | 392.611 |

The numbers of the branched rays in the dorsal fin are represented in Table 4. It is a conservative character and the variations in *Alburnoides* are usualy very small. They are usually 8, but in some species 7, rarely they could vary between 6 to 9 [17]. In our survey we have received similar results. In the species *A. strymonicus* and *A. thessalicus* we found mainly 8 branched rays (mean 7.75 and 7.86, respectively), while in the species

*A. economoui* we found mainly 7 (mean 7.33). In *A. tzanevi* they are almost equally 7 and 8 (mean 7.52) and this is the only species in which we observed six branched rays. Nine branched rays are observed only in *A. thessalicus*.

**Table 4. Number of branched rays in dorsal fin in the surveyed four *Alburnoides* species**

| branched rays in D | 6 | 7 | 8 | 9 | n | Mean |
|---|---|---|---|---|---|---|
| *A. tzanevi* | 2 | 29 | 38 | | **69** | **7.52** |
| *A. strymonicus* | | 13 | 40 | | **53** | **7.75** |
| *A. thessalicus* | | 11 | 36 | 4 | **51** | 7.86 |
| *A. economoui* | | 6 | 3 | | **9** | **7.33** |

The numbers of the branched rays in the anal fin are represented in Table 5. This character was treated for a long period of time as the most diagnosable among the *Alburnoides* representatives. Bogutskaya & Coad found between 8 and 17 rays in the different species, surveyed by them [17]. Such a big variation is not observed by us and we established between 10 and 15 branched rays in the anal fin. The lowest number we found in the southernmost species – *A. economoui* (mean 10.78). In all other species the mean of this character varies between 11.25 and 11.75 (Table 5).

**Table 5. Number of branched rays in the anal fin in the surveyed four *Alburnoides* species**

| branched rays in A | 10 | 11 | 12 | 13 | 14 | 15 | n | Mean |
|---|---|---|---|---|---|---|---|---|
| *A. tzanevi* | 11 | 31 | 26 | 1 | | | **69** | **11.25** |
| *A. strymonicus* | 3 | 25 | 23 | 2 | | | **53** | **11.45** |
| *A. thessalicus* | 2 | 19 | 21 | 8 | 1 | | **51** | **11.75** |
| *A. economoui* | 2 | 7 | | | | | **9** | **10.78** |

The number of the vertebrae is an important character for the subfamily Leuciscinae. Bogutskaya & Coad found betwen 36 and 43 vertebrae in the different species of *Alburnoides,* surveyed by them [16]. In our survey we found lesser variation in this character – between 40-43 vertebrae. A smaller number of vertebrae we found in *A. economoui* and *A. thessalicus* with mean values of 40.67 and 40.77, respectively. The highest number we found in *A. tzanevi* (mean 41.46) and the species *A. strymonicus* has an intermediate position with a mean value of 41.17 (Table 6).

In our survey we divided the vertebrae in abdominal and caudal and counted them separately as well. We found that the number of the abdominal vertebrae varies between 19 and 22 and it is highest in *A. tzanevi* (mean 21.12). In all other species it is considerably lower (mean 20.00) and in *A. strymonicus* we didn't find any variation in this character (Table 6). The number of caudal vertebrae, which we found in the different species is also between 19 and 22. It is highest in *A. strymonicus* (mean 21.17), followed by *A. thessalicus* and *A. economoui* with mean values of 20.77 and 20.67, respectively. The smallest number of caudal vertebrae we found in *A. tzanevi* (mean 20.34).

**Table 6. Number of abdominal, caudal and total vertebrae in the surveyed four *Alburnoides* species**

| | abdominal vertebrae | | | | | caudal vertebrae | | | | | total vertebrae | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **vertebrae** | **19** | **20** | **21** | **22** | **mean** | **19** | **20** | **21** | **22** | **mean** | **40** | **41** | **42** | **43** | **n** | **Mean** |
| *A. tzanevi* | | 1 | 34 | 6 | **21.12** | 2 | 23 | 16 | | **20.34** | | 23 | 17 | 1 | 41 | **41.46** |
| *A. strymonicus* | | 18 | | | **20.00** | | | 15 | 3 | **21.17** | | 15 | 3 | | 18 | **41.17** |
| *A. thessalicus* | 2 | 26 | 2 | | **20.00** | | 8 | 21 | 1 | **20.77** | 9 | 19 | 2 | | 30 | **40.77** |
| *A. economoui* | 1 | 7 | 1 | | **20.00** | | 4 | 4 | 1 | **20.67** | 3 | 6 | | | 9 | **40.67** |

The only species, which possess mainly 21 abdominal vertebrae, is *A. tzanevi*. In all other species the dominating number is 20. In *A. tzanevi* the dominating number of caudal vertebrae is 20, while in *A. strymonicus* and *A. thessalicus* they are mainly 21. In *A. economoui* both 20 and 21 are equally often found (Table 6).

In our survey we paid attention not only to the absolute values of the vertebrae in the abdominal and caudal regions but also to their relative length. In most leuciscine cyprinid fishes the abdominal region is usually 3-4 vertebrae longer than the caudal region, which may be considered as a primitive feature [17]. In the Alburnini tribe the caudal region is relatively elongate and is often longer than the abdominal region. A primitive character state (the difference between the abdominal and caudal region is positive, from +3 to +1, or the caunt are equal) was found by us in *A. tzanevi* (mean +0.78). The most specialized pattern (the difference between the abdominal and the caudal count is negative, -1 to -2) we found in *A. strymonicus* (mean -1.17). Similar state of this character we found also in the species *A. thessalicus* and *A. economoui*, with mean values of -0.77 and -0.67, respectively (Table 7).

**Table 7. Difference between the abdominal and caudal vertebrae number in the surveyed four *Alburnoides* species**

| | **-3** | **-2** | **-1** | **0** | **1** | **2** | **3** | **n** | **Mean** |
|---|---|---|---|---|---|---|---|---|---|
| *A. tzanevi* | | | 1 | 14 | 21 | 3 | 2 | 41 | **0.78** |
| *A. strymonicus* | | 3 | 15 | | | | | 18 | **-1.17** |
| *A. thessalicus* | | 3 | 18 | 8 | 1 | | | 30 | **-0.77** |
| *A. economoui* | 1 | | 4 | 3 | 1 | | | 9 | **-0.67** |

The obtained results clearly demonstrate the differences in morphology of the four different molecular lineages described by Stierandová et al. (2016). The pattern of morphological variation well corresponds to the published molecular lineages and supports the taxonomic validity of the species *A. economoui*, *A. thessalicus*, *A. strymonicus* and *A. tzanevi*. Moreover, they are well geographically isolated and have alopatric distribution, as follows: *Alburnoides tzanevi* inhabits the rivers, flowing in the South-eastern part of Black Sea in Bulgaria and European part of Turkey; *Alburnoides strymonicus* inhabits the rivers Struma and Mesta, flowing into the Aegean Sea in Bulgaria and Greece; *Alburnoides*

*thessalicus* inhabits the rivers Vardar, Aliakmon and Pinios, flowing into the Aegean Sea in FYROM and Greece and *Alburnoides economoui* is endemic for the Sperchios River, flowing into the Aegean Sea in Greece.

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## Appendix: Morphometric data for all species. Abbreviations used in the table: x – mean; m – standard error; n – number of studied specimens

| species | *Alburnoides tzanevi* | | | *Alburnoides strymonicus* | | | *Alburnoides thessalicus* | | | *Alburnoides economoui* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **character** | **limites** | **x – m** | **n** | **limites** | **x – m** | **n** | **limites** | **x – m** | **n** | **Limites** | **x – m** | **n** |
| [Bl] | 52.7 – 102.8 | 72.39 | 69 | 59.3 – 91.6 | 74.08 | 53 | 51.0 – 79.1 | 68.96 | 51 | 59.3 – 78.9 | 66.00 | 9 |
| In % of Bl | | | | | | | | | | | | |
| [lc] | 23.53 – 26.66 | 24.81 – 0.0895 | 69 | 22.81 – 25.58 | 23.76 – 0.0840 | 53 | 23.06 – 25.58 | 24.18 – 0.0813 | 51 | 23.71 – 24.88 | 24.45 – 0.1402 | 9 |
| [lpc] | 22.07 – 26.38 | 23.78 – 0.1087 | 69 | 22.39 – 26.95 | 24.94 – 0.1369 | 53 | 19.65 – 25.32 | 23.44 – 0.1611 | 51 | 22.94 – 24.72 | 23.95 – 0.1634 | 9 |
| [H] | 22.64 – 27.90 | 24.96 – 0.1307 | 69 | 22.85 – 28.27 | 25.81 – 0.1692 | 53 | 23.44 – 28.55 | 25.75 – 0.7131 | 51 | 25.29 – 28.37 | 27.59 – 0.3441 | 9 |
| [h] | 9.20 – 11.47 | 10.19 – 0.0510 | 69 | 9.63 – 11.28 | 10.65 – 0.0503 | 53 | 10.06 – 11.32 | 10.63 – 0.0427 | 51 | 10.75 – 11.60 | 11.13 – 0.1112 | 9 |
| [pD] | 50.82 – 54.94 | 52.74 – 0.1159 | 69 | 49.24 – 53.59 | 51.37 – 0.1242 | 53 | 50.55 – 54.17 | 52.31 – 0.1358 | 51 | 50.07 – 54.45 | 52.33 – 0.4211 | 9 |
| [poD] | 35.65 – 41.19 | 38.52 – 0.1261 | 69 | 37.41 – 41.79 | 39.38 – 0.1150 | 53 | 35.03 – 39.84 | 37.49 – 0.1557 | 51 | 35.87 – 38.70 | 37.65 – 0.3332 | 9 |
| [pV] | 43.29 – 48.46 | 45.90 – 0.1280 | 69 | 42.86 – 47.01 | 45.15 – 0.1121 | 53 | 43.34 – 49.32 | 45.70 – 0.1697 | 51 | 44.18 – 47.07 | 45.40 – 0.2744 | 9 |
| [pA] | 60.48 – 66.06 | 63.16 – 0.1458 | 69 | 59.10 – 64.02 | 61.54 – 0.1318 | 53 | 60.52 – 65.72 | 62.40 – 0.1522 | 51 | 60.70 – 64.26 | 62.58 – 0.4528 | 9 |
| [P-V] | 19.78 – 24.22 | 21.94 – 0.1168 | 69 | 19.61 – 24.34 | 22.33 – 0.1324 | 53 | 19.66 – 23.39 | 21.73 – 0.1131 | 51 | 19.73 – 22.03 | 21.03 – 0.2352 | 9 |
| [V-A] | 16.39 – 21.84 | 18.07 – 0.1038 | 69 | 15.99 – 19.13 | 17.73 – 0.1132 | 53 | 15.10 – 20.31 | 18.03 – 0.1453 | 51 | 16.48 – 19.87 | 18.26 – 0.3676 | 9 |
| [lD] | 10.65 – 14.18 | 12.66 – 0.1008 | 69 | 11.34 – 14.32 | 12.79 – 0.0986 | 53 | 12.12 – 15.56 | 13.47 – 0.0961 | 51 | 13.32 – 14.90 | 14.15 – 0.2016 | 9 |
| [hD] | 19.76 – 24.68 | 22.85 – 0.1236 | 69 | 21.06 – 25.11 | 22.95 – 0.1390 | 53 | 21.15 – 24.85 | 22.87 – 0.1187 | 51 | 22.24 – 24.56 | 23.22 – 0.2944 | 9 |
| [lA] | 13.98 – 18.86 | 16.46 – 0.1267 | 69 | 14.55 – 18.83 | 16.43 – 0.1513 | 53 | 15.34 – 19.63 | 17.43 – 0.1258 | 51 | 15.51 – 18.91 | 16.89 – 0.3951 | 9 |
| [hA] | 16.05 – 20.00 | 18.26 – 0.1026 | 69 | 17.07 – 20.22 | 18.45 – 0.1127 | 53 | 17.14 – 20.06 | 18.55 – 0.0898 | 51 | 16.64 – 19.49 | 18.05 – 0.3022 | 9 |
| [lP] | 17.35 – 21.82 | 19.97 – 0.1185 | 69 | 18.67 – 22.19 | 20.26 – 0.1136 | 53 | 17.82 – 22.06 | 19.89 – 0.1178 | 51 | 18.61 – 21.35 | 20.22 – 0.3064 | 9 |
| [lV] | 14.51 – 18.21 | 16.44 – 0.1043 | 69 | 14.82 – 18.32 | 16.60 – 0.1163 | 53 | 14.51 – 17.53 | 15.87 – 0.0919 | 51 | 14.73 – 18.23 | 16.39 – 0.3975 | 9 |
| In % of lc | | | | | | | | | | | | |
| [hc] | 66.01 – 75.38 | 70.92 – 0.2183 | 69 | 69.93 – 81.14 | 76.04 – 0.3253 | 53 | 70.06 – 80.52 | 75.80 – 0.3382 | 51 | 73.78 – 79.39 | 75.87 – 0.6957 | 9 |
| [prO] | 28.72 – 34.12 | 31.24 – 0.1338 | 69 | 28.05 – 35.58 | 31.69 – 0.2260 | 53 | 27.81 – 32.37 | 29.82 – 0.1487 | 51 | 29.14 – 33.12 | 30.57 – 0.4044 | 9 |
| [do] | 27.74 – 31.76 | 29.58 – 0.1194 | 69 | 26.83 – 32.69 | 29.64 – 0.1719 | 53 | 28.48 – 34.73 | 32.43 – 0.1864 | 51 | 27.88 – 32.20 | 29.44 – 0.4272 | 9 |
| [poO] | 41.42 – 47.94 | 44.93 – 0.1756 | 69 | 42.59 – 48.21 | 45.19 – 0.1699 | 53 | 41.13 – 48.02 | 44.46 – 0.1927 | 51 | 43.90 – 47.59 | 45.82 – 0.4671 | 9 |
| [io] | 30.60 – 37.34 | 33.29 – 0.1872 | 69 | 32.96 – 40.22 | 36.82 – 0.2205 | 53 | 31.46 – 39.55 | 35.67 – 0.2616 | 51 | 31.13 – 38.56 | 34.69 – 0.8290 | 9 |

# ECOLOGICAL STATUS ASSESSMENT OF MOUNTAINOUS AND SEMI-MOUNTAINOUS STREAMS VIA DIFFERENT BIOTIC INDICES BASED ON BENTHIC MACROINVERTEBRATES – THE CASE STUDY OF MACEDONIAN-BULGARIAN CROSS-BORDER TERRITORY

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**Abstract**

**Aim**: The aim of this study was to provide indicative ecological status assessment of mountainous and semi-mountainous streams of a poorly studied cross-border territory, based on benthic macroinvertebrates indices according to the requirements of the EU Water Framework Directive (WFD).

**Material and Methods**: The sampling was conducted in October 2017 at 10 sites. The bottom macroinvertebrates were collected by hydrobiological hand net applying kick & sweep multihabitat procedure. For the indicative ecological status assessment, the following metrics were used: BMWP, ASPT, Irish Q-scheme and EPT.

**Results**: The water quality of the examined sites was assessed from high to moderate (I – III class). Predicting that presence of any kind of habitat degradation or pollutants at any level causes observable changes over benthic communities, a comparison between the affected and clear sites was done.

**Conclusion**: Our results contributed to the knowledge on the current ecological state of some rivers in a poorly studied area and assisted the selection of appropriate metrics for water quality assessment based on macroinvertebrates as BQE.

**Keywords**: Macrozoobenthos, Mountainous and semi-mountainous streams, BMWP, ASPT, Irish Q-scheme, EPT.

## Introduction

The ecological monitoring especially in the aquatic ecosystems requires the development and implementation of the varied biotic indices. Based on the ecological sensitivity and richness of the present taxa, they all classify water quality by numeric expressions. This data processing reduces ecological information, but makes the results more accessible to a non-biologist who needs the data before making decision involving the management of water bodies [1]. In the environmental processes the benthic macroinvertebrates assemblages occupy central role in the aquatic ecosystems (by participating in the decomposition of organic matter; by constituting the major food

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source for other aquatic invertebrates, fishes, and some birds) [2, 3]. Thus, the bottom fauna is the key why they present essential element for their establishment in many biotic indices [4, 5, 6, 7, 8, 9].

According to the Water Framework Directive (WFD), which is obligatory for examining water quality in the EU countries, besides phytoplankton, phytobenthos, macrophytes and fishes, macroinvertebrates are a key element used for water quality assessment [10]. In that order, the aim of this study was to provide indicative ecological status evaluation of mountainous and semi-mountainous streams in a poorly studied Macedonian-Bulgarian cross-border territory, based on benthic macroinvertebrates communities/assemblages.

## Materials and Methods

Bottom macroinvertebrates were sampled at 10 river localities in a cross-border Macedonian-Bulgarian territory (Maleshevska and Ograzhden Mtn.) in October 2017 (Fig. 1, Table 1). The examined river sections were defined as R5 river type – mountainous and semi-mountainous rivers in Eastern Balkans (Ecoregion 7). The sampling sites selection was done considering two main criteria, namely - natural or closed to natural conditions or the presence of a different anthropogenic impact on the freshwater ecosystems.

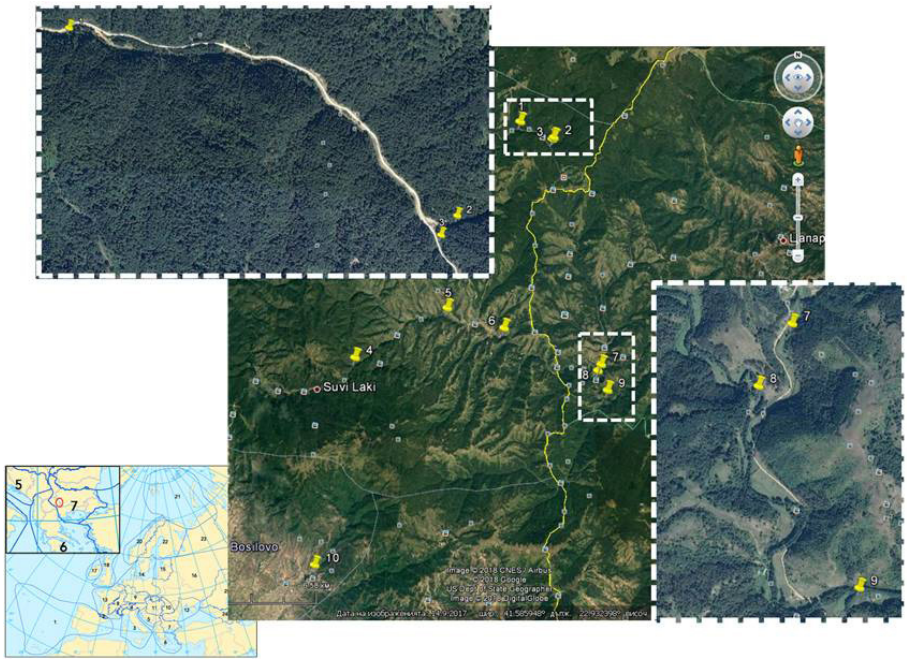


**Fig. 1. View of the study area and location of the sampling sites**

The bottom macroinvertebrate samples were collected with a standard hydrobiological hand net (mesh size 500 μm) applying a kick & sweep multihabitat procedure. The methodology

for collection of bottom fauna was according to EN 28265:1994, EN 27828:1994, EN 9391:1995. At the laboratory macroinvertebrate specimens were elutriated from inorganic substrates, passed through nested 1 mm and 0.5 sieves. All samples were sorted and labeled and the collected taxa identified to the possible lowest level. All collected and analyzed specimens were preserved in 70% ethanol and stored in the Institute of Biodiversity and Ecosystem Research – Bulgarian Academy of Sciences (IBER-BAS), Sofia, Bulgaria.

For the purposes of the current study – indicative ecological status assessment - the following metrics were used: Biological Monitoring Working Party Score – BMWP (Hungarian version), Average Score per Taxon – ASPT (Hungarian version), Irish Quality Rating System – QRS (Adapted version) [12] and EPT index (Ephemeroptera, Plecoptera and Trichoptera taxa richness). BMWP, ASPT and EPT indices were calculated using the ASTERICS software package [13].

To obtain the BMWP score identification to family level is sufficient, so the score per site was calculated by summing the individual scores of all the family present [1]. Gained score values for individual families reflect their pollution tolerance based on current knowledge of distribution and abundance, where the highest scores are given for intolerant families and the lowest scores for tolerant ones [1] (Table 2).

**Table 1. List of the sampling sites**

| Site № | River | Locality | Altitude (m) | Latitude | Longitude |
|---|---|---|---|---|---|
| **1** | *Klepalska | Downstream 2nd hydropower plant | 1122 | 41°40'27.88" | 22°56'34.24" |
| **2** | Ambarska | Upstream small hydropower plant | 1165 | 41°40'2.44" | 22°57'44.02" |
| **3** | Klepalska | Upstream small hydropower plant | 1165 | 41°39'59.81" | 22°57'41.25" |
| **4** | Dvorishka | Upstream closest to spring region | 1138 | 41°34'18.90" | 22°50'50.36" |
| **5** | *Dvorishka | Upstream Dvorishte vlg. | 944 | 41°35'36.77" | 22°54'1.65" |
| **6** | *Dvorishka | Downstream Dvorishte vlg. | 901 | 41°35'5.33" | 22°56'0.84" |
| **7** | Dvorishka | Inflow in Lebnitsa R. | 796 | 41°33'32.65" | 22°59'18.53 |
| **8** | Tributary of Lebnitsa R. | Inflow in Lebnitsa R. | 810 | 41°32'48.92" | 22°59'32.71" |
| **9** | *Lebnitsa | Downstream Dobri Laki v. | 797 | 41°34'8.36" | 22°59'24.88" |
| **10** | Spring above Stuka vlg. | Inflow in Reservoir above Stuka vlg. | 379 | 41°28'44.38" | 22°49'15.63" |

**Legend:** *Sites with a different kind of negative impact

The ASPT taxa score was obtained by dividing the BMWP score by the total number of the scoring taxa. The interpretation of the scores is similar to the BMWP values [1], where higher values reflects clean upland sites containing relatively large numbers of high scoring taxa and opposite obtained lower scores are typical for disturbed lowland where pollution tolerant taxa exist (Table 2).

The calculated EPT index reflects the taxonomic richness of intolerant taxa belonging to the insect's groups of Ephemeroptera, Plecoptera and Trichoptera [11]. The highest values scores show clean upland sites where higher diversity of intolerant taxa exist and lover scores presents higher disturbance of the sites (Table 2).

The QRS index provides an integrated water quality assessment, based on the relative abundance and diversity of key groups of organisms with certain sensitivity to

pollution [4]. The rating scale ranges from 1 to 5 as the higher values reflect the referent conditions and lowest (1) – the most polluted waters.

**Table 2. Class boundaries for the used biotic indices**

| BMWP | ASPT | EPT | QRS Adapted version | Ecological status/Class |
|---|---|---|---|---|
| > 150 | 6.01 - 7.00 | >10 | 4-5 | high/Class I |
| 100 - 150 | 5.01 - 6.00 | 6-10 | 3.5 | good/Class II |
| 50 - 99 | 4.01 - 5.00 | 2-5 | 2.5-3 | moderate/Class III |
| 25 - 49 | 3.01 - 4.00 | <2 | 2 | poor/Class IV |
| < 25 | 2.00 - 3.00 | <2 | 1-2 | bad/Class V |

## Results

A total of 124 macroinvertebrate taxa, belonging to 13 systematic groups were found (Chironomidae and Oligochaeta were not identified at lower level) (Table 3). The most dominant groups (as taxa richness) were the insect orders – Ephemeroptera, Plecoptera, Trichoptera and Diptera. The highest richness of the systematic groups was observed at sites selected as referent/near natural (sites 2, 3, 4, 8, 10; Table 1), respectively.

**Table 3. Taxa richness (number of taxa established per site) in the sampled area during the study**

| Systematic groups | Number of taxa | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Site 1 | Site 2 | Site 3 | Site 4 | Site 5 | Site 6 | Site 7 | Site 8 | Site 9 | Site 10 |
| Turbellaria | | | 1 | | | | | | | |
| Nematoda | | | | | | 1 | 1 | | | |
| Oligochaeta | | | | | 1 | 1 | 1 | 1 | 1 | 1 |
| Gastropoda | | 1 | | | | | 1 | | 2 | |
| Crustacea | 2 | 2 | 1 | 1 | 1 | | 1 | 2 | | 3 |
| Ephemeroptera | 5 | 6 | 8 | 7 | 15 | 8 | 12 | 15 | 16 | 5 |
| Odonata | | | | 1 | 1 | | 5 | 1 | 2 | 3 |
| Plecoptera | 1 | 6 | 7 | 7 | 7 | 1 | 4 | 8 | 1 | 7 |
| Heteroptera | | | | | | 1 | 1 | 1 | 4 | |
| Megaloptera | | | | | 1 | | | | | |
| Coleoptera | | 4 | 2 | 1 | 2 | 2 | 2 | 9 | 9 | 1 |
| Trichoptera | 2 | 5 | 7 | 5 | 5 | 1 | 5 | 3 | 12 | 4 |
| Diptera | 2 | 2 | 2 | 3 | 4 | 3 | 6 | 9 | 13 | 4 |

Water quality of the studied sites, examined by the BMWP, ASPT, EPT and QRS was assessed from high (I class) to moderate (III class) (Table 4). Predicting that presence of any kind of habitat degradation or pollutants at any level causes domination of more tolerant taxa over benthic composition. Comparison between potential affected and clear sites was done, tracking especially the situation with the Dvorishka River from the most upstream locality (site 4) to its inflow at river Lebnitsa (site 7). It's evident that the water quality changes in a longitudinal gradient, where on site 4 and 5 the BMWP belongs to

good and QRS to high ecological status; on site 6 indices get lower water quality (class III for BMWP and QRS) and on the last site 7 already the quality improves back to good-BMWP and high-QRS ecological status (as at the non-affected upstream sites) (Table 4).

Values of used indices in this study (Table 4) reviled the sensitivity to organic pollution and habitat loss at selected sites. In literature [1, 4, 11] it is familiar that BMWP and QRS scores are more sensitive than EPT and ASPT. That is why in this study always the class boundaries of EPT and ASPT (they present the species richness of the studies sites and not their pollution tolerance) were higher than BMWP and QRS with one class. Only on sampling site 9 the BMWP score had the class I category, although the ASPT was pointed out on II class, but the collected macroinvertebrates taxa belong to the higher scores of the BMWP score system [1].

**Table 4. Values of the calculated indices per studied sites**

| Site number | St_13 MK | St_12 MK | St_11 MK | St_6 MK | St_9 MK | St_8 MK | St_11 BG | St_10 BG | St_12 BG | St_5 MK |
|---|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** |
| **Number of Taxa** | 12 | 26 | 28 | 25 | 37 | 18 | 39 | 49 | 61 | 28 |
| **BMWP** | 46 | 110 | 125 | 140 | 144 | 53 | 146 | 143 | 156 | 120 |
| **Class** | III-IV | II | II | II | II | III | II | II | I | II |
| **QRS** | 3 | 3,5 | 4 | 4 | 4 | 3,5 | 4,5 | 4,5 | 4,5 | 4 |
| **Class** | III-IV | II | I | I | I | III | I | I | I | I |
| **ASPT** | 5.75 | 6.87 | 6.94 | 7.37 | 6.54 | 5.3 | 6.08 | 6.22 | 5.57 | 6.32 |
| **Class** | II | I | I | I | I | II | I | I | II | I |
| **EPT Taxa** | 6 | 12 | 15 | 14 | 22 | 9 | 16 | 23 | 17 | 12 |
| **Class** | II | I | I | I | I | II | I | I | I | I |

## Discussion

From the results presented in Table 4, it is obvious that the highest taxa richness was detected on the sampling sites 5 and 9. As expected, the lowest taxa richness was observed on site 1. This situation occurs as a result of the damming and capturing/or slowing the water flow of the small mountainous rivers (Klepalska Reka and Ambarska Reka), in which the lowest quality of the water (Table 2) was result of the mechanical degradation of the river bed (existence of 2 small hydropower stations on a less than 3km distance), but not as a result of water pollution with organic matter (the index values of EPT and ASPT belongs to class II).

The similar situation was observed on site 6 where the BMWP and QRS scores pointed out on higher disturbance (moderate water quality) of the macroinvertebrate's communities, situation that comes as direct consequences of the organic pollution of the river Dvorishka Reka by the local inhabitants of the Dvorishte vlg. (Fig. 1, Table 4). This site reflects the organic input (pollution) of the river sector of the river Dvorishka, changes that couldn't be measured by the ASPT and EPT indices (good ecological status), because they are less sensitive to water pollution, and more sensitive to present taxa [1, 4]. Contrary to the results

gained from the Macedonian territory, on the sampling site of Dvorishka Reka (Site 7) before its inflow at river Lebnitsa (site 9) on Bulgarian territory, the water quality was improved to the II class for BMWP, i.e. I class for QRS, ASPT and EPT indices. The observed results pointed out on the measured distances between the two sampling sites, where in, the non-disturbed environment of the river ecosystem has enough time for self-purification of the organically disturbed stretch of the river bed (Table 2) and reaches the close to referent conditions state of the site 4 and 5 (the closest site to the spring region of the Dvorishka Reka – Fig. 1).

Bearing in mind the observed situation at the selected sampling sites and the possible selection of the reference sites, we could name as referent the sites 2, 3, 4, 10 (Macedonian territory) and 8 (Bulgarian territory) in this preliminary study of the R5 type – mountainous and semi-mountainous streams in a transboundary area (Maleshevska and Ograzden Mtn.).

**Conclusion**

In summary, several recommendations could be listed in order to be considered in future studies: (i) type and stressor specific system should be developed; (ii) the number of studied sites should increase; (iii) identification of reference and "near natural" sites and (iv) type-specific macroinvertebrates communities were clearly defined. The application of the proposed measures will enable to achieve the environmental goals, that will contribute to improve the quantitative and qualitative status of water resources and consequently will ensure the preservation of higher species diversity.

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# NEW AND AMONG THE LARGEST POPULATIONS OF THE ENDANGERED ORCHID *GOODYERA REPENS* (L.) R. BR. IN BULGARIA – STATUS

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**Abstract**

**Aim**: *Goodyera repens* is a small relict boreal orchid included in the Red Data Book and Biodiversity Act. The study aimed to explore and evaluate its distribution and bioconservatory status in the Trigrad-Yagodina plateau.

**Materials and Methods:** Exploration and observations of *G. repens* populations in the plateau was done from 2013 till 2017. Shoot count and GPS coordinates caching were performed for distribution mapping.

**Results**: During the period of the study six new locations of *G. repens* for Bulgaria were found on an area of several square kilometers in the coniferous belt between the villages Trigrad and Yagodina. However by the end of the period four locations were destroyed by wood cutting and forest clearing activities. Parts of the largest survived locations were in suppressed state due to anthropogenic factors.

**Conclusion**: The new data may cause reevaluation of the conservatory status of the species, however the presented observations necessitate carefulness and further study and monitoring, and possibly giving to some of these habitats protected status.

**Keywords**: *Goodyera repens*, Bioconservation, Orchidaceae, population

## Introduction

*Goodyera repens* (L.) R. Brown (commonly called Creeping Lady's Tresses by anglophones) is a small inconspicuous relict boreal orchid found mostly in century old

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forests. The individual partial shoots are monocarpic, live 5-8 years, have rosette leaves, and in the year bloom is formed on a flowering shoot up to 25 cm high with several reduced leaves [1, 2]. Traditionally, *G. repens* has been referred to as a semi-saprophyte. Under strong eclipse or shade, it has the ability to switch to a saprophytic nutrition, loosing the aboveground part [1]. For its development and growth it depends on few mycosymbionts as *Ceratorhiza goodyerae-repentis* and *Ceratobasidium cornigerum* [3] which significantly limits and affects the possibilities for its distribution.

Typical habitats for *G. repens* are shady and old pine woodlands (as *Pinus sylvestris* and *P. nigra*), less common are the mixed ones with spruce, fir or birch. It prefers mossy sites, and its successful establishment depends largely on the presence and persistence of the green moss cover [2, 4].

*G. repens* is a circumboreal species that occurs in Europe, Caucasus, North and East Asia, the Himalayas and North America. In Europe it extends northwards to northern the Scandinavia and the Murmansk Region (northwestern Russia), the British Isles, while southwards it reaches Spanish and French Pyrenees, central Italy and north Greece [2, 4, 5, 6]. In Bulgaria the distribution is very fragmented which is typical for relict species and is reported for the following locations: Mt. Stara planina – above Stoevci village, Mt Rhodope West: Brashten, Mt Rhodope Central: Dobrostan area, Zarenishki rid and Bjala Cherkva, in the surroundings of the villages Dobralak, Sitovo, Slavejno, Orehovo, Hvojna. It occurs from 800 to 1500 m.a.s.l. [5].

The plant is in the IUCN Red List with a status of Least Concern (LC) of the EU 27 regional assessment however due to its peculiar biology and environmental requirements it is included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) [5, 6]. In Bulgaria it fulfills the IUCN criteria for endangered [EN B1ab(iii)+2ab(iii)] therefore is included in the Red Data Book and Biodiversity Act. Only few locations are in protected areas as the ones in Chervenata Stena Strict Nature Reserve in Rhodope Mt. and Bulgarka Nature Park in Mt. Stara planina, some areas being part of the NATURE 2000 network [5].

**Materials and Methods**

In 2013 during a floristic study, shoots of *G. repens* were found at the Buynovsko gorge near Yagodina village (Rhodope Mts (Central) floristic region) [7]. Further extended exploration and observation of *G. repens* populations in Trigrad-Yagodina karst plateau was done from 2013 till 2017 during the flowering-fruiting season (August-October). A voucher specimen of the species is deposited in the Herbarium of the Sofia University (SO 107607).

Shoot and group of stems (group of leaf rosettes with proximity less than 10 cm from each other, probably ramets) count and a GPS coordinates caching were performed for distribution mapping. The marking of the populations of the species was done using GPS receiver Garmin Colorado 400 and/or Solmeta 2 Pro Geotagger. The mapping and the measurement of the distances were done with the software Garmin BaseCamp ver 4.6 and SASPlanet ver. 15.

The data were processed with Originlab Origin Pro ver. 7 and Microsoft Office ver. 2007.

The species in the habitats were determined using the Identification guide of the plants in Bulgaria [8], Guide to the mosses of Bulgaria [9]. The soil determination was according to Soils of Bulgaria [10]. The vegetation structure was characterized according to Edwards' structural classification [11] and the other characteristics of the vegetation cover were estimated according to the Braun-Blanquet method [12].

**Results and Discussion**

During the exploration six new locations of *G. repens* for Bulgaria were found in the coniferous forest belt between the villages Trigrad and Yagodina (Fig. 1).

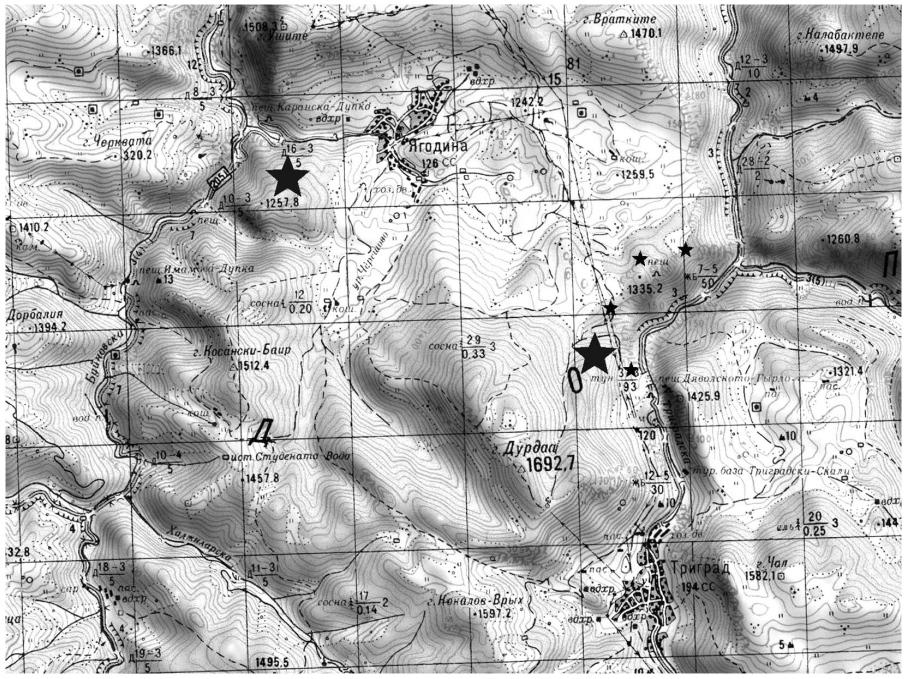


**Fig. 1. Distribution map of the locations with the populations of *Goodyera repens* found in the Trigrad-Yagodina karst plateau. With big stars are marked the presently existing locations, with small stars are marked the extinct ones. Grid is 1 km**

The GPS coordinates as latitude, longitude and altitude of the center point of the locations are presented in Table 1.

**Table 1. GPS coordinates of the locations of *Goodyera repens* found in Trigrad–Yagodina karst plateau**

| Location | Latitude | Longitude | Altitude | Slope |
|---|---|---|---|---|
| Yagodina location | N41°37'59.07" | E24°20'26.58" | 1160 m a.s.l. | North |
| Trigrad location_1 | N41°37'06.28" | E24°22'22.71" | 1360 m a.s.l. | North |
| Trigrad location_2 | N41°36'59.00" | E24°22'35.07" | 1270 m a.s.l. | East |
| Trigrad location_3 | N41°37'27.07" | E24°22'42.02" | 1300 m a.s.l. | North |
| Trigrad location_4 | N41°37'42.66" | E24°22'58.55" | 1280 m a.s.l. | North-East |
| Trigrad location_5 | N41°37'16.10" | E24°22'27.96" | 1320 m a.s.l. | North-East |

The locations near the Trigrad village are located near the edge of the cliffs of the Trigrad gorge. Two of the locations Near Trigrad have a north facing slope exposure namely location 1 and 3. Another two locations – location 5 and 4 have North-East facing slope exposure; and location 2 has east facing slope exposure. The location near Yagodina village is located near the edge of the cliffs of the Buynovsko gorge and has a north facing slope exposure. All these locations have slope orientations which either receive reduced solar radiation compared to the other orientations as is the case with the north and north-east ones, or the largest amount of solar radiation is received in the cooler parts of the day as is the case with the east facing slopes [13]. This along with the canopy effects of the coniferous forest especially the ones of closed type which further greatly reduce surface energy budget provide cooler microclimate.

Soils are brown forest type (eutric and dystric cambisols) over calcareous rocks.

All of the locations are located in coniferous forests. The habitats at Trigrad location 1 and 2 are closed coniferous forest with dominant *Abies alba*, location 5 is open coniferous forest with dominant *Pinus nigra*, and locations 3 and 4 are mixed closed coniferous forests with *Pinus nigra, Picea abies* and etc. The habitat at Yagodina location is an open coniferous forest with dominant *Pinus nigra* and fewer *Abies alba*. At all of the locations the plants grow among rather thick layer of mosses comprising of such tall and large species as *Rhytidiadelphus triquetrus*, *Hylocomium splendens* etc. whose thickness can reach of up to 20cm or even more. Considering the big thickness of the moss layer at most of the locations and the fact that *G. repens* has relatively short roots 2.5-8cm [14] then this is the second Bulgarian orchid after *Hammarbia paludosa* which can lead epiphytic lifestyle under the conditions of this karst plateau.

The herbaceous layer of the habitat at Yagodina location is poorly developed and comprises of such taxa as *Sesleria latifolia, Euphorbia amygdaloides, Geranium sanguineum, Hieracium murorum, Leontodon crispus, Monotropa hypopitys*, etc. and many orchids as the rare orchid – *Ophrys insectifera* [13], *Gymnadenia conopsea, Listera ovata, Neottia nidus-avis, Epipactis heleborine*, etc. The shrub stratum is also poorly developed and comprises of such taxa as: *Juniperus communis*, *Ostrya carpinifolia*, At the largest Trigrad location (location 1) the herbaceous layer is almost absent with sparse plants of such taxa as *Euphorbia amygdaloides, Pyrola chlorantha, Monotropa hypopitys, Ajuga reptans* etc. and few heterotrophic orchids as *Neottia nidus-avis* and *Corallorhiza trifida*.

The shrub layer is also nearly absent, presented with only young *Abies alba* plants at the few spots of fallen or cut trees.

At each location shoot number ranged from few tens to several hundred leaf rosettes as presented in Table 2. The approximate size of the groups is also presented as an indirect measure of the level of the vegetative reproduction.

**Table 2. Size of the populations of *Goodyera repens* found in the Trigrad–Yagodina karst plateau in 2013 and 2017**

| Location | Number of groups | Total number shoots | Mean size of groups |
|---|---|---|---|
| Yagodina location (2013) | 61 | 1169 | 19.16±13.24 |
| (2017) | 64 | 1231 | 19.23±12.95 |
| Trigrad location_1 (2013) | 32 | 693 | 21.66±11.12 |
| (2017) | 14 | 271 | 19.36±13.29 |
| Trigrad location_2 (2013) | 2 | 25 | 12.5 |
| (2017) | Not found | - | - |
| Trigrad location_3 (2013) | 4 | 43 | 10.75±4.57 |
| (2017) | Not found | - | - |
| Trigrad location_4 (2013) | 4 | 46 | 11.5±4.65 |
| (2017) | Not found | - | - |
| Trigrad location_5 (2013) | 3 | 32 | 10.67±2.52 |
| (2017) | Not found | - | - |

The distance to the nearest forestry road or touristic trail as a factor of direct antropogenic activities and influence to the locations are as follows: Trigrad location 1 – 25 m; location 2 – 35 m; location 3 – 350 m; location 4 – 700 m; location 5 – 30 m; and Yagodina location – 1 m. No grazing by herds was observed at the locations despite the proximity to Trigrad – 1700 m and Yagodina village – 570 m. However at 2015 four locations namely Trigrad locations 2, 3, 4 and 5 were destroyed by wood cutting and forest clearing activities. At the Trigrad location over 60% of the shoots and occupied area were destroyed especially by the transportation of the trees during which the ground surface cover is severely damaged and by leaving the unused wood material which decomposes and prevents the access to light, aeration etc. for the plants underneath (Table 2).

Although the Yagodina location is within the Byunovsko gorge protected area, the population is located near the touristic trail and is impacted by antropogenic activities. Along with this trail during the period of the study new tracks were made by unaware tourists and residents through the moss layers and shrubs thus destroying a crucial for this species part of this delicate ecosystem. This also causes a change of the light regime near the ground. One of the indirect impacts of the anthropogenic activities at both of the undestroyed locations are the changes of the light regime. The species is a pronounced sciophyte and prolonged exposure to direct sunlight damages its leaves and suppresses its development as it was observed after the woodcutting or near the new man-made tracks.

## Conclusions

The presented data extend the local range and number of occurrence of this species and probably may decrease the level and estimate of the vulnerability of the species. However this should not pacify us too much. Bulgaria is near the southernmost border of the species' global distribution areal which puts high stress on population survival and distribution. Two of these locations are among the largest known in Bulgaria, however regardless the high count, the population's genetic diversity is probably lower due to dominantly vegetative reproduction as was found through genetic studies in some other species from this genera [15]. Additionally a large portion of the newly found locations as of 2017 were completely or severely destroyed by anthropogenic activities, which probably necessitate appropriate change in the woodcutting and clearing activities or restriction of anthropogenic activities in these habitats and giving to some of them protected status. Along with that, actions for informing the local communities and forestry agencies would be greatly helpful for the conservation and the prevention of further population damage of this endangered species and its specific habitats.

However due to the complex biology of the species further studies are necessary encompassing other ecological factors as climate fluctuations, soil diversity, mycosymbiont relations and influences and etc. to provide a better action plan for the populations' bioconservation of this threatened plant.

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# SHORT COMMUNICATIONS

## Topic: BIODIVERSITY AND CONSERVATION BIOLOGY

## PHENOLIC ACIDS PROFILE ON *CLINOPODIUM VULGARE* L. (LAMIACEAE)

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**Ethnopharmacological relevance:** *Clinopodium vulgare* L. (Lamiceae) is a traditional plant in Bulgarian folk medicine for treatment of wounds and injuries, gastric ulcers, as a heart tonic and an expectorant. Aerial parts alleviate symptoms associated with mastitis and prostatitis. Some previous *in vitro* studies showed antitumor activity against A2058, HEp-2 and L5178Y cell lines, and strong antioxidant capacity and DNA (deoxyribonucleic acid) protective activity of aerial part water extracts of the plant. Reduction of neoplastic diseases has been demonstrated with *in vivo* experiments on hybrid mice BDF1 [1]. The content of some phenolic acids in extracts of the plant is described as well, using gas chromatography after derivatization [2]. The literature however is poor on data about the active ingredients in the *Clinopodium vulgare* plant extracts, with reliable confirmation of their chemical structure by modern analytical techniques as HPLC/HRMS.

**Aim of the study:** To amplify the information about the phenolic acids content of *Clinopodium vulgare* L. lyophilized water extract by UHPLC (ultrahigh performance liquid chromatography) combined with HRMS (high resolution mass spectrometry).

**Material and Methods:** *Clinopodium vulgare* L. aerial parts were harvested in July 2017 from village German region near Sofia, Bulgaria (voucher specimen SO 107606). A lyophilized hot water extract was analyzed by UHPLC-HRMS using a quaternary pump

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and a hybrid quadrupol-"Orbitrap" high resolution "Q-Exactive" mass spectrometer coupled with a HESI (heated electrospray ionization) probe. The chromatographic separation was performed on RP (reversed phase) "Poroshell" C18 3 x 150 mm 2.7 µm column with gradient of 10÷95% acetonitrile in 0.08% formic acid as mobile phase at flow rate 250 µl/min, and on Silica-HILIC (hydrophilic interaction liquid chromatography) on 150 x 3 mm 2.6 µm "Kinetex" column with 5÷50% gradient of water in acetonitrile with 10 mM ammonium formate pH 4.6 at flow rate 450 µl/min. HESI worked at 250°C, spry voltage 3 kV, ion transfer tube at 300°C, sheath gas pressure 45 Psi and mass tolerance of 5 ppm.

The LC-MS method was operating in "Full scan-data dependent MS/MS Top-5" scan mode. It was performed in negative ion monitoring and MS/MS spectra were recorded at different collision energy (hcd). The identification of the studied compounds was based on the accurate masses, MS/MS data and comparison with fragmentation fingerprints observed for the reference standards, and literature data [3]. The retention times of the assayed compounds in the RP UHPLC separation were: 15.74 min (**1**), 18.44 min (**2**); 19.42 min (**3**), 20.05 min (**4**), 25.77 min (**5**), 26.99 (**6**).

**Results:** In this study, IUPAC numbering system was used for the caffeoylquinic acids. They were identified according to the hierarchical key for identification of phenolic acids of Clifford (2003) [3]. The hcd (25) induced dissociation corresponded to $MS^3$ spectra of Cliford's hierarchica key. Three isobaric compounds (**1**, **2** and **3**) shared the same deprotonated molecule $[M-H]^-$ at *m/z* 353.088. They gave typical fragment ions at *m/z* 191.055 $[quinic\ acid-H]^-$ and 179.034 $[caffeic\ acid-H]^-$ indicating that they are derivatives of quinic and caffeic acid. In MS/MS spectra **1** and **3** (produced a base peak (100%) at *m/z* 191 corresponding to the loss of caffeoyl moiety. In **1** abundant fragment ions at *m/z* 179.034 (57.40%) $[M-H-174]^-$ and 135.0436 (27.92%) $[M-H-(174+CO_2)]^-$ were also observed. Regarding **3**, the aforementioned fragments showed lower abundance (below 2%). By comparison with reference standards, **1** and **3** were identified as neochlorogenic (3-caffeoylquinic) acid and chlorogenic (5-caffeoylquinic) acid, respectively. The third isomer was distinguish by its "dehydratated" base peak at *m/z* 173.044 $[M-H-(162+H_2O)]^-$ supported by the abundant fragment ions at 191.055 (52.13%) and 179.034 (64.31%). Based on the MS/MS data and elution order, it can be assumed that **2** is 4-caffeoylquinic acid. Compounds **4** and **5** had the fragmentation pattern of dicaffeoylquinic acids with $[M-H]^-$ at *m/z* 515.121 and fragment ions of caffeoylquinic and quinic acid at *m/z* 353.088 and 191.055, respectively. The absence of the base peak at *m/z* 173 clearly defined **4** as lacking substitution at position 4 on the quinic moiety. The base peak at *m/z* 191.055 indicated either 3- or 5- quinic acid substitution. Fragmentation of **4** yielded an abundant ion at *m/z* 179.034 (82.66%) supported by the ions at *m/z* 353.088 $[M-H-162]^-$ (56.03%) and 135.044 $[M-H-(162+174+CO_2)]^-$ (37%). Based on this observation and low abundant fragment ion at *m/z* 335.0780 $[M-H-(162+H_2O)]^-$ (11.74%), **4** was assigned accordingly as 1,3 dicaffeoylquinic acid. **5** afforded a base peak at *m/z* 173.044 as was observed for 4-substituted acylquinic acids [4]. Abundant fragment ions at *m/z* 353.088 $[M-H-162]^-$ (95.19%) and 179.034 $[M-H-(162+174)]^-$ (67.31%) were also observed. Consistent with the hierarchical key, the two *vic* dicaffeoylquinic acid isomers differ with regard to the

abundance of "dehydrated ion" at *m/z* 335.0775 (1.36%) $[M-H-(162+H_2O)]^-$. The latter ion is undetectable in 4,5-dicaffeoylquinic acid. Thus, the fragmentation pattern of **5** corresponded to 3,4-dicaffeoylquinic acid. The major component in the studied lyophilized *Clinopodium vulgare* water extract demonstrated deprotonated molecule $[M-H]^-$ at *m/z* 359.077. Compound **6** produced base peak at *m/z* 161.022 corresponding to the "dehydrated" caffeic acid and fragment ions at *m/z* 197.044 (23.06%) [M-H-162], 179.033 (17.71%) $[CA-H]^-$ and 135.043 (10.23%) $[CA-H-CO_2]^-$. Based on these data, as well as comparison with reference standards and literature data compound **6** was identified as rosmarinic acid.

**Conclusion:** A modern UHPLC/HRMS technique together with literature data about principals of mass spectral fragmentation was applied to insight into content of *Clinopodium vulgare* L. lyophilized water extract. As a result, 6 caffeic acids derivatives were reliable identified, including rosmarinic acid, mono- and dicaffeoylquinic acids. Rosmarinic acid was the major compound in the lyophilized water extract. Most of identified ingredients are known as bearers of biological activity. The results highlighted *Clinopodium vulgare* L. as a valuable source of bioactive compounds, extracted with water in more than 20% from dried aerial parts of the plant.

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**Keywords:** *Clinopodium vulgare,* UHPLC, HRMS, MS/MS

**References:**

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## CONDITIONS OF SHOOT SURFACE STERILIZATION OF THE WILD SPECIES *MENTHA PULEGIUM* L.

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The intensive use of plant resources of medicinal plants has led to real danger of extinction of many populations and reduction in genetic diversity. The use of *Mentha*

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*pulegium* L. by domestic animals grazing along the Stryama River leads to fragmentation of their populations. There is no data about *in vitro* introduction of the species.

**Aim:** The aim of the present study was to apply biotechnological methods in order to introduce *Mentha pulegium* L. *in vitro*.

**Material and Methods**: The experimental work was carried out in 2017 in the Laboratory of the Tissue Culture of IRGR "Konstantin Malkov" – Sadovo. The determination of the species *Mentha pulegium* L. was done by assoc. prof. Katya Uzundzhalieva. The aerial part of several plants was taken during an expedition along the Stryama River.

The sterilization procedure started with decontamination of the fresh plant material by treatment of shoot segments 3-4 cm long and 2 with lateral buds each, with 0.3% $HgCL_2$ solution for 1, 2 and 3 minutes, as a routine practice of the laboratory for wild plants. Treatment was followed by flushing with sterile distilled water, drying with sterile filter paper and soaking 20 minutes in sterile distilled water with 0.5 ml/l Cytrosept added. Explants were then immersed in 98% ethyl alcohol for 3 s and washed three times in sterile distilled water finishing with drying on sterile filter paper.

The number of explants was 100 for each variant. Survey of surviving, necrotic and contaminated explants was performed every 5 days over a 30-day period.

The sterilized explants were cultured on MS medium [1] without added plant growth regulators. Sucrose (30 g/l) was used as carbohydrate source, and agar (7.0 g/l) as hardener. Explants were placed in test tubes and cultivated in a culture room under temperature of 24±2°C and a photoperiod of 16 hours of light with 3000 lx and 8 hours of darkness.

**Results and Discussion:** Wild-growing plants are difficult to pass into *in vitro* conditions due to the usual heavy microbial contamination. In addition to the specific sterilization procedure, they also have requirements regarding the environmental conditions [2]. The use of exactly 0.3% $HgCL_2$ was based on the previous experience of the Laboratory on decontamination of plant material taken from wild plants [3]. In recent years, due to its toxicity, it is not being widely used as a sterilizing agent; however, the softer ones were ineffective.

In the variant with 3-minute exposure scenario with 0.3% $HgCL_2$ drastic dying and contamination of the explants was observed early in the trial period. Most of the explants were brownish in color, due to the pretreatment with mercury compound. After the $15^{th}$ day of the study, pathogen-free explants were observed which were necrotizing and brown due to the long duration of the treatment with 0.3% $HgCL_2$.

In the 2-minute exposure scenario with 0.3% $HgCL_2$ after the $10^{th}$ day of the trial, a few pure explants were obtained. At day $15^{th}$, 40 survived explants were noticed, and after the $30^{th}$ day only 30 pure and vital explants were obtained. This could be due to a larger number of micro-organisms, on the one hand, and to the sensitive tissue of *M. pulegium* on the other hand, which leads to greater difficulty when choosing a detergent and the duration of its use.

The efforts made to introduce *M. pulegium* in *in vitro* conditions showed that the largest number of pure and green vital explants was noticed in the 1-minute exposure variant of the sterilizing agent (0.3% $HgCL_2$) – 70 survival explants. This variant provided the smallest number of contaminated and necrotic explants compared to the other exposures. Obtaining of 70% vital explants, free of contamination, was perspective for *in vitro* culture initiation.

**Conclusion:** As a result of the experiments for the *in vitro* culture of *Mentha pulegium* L., successful sterilization of fresh plant material was obtained using a 0.3% solution of $HgCL_2$ for 1 minute exposure. Survival explants will be used for further *in vitro* multiplication of the species.

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# REPORTS

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

# INTERRELATIONSHIP BETWEEN FRESH AND DRY WEIGHT OF INTERNAL TARGET ORGANS OF ROE DEER (*CAPREOLUS CAPREOLUS*): APPLICATION FOR THE PURPOSES OF BIOMONITORING OF THE ENVIRONMENT

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**Abstract**

**Aim:** The present research aim to evaluate the nature and the degree of relationship between fresh and dry weight of the internal organs (liver and kidneys) of the European roe deer (*Capreolus capreolus*), which would allow to comparatively assess the analytical concentrations of toxic elements in them, obtained by different methods of preparation of these tissues for analysis.

**Materials and Methods:** The tissues from the liver (n=38) and kidneys (n=33) were taken from adult roe deer (over 2 years) inhabiting areas with different anthropogenic impact in Bulgaria.

**Results:** Under laboratory conditions, the fresh and the dry weight of the investigated tissue samples were weighed with accuracy to 0.001g. The established correlation (R = 0.981 in the liver and R = 0.884 in the

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kidney) and regression relationships (linear regression models) between the fresh and the dry weight of the studied samples, as well as the percentage of liquid content in them (71.75% in the liver and 79.75% in the kidney), allow a comparison of the analytical concentrations of heavy metals measured in fresh and in dry tissues of the liver and kidney of the roe deer.

**Conclusion:** This new opportunity for the comparative assessment of the analytical concentrations of toxic elements in examining target organs strongly supports the comparative analysis of the accumulation of priority natural pollutants in the roe deer's body and its use as a bioindicator for the assessment of the environmental quality in both geographical and time aspects.

**Keywords:** weight of tissue samples, biomonitoring, European roe deer, *Capreolus capreolus*

## Introduction

To keep the natural ecosystems function normally, information allowing assessing and prognosticating the changes in the natural cycles of biologically active substances is needed. One of the main aims of the biological monitoring for assessment of the environmental quality is to deduce the normative characteristics of biological components in natural and artificial ecosystems under normal and disturbed conditions. Game mammals have been traditionally used as zoo-monitors of the anthropogenic pollution in biological monitoring of the environment. They have proven to be of high bio-indicating value in the assessment of environmental pollution by heavy metals, radionuclides and organic chemicals in agricultural, partially urbanized and natural forest areas [1, 2, 3, 4]. The European roe deer (*Capreolus capreolus*) as a representative of the game mammals, whose populations are successfully developing both in natural ecosystems and in ones with different anthropogenic impact, was identified as suitable for zoo-monitor. With a view to the assessment of environmental quality, the roe deer was recommended in the National Biomonitoring Program of Bulgaria (1999) as a bio-indicator species for the accumulation of toxic pollutants in animals' bodies from the environment. Examination of the analytical concentrations of priority pollutants, such as heavy metals in target organs of the roe deer, has proven to be of high informative value and has been widely introduced into bio-indicator studies in many European countries [1, 5-9].

Diverse researchers in different time periods or country used different methods [10] to estimate their relative content defining them in fresh or in dry tissue. At the moment, the available information on bioaccumulation of toxic elements in roe deer organs as zoo monitor contain data presenting their relative content in fresh or in dry tissue. This makes it quite difficult to carry out a comparative analysis of the state and changes in the natural environment and its biota caused by anthropogenic impact both in geographic and temporal terms.

In view of this, the aim of the present study is to evaluate the nature and degree of relationship between fresh and dry weight of target organs (liver and kidneys) of the European roe deer which would allow to assess comparatively the analytical concentrations of toxic elements in the internal organs of this zoo-monitor, obtained by different methods of preparation of the examined tissues.

## Material and Methods

The tissue samples for analysis of the relationship between the fresh and dry weight of the target organs were taken from 38 (liver) and 33 (kidneys) adult roe deer (over 2 years) from habitats with different anthropogenic impact in Bulgaria.

The fresh and the dry weight of tissue samples from the dissected internal organs – liver and kidneys were weighed under laboratory conditions with accuracy to 0.001g. The initial weight parameters (dry and fresh weight) and the percentage of liquid content in both organs were tested for normality using the Kolmogorov-Smirnov D-statistics, and for homogeneity of variances using the Levene's test. The obtained values showed that the studied samples of fresh and dry weight and the percentage of liquid content in both organs had normal distribution. This conclusion allowed applying "Least squares method for linear regression" for analysis of the correlation and regression relationships between the independent variable "fresh weight" and the dependent variable "dry weight". The relationship between the independent and the dependent variable was assessed through the F-test. The degree of relationship between the studied variables was characterized using the Pearson correlation coefficient (R) and the derivative coefficient of determination ($D = 100\ R^2$). The percentage of the liquid content in both organs was described by its basic descriptive statistics. All calculations were performed using the statistical package STATISTICA [11].

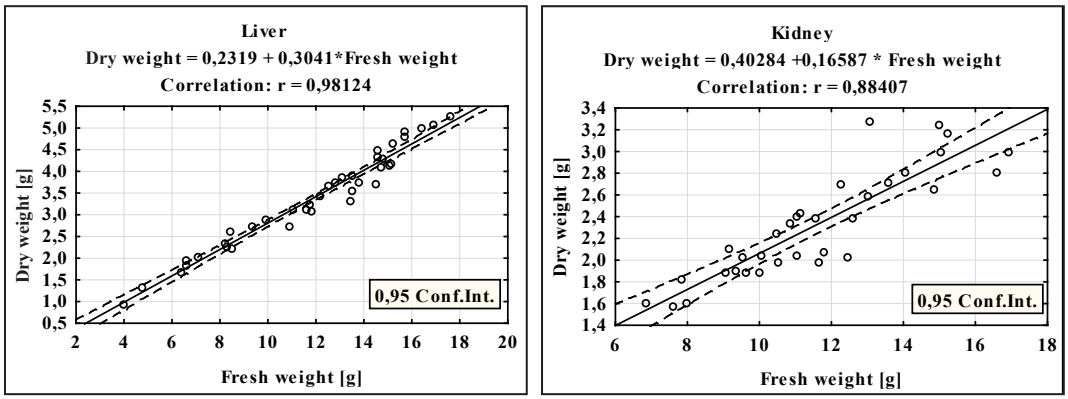
## Results

The parameters describing the degree of relationship between the fresh and the dry weight of the examined organs and showing how adequate are the deduced regression models to the empiric data are presented in Table 1.

**Table 1. Parameters of the linear regression models describing the degree of relationship between the fresh and the dry tissue weight of the internal organs of roe deer (*Capreolus capreolus*): R – Pearson correlation coefficient; D – Coefficient of determination; F, d.f., p – parameters of F-test, S – Standard error of estimate**

| Organ | R | D | F | d. f. | p | S |
|---|---|---|---|---|---|---|
| **Liver** | 0.981 | 96.28 | 932.71 | 1. 36 | 0.000000 | 0.2168 |
| **Kidney** | 0.884 | 78.15 | 110. 92 | 1. 31 | 0.000000 | 0.2317 |

The deduced linear regression relationships between the fresh and the dry tissue weight of the examined internal organs together with the 95% confidence interval are presented graphically and analytically ($Y_{\text{dry weigh}} = a + b * X_{\text{fresh weight}}$) in Fig. 1.




A B

**Fig. 1. Graphic and analytic model of linear regressive dependency between fresh and dry weight of internal organs of roe dear (*Capreolus capreolus*): A – liver; B – Kidney**

The percentage of liquid content in both internal organs is described by their basic descriptive statistics in Table 2.

**Table 2. Liquid content values [%] of the internal organs of roe deer (*Capreolus capreolus*) and their basic statistics**

| Organ | N | Mean | Std. Error | Std. Dev. | Coef. Var. |
|---|---|---|---|---|---|
| **Liver** | 38 | 71.75 | 0.3092 | 1.9062 | 2.65 |
| **Kidney** | 33 | 79.75 | 0.3512 | 2.0178 | 2.53 |

## Discussion

The performed analysis revealed a strong correlation between the fresh and the dry weight in both target organs of roe deer (liver and kidneys), which was better manifested in the liver. Like the strongly correlated relationship between the studied "cause" – fresh tissue weight – and "effect" – dry tissue weight, coefficients of determination of high values were obtained. The high values of the F-test obtained in assessment of the relationship between the fresh and dry weight of the studied organs showed that the analytically deduced model replicated well the observed empirical data, that being confirmed also by the assessment of the parameter "Standard error of estimate". It is typical that both models of regression relationships were deduced within a narrow 95% confidence interval.

The statistical estimate of the basic descriptive statistics of the mean percentage of liquid content in both studied internal organs revealed a low absolute and relative variation as well as a rather low standard error.

The established characteristics of the correlation between the fresh and dry weight in both target organs as well as the baseline descriptive statistics on the mean percentages of

liquid content in them allow recalculating the relative heavy metal concentrations found in fresh or in dry tissue using the dependence:

$$D * (100 - C) * 100^{-1} = W$$

where:

D – dry weight element concentration;
C – specific to the each organ liquid content (%);
W – wet weight element concentration.

**Conclusion**

The deduced patterns in the weight ratios of the fresh and dry tissue of the "target organs" (liver and kidney) in European roe deer reveal a new possibility to perform a comparative analysis of the bioaccumulation of toxic elements in them. They allow for broadening of the possibilities for anthropogenic impact assessment of the natural environment by comparing the analytical concentrations of heavy metals in the internal organs of this primary zoo monitor evaluated in both fresh and dry tissues.

This new opportunity for comparative assessment of the analytical concentrations of toxic elements in the internal organs of this zoo monitor, obtained through various methods of preparation and analysis of the studied tissues, strongly supports the comparative analysis of the accumulation of priority natural pollutants in the body of the European roe deer and its use as a bio-indicator species for the assessment of the environment quality in both geographical and comparative time aspects.

**Acknowledgements:** This study was supported by the project № ДФНП-17-50/26.07.2017 "Study of the roe deer (*Capreolus capreolus* L.) in Bulgaria as accumulation bio indicator of heavy metals in the environment" developed in the "Program to support young scientists and doctoral students of BAS-2017".

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# EUROPEAN ROE DEER (*CAPREOLUS CAPREOLUS*) AS A BIOMONITOR FOR THE CURRENT ENVIRONMENT HEAVY METALS CONTAMINATION IN AN AGRICULTURAL REGION IN BULGARIA

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**Abstract**

**Aim:** The aim of the study was to evaluate the current contamination of liver and kidney tissues of European roe deer (*Capreolus capreolus*) inhabiting typical agricultural region in Bulgaria by priority pollutants of the heavy metal group (Pb, Cd, Cu and Zn).

**Materials and Methods:** All investigated 15 adult male roe deer (between 2 and 5 years) inhabited an area covered by forests and arable lands. The roe deer's liver and kidneys were used as test systems for determination of analytic concentrations of the studied heavy metals by an inductively coupled plasma atomic emission spectrometry (ICP-AES) using a Perkin Elmer Optima 7000 DV.

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**Results:** The concentrations of the tested metals, specific for each organ, characterized by the width of the range about the median that includes 95% of the cases demonstrate the limits of their variability. In the liver, the limits of variability for Cu were from 25.988 to 269.853 [mg/kg dry tissues]; for Zn from 70.81 to 1115.631[mg/kg dry tissues]; for Pb from 0.8081 to 9.755mg/kg dry tissues and for Cd from 0.859 to 5.314 [mg/kg dry tissues]. In the kidneys, respectively they were for Cu from 46.488 to 83.068mg/kg dry tissues; for Zn from 116.286 to 1542.518 [mg/kg dry tissues]; for Pb from 0.713 to 8.401mg/kg dry tissues and for Cd from 8.113 to 74.92 [mg/kg dry tissues].

**Conclusion:** These results create a baseline for estimation of the current heavy metal accumulation in roe deer and provide an opportunity to use it as a bioindicator of future potential anthropogenic negative influence on the environment in agricultural regions in the country, under the conditions of modern agricultural activities in them.

**Keywords:** Heavy metals, biomonitoring, European roe deer

## Introduction

European roe deer (*Capreolus capreolus* L.) is one of the key species of European terrestrial ecosystems and so, it is of great importance to forest and agricultural ecosystems. It also represents the most important game-management species in the vast majority of European countries [1, 2].

The roe deer is common for the whole of Bulgaria and is a typical game species in the terrestrial ecosystems of the country. The potential inhabitable territory in the country is about 7.55 million hectares, of which 3.77 million ha are field resources and include biotopes with various ecological conditions. The taxation of the game in Bulgaria conducted in the spring of 2017 indicates that there are about 110000 roe deers in the country [3].

The real risk presented by toxic metals to wildlife and humans is mostly demonstrated as a chronic or sub-lethal effect, e.g. nephrotoxicity, carcinogenicity, teratogenicity, endocrine and reproductive toxicities [4]. The main threats to wildlife health from heavy metals are associated with exposure to not essential elements, such as lead and cadmium, but the anthropogenic pollution of ecosystems with essential heavy metals (Zn and Cu) is also problematic at some sites. These latter two metals can be toxic to wildlife if the concentration of available metal in it is high enough [5]. Therefore, it is necessary and important to monitor heavy metals concentrations in the environment, as well as in areas that are not directly related to pollution sources.

Identification of heavy metal concentrations in the organs of free-living animals provides an indirect measure of environmental pollution while making it possible to determine the extent of exposure of animals to these elements, that's why environmental studies often use free-living animals as indicators of environmental pollution with toxic metals [6, 7].

Bioindicator is given to a living entity or group of organisms that shows the information, either based on the environment or a constituent of it [8]. They are key tools to mitigate human impact on biota and offer the potential for assessing ecosystem health state before this is functionally compromised, and are responsible for showing progressive impacts of different types of pollutants [9].

Wild animals, especially game species, such as roe deer, are suitable as bioindicators [10] due to their large geographical distribution, a residential way of life, feeding habits, relatively long lifespan and easy sample collection (regular hunting activities).

For sustainable use of the potential of roe deer as a natural resource and for balanced development of terrestrial ecosystems, which it inhabits, it is crucially necessary to assess the degree of contamination of its internal organs (liver and kidneys) with toxic elements [11].

The aim of the study was to check the loading of liver and kidney tissues of roe deer inhabiting a typical agricultural region in the country by priority pollutants of the heavy metal group (Pb, Cd, Cu and Zn). The established in the current survey reference values of their residuals in roe deer would provide an opportunity to use this species as a bioindicator of future potential anthropogenic negative influence on the environment in agricultural regions in the country, under the conditions of modern agricultural activities in them. This monitoring is also important because people consume the meat and internal organs of this species. The main reason for such a kind of investigation resides in significant differences in natural concentrations of the investigated metals in Bulgaria, but also in the difference in industrial, agricultural, and traffic contaminations in different habitats of the roe in the country.

## Materials and Methods

The roe deer included in this study were legally hunted, in the course of plans for hunting economic exploitation of its population as bio resource during the hunting season of 2017 in Northeastern Bulgaria, from different areas in the Danube Plain – South Dobrudzha and the northern stretches of Ludogorie, where the forests form separate complexes and massifs were separated from arable lands. The study area represents an agricultural region characterized by relatively intensive agriculture and well-developed road network in the central part of Northeastern Bulgaria (Fig. 1).


**Fig. 1. Location of the study. The sampling area is marked as „investigated territory“**

All investigated 15 adult male roe deers (between 2 and 5 years) inhabited an area covered by forests and arable lands. According to the initial veterinary health inspection, carried out after harvesting, all individuals were in normal physiological condition.

The liver and the kidneys were used as target organs for the determination of the analytic concentrations of the studied heavy metals (Pb, Cd, Cu and Zn) in the roe deer specimens. A small piece of liver and kidney tissues, around 3 g, without external contamination was immediately removed from each roe deer, after its shooting. The collected samples were cooled in a portable cooler, transported to the laboratory, and stored at −20°C in a freezer until analysis.

The preliminary preparation of samples to establish the analytical concentration of the investigated heavy metals, by the methods of atomic spectrometry, includes the following steps: a) drying to air dry weight and grinding to a homogenized mass; b) dissolving the sample with a mixture of $HNO_3$ and $HClO_4$ for 24 hours at room temperature; c) evaporating it to a wet residue on a sand bath and d) quantitatively transferring it to a test tube and bringing it to a standard final volume with 1N $HNO_3$. The content of Pb, Cd, Zn and Cu in the analyzed samples was determined using an inductively coupled plasma atomic emission spectrometry (ICP-AES) in a Perkin Elmer Optima 7000 DV instrument. The residual amounts of the studied elements were established using an atomic-absorption analysis. The Concentrations of the elements in the liver and kidney tissues were expressed as mg/kg of dry analyzed tissue.

The reference interval of the residues of all investigated heavy metals in both internal organs was calculated using a non-parametrical percentile method. All concentrations were described by their basic descriptive statistics (Median; Std. Dev. and 2.50 and 97.5 percentile value). They were calculated by the algorithm of statistical analysis "Nonparametric Methods". The range of each variable was determined by the values of the $97.5^{th}$ and $2.50^{th}$ percentiles – width of the range about the median that includes 95% of the cases.

To assess the difference of the observed concentrations of the studied heavy metals in both target organs, a nonparametric test (Man-Whitney test) for independent samples was applied using the median as a goal and a means to perform their comparison; the p-value of 0.05 was treated as a "border-line acceptable" error level. All calculations were performed using the statistical package STATISTICA version 10.0 [12].

**Results**

Descriptive statistics for Pb, Cd, Zn and Cu in the liver and kidney tissue of the investigated roe deer is presented in Table 1.

**Table 1. Values of basic statistical characteristics of the concentrations of Zn, Cu, Pb and Cd in liver and kidney of adult male roe deer inhabiting Northeastern Bulgaria**

| Metal | Mean | Median | Minimum | Maximum | 2.5 percen. | 97.5 percen. | Std. Dev. |
|---|---|---|---|---|---|---|---|
| Concentration in liver tissue [mg/kg dry tissues] | | | | | | | |
| Cu | 57.0 | 55.8 | 46.5 | 83.1 | 46.5 | 83.1 | 9.8 |
| Zn | 530.4 | 310.5 | 116.3 | 1542.5 | 116.3 | 1542.5 | 526.8 |
| Pb | 6.1 | 6.9 | 0.7 | 8.4 | 0.7 | 8.4 | 2.6 |
| Cd | 29.4 | 26.5 | 8.1 | 74.9 | 8.1 | 74.9 | 19.8 |
| Concentration in kidney tissue [mg/kg dry tissues] | | | | | | | |
| Cu | 143.2 | 114.5 | 144.5 | 441.7 | 25.90 | 4417 | 126.1 |
| Zn | 749.50 | 504.2 | 504.2 | 2099.2 | 70.80 | 2099.2 | 734.2 |
| Pb | 6.35 | 6.5 | 0.81 | 9.75 | 0.81 | 9.7 | 2.8 |
| Cd | 2.91 | 2.8 | 0.86 | 5.31 | 0.86 | 5.3 | 1.4 |

The results of the Man-Whitney test showed that the differences of the medians of the observed heavy metals' concentrations in both target organs were of different statistical significance: for copper it is p=0.0985; for zinc: p=0.486; for plumbum: p=0.1976; for cadmium: p=0.0668. As could be seen, all the established significance levels are higher than p-value of 0.05; the concentrations of all investigated elements were specific to each one of the target organs. The concentrations of the tested metals, specific for each organ, characterized by the width of the range about the median that includes 95% of the cases demonstrate the limits of their variability (Table 2).

**Table 2. The limits of variability of the concentrations of the investigated heavy metals in liver and kidney of adult male roe deer inhabiting in Northeastern Bulgaria**

| Liver | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Metal | Cu | | Zn | | Pb | | Cd | |
| Concentration limits [mg/kg dry tissues] | From | To | From | To | From | To | From | To |
| | 25.988 | 269.853 | 70.81 | 1115.631 | 0.8081 | 9.755 | 0.859 | 5.314 |
| Kidney | | | | | | | | |
| Metal | Cu | | Zn | | Pb | | Cd | |
| Concentration limits [mg/kg dry tissues] | From | To | From | To | From | To | From | To |
| | 46.488 | 83.068 | 116.286 | 1542.518 | 0.713 | 8.401 | 8.113 | 74.92 |

To reveal the specificity of heavy metals' levels in the internal organs of roe deer inhabiting agricultural landscape in Bulgaria, they were compared to the levels in the liver and kidney of roe deer inhabiting the north-western part of Poland in an area

dominated by agriculture and industry. It is characterized by a medium air pollution level. In the northern and eastern part of the province, the pollutant concentrations are low and permissible levels of air pollutants are not exceeded. Almost half of this area is occupied by agricultural land (arable land, meadows and pastures), and over 35% by forests and forest lands [13].

The comparison of concentrations of tested metals in the agro-regions of Northeastern Bulgaria and north-western part of Poland, specific for each organ of the studied elements, shows their specificity in loading in the liver and kidneys in the roe deer from the two agro regions. The observed mean concentrations of Cu (14.34 mg/kg dry tissues), Zn (55.73 mg/kg dry tissues), Pb (0.946 mg/kg dry tissue) and Cd (0.877 mg/kg dry tissue) in the liver and kidneys Cu (17.09 mg/kg dry tissues), Zn (78.55 mg/kg dry tissues), Pb (0.610 mg/kg dry tissues) and Cd (9.177 mg/kg dry tissues) of the roe deer from Poland are considerably lower than those in the roe deer inhabiting in Northeastern Bulgaria. At the same time, they fall within the limits of the variability of the investigated heavy metals in the liver and kidney of adult male roe deer from North – Eastern Bulgaria.

**Conclusion**

Our results provide an opportunity the roe deer in Bulgaria to be used as a bioindicator of future potential anthropogenic negative influence on the environment in agricultural regions in the country, under the conditions of modern agricultural activities in them. They confirm that the availability and abundance of roe deer in the agroecosystem makes them suitable bioindicators for environmental contamination in them.

It has been demonstrated that roe deer in the investigated area are laden with lead and cadmium and are at a risk for bioaccumulation of trace metals in their organs. The finding of highly toxic lead and cadmium in the studied roe deer, which in wild animals may be due to anthropogenic pollution of the environment [14], confirmed the necessity of assessment of the bioaccumulation of priority environmental toxicants in herbivores mammals that are typical for the agroecosystem. Therefore, further research concerning the determination of the level of these elements in animal tissues and in abiotic components of the agro environment is needed to make a full assessment of the environmental risk.

The results obtained from the investigated agricultural area, which includes a large part of the stock of roe deer in Bulgaria can be used not only for evaluation of the physiological condition of the individuals, but also as a bio-indicative marker for evaluating the quality of the natural environment, which they inhabit. In the future, they can be compared with similar results from other characteristic game habitats located in Bulgaria, namely mountain habitats. Further studies should address the sources of contamination for roe deer and different factors such as age, seasonal variations, food preferences and diet composition. Heavy metal concentrations determined within this study should be considered as baseline values for comparison in future investigations, such as those that may determine if biomagnification of heavy metals has occurred in roe deer food chains. In addition, our results should be included in a database for metal loading in European ungulates.

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# COMMUNITY STRUCTURE AND INDIVIDUAL RESPONSE OF CHIRONOMIDAE (DIPTERA) LARVAE IN ANTHROPOGENICALLY IMPACTED SITES OF THE DANUBE RIVER, NORTH EAST BULGARIA

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**Abstract**

**Aim:** The aim of the study is to establish the response of Chironomidae to anthropogenic impact in Danube River.

**Material and Methods:** The larvae of the family Chironomidae were determined by external morphology of larva head and cytotaxonomically. Head capsule deformities were presented in percentage. Physical and chemical variables of water and sediment were analyzed.

**Results:** The concentrations of some heavy metals and nutrients exceeded the reference data. The density of the genera *Cricotopus* and *Polypedilum* correlated ($P<0.05$) with alkalinity, Cu and nutrients. Somatic alterations (S index-3.66) were established in the salivary gland chromosomes of *Chironomus piger* Strenzke.

**Conclusions:** The results showed the anthropogenic impact on Chironomid taxa. The potential of midge deformities as a biomonitoring tool is shown, however they should be used very carefully because a number of abiotic and population factors can affect their occurrence.

**Keywords:** Chironomidae, Danube, deformities, environmental variables, anthropogenic impact

## Introduction

The ripal zone of the Bulgarian stretch of the Danube River is highly influenced by a different anthropogenic pressure which changes the composition and functioning of the benthic communities. The larvae of the family Chironomidae (Diptera) show large ecological plasticity and can be found in different water habitats and variable environmental conditions [1]. They dominate in benthic communities, are exposed directly to the influence of environmental factors, participate in the transformation of the organic matter and in the processes of water self-purification. These biological characteristics make them suitable for an assessment of the anthropogenic impact on the freshwater ecosystems [2]. Very sensitive to stress agents in the environment is the external morphology of the larvae: mandibles, antenna, mentum, epypharings [1]. The biomarkers on phenotype and genome level are an important signal for detecting processes of disturbance in community and population.

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Morphological deformities in the mouthparts of chironomid larvae were demonstrated with correlations between their frequencies and contamination in aquatic ecosystems. They can be used for a long time as phenotype markers for toxicity assessment [3]. However, they could emerge under the influence of other factors of the environment as well [4]. Many data indicated that Chironomid genome is more sensitive to the contaminations in the aquatic ecosystems than the external morphology [5, 6]. Chironomid larvae (stage which is exposed to the contaminants) possess very well banded salivary gland chromosomes which make them prospective subjects for cytogenetic monitoring. Through the analysis of the chromosome aberrations chironomids can be used for tracing the mutagens effects of a number of stress agents in the environment [2, 6].

The aim of the present study is to evaluate the response to the contaminants in the environment of the Danube River of the chironomids at community, phenotype and genome level.

**Material and Methods:**

**1. Study area**

The sampled sites were located in three stations along the Danube River: after the tributary Rusenski Lom (RusLom), the industrial area of the cities of Ruse (Marten) and Silistra (Silistra) (Fig. 1). The samples were collected in the low water period (July-September), years 2013 and 2014.

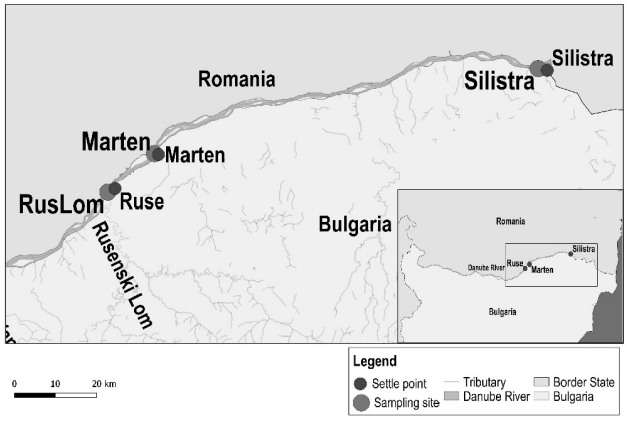


**Fig. 1. Map of sampling sites**

**2. Physical and chemical analysis of the water**

Alkalinity (pH), conductivity (Cond), water temperature (Temp), dissolved oxygen (DO) and oxygen saturation (Osat) were measured *in situ*. In laboratory were analyzed the nutrients ($NH_4$-N, $NO_2$-N, $NO_3$-N, $PO_4$-P, Total P) and heavy metals (Cu, Pb, Zn, Cd). As reference data we used those appointed by Regulation N-4/23.09.2014 and Directive 2013/39/EU. The analytical methods were used as follows: water temperature (°C) – BSS 17.1.4.01:1997 (Method Detection Limit-0.1); alkalinity (pH) – BSS ISO 10523:2012 (MDL-0.00); conductivity (µS.cm$^{-1}$) - BSS EN 27888:2000 (MDL-0.0); dissolved oxygen/oxygen saturation (mg. l$^{-1}$/%) – BSS/EN/ISO 5814:2012 (MDL-0.00); ammonium nitrogen – $NH_4$-N (mg. l$^{-1}$) – BSS EN ISO 11732:2006 (MDL-0.01); nitrite nitrogen – $NO_2$-N (mg. l$^{-1}$) – BSS EN ISO 13395:2001 (MDL-0.002); nitrate nitrogen – $NO_3$-N (mg. l$^{-1}$) – BSS EN ISO 13395:2001 (MDL- 0.2); P - orthophosphate – $PO_4$-P/total phosphorus – Total P ($PO_4$-P) (mg. l$^{-1}$) – BSS EN ISO 15681:2005 (MDL-0.01); Cu, Pb, Zn, Cd (µg. l$^{-1}$) – BSS 16777:1987. Water samples were collected and preserved according to BSS EN ISO 5667-6:2016.

**3. Chemical analysis of the sediment**

In the samples were analyzed the nutrients ($NH_4$, $NO_2$, $NO_3$, $PO_4$, Total P) and heavy metals (Cu, Pb, Zn, Cd, Fe, As) according to the methods described in the standards: Pb ($mg.kg^{-1}$) – BSS 17.4.4.02/1980 Amendment 1981; Cu ($mg.kg^{-1}$) – BSS 17.4.4.03/1980 Amendment 1981; Cd ($mg.kg^{-1}$) – BSS 17.4.4.04/1980; Zn ($mg.kg^{-1}$) – BSS 17.4.4.05/1980; As ($mg.kg^{-1}$) – BSS 17.4.4.02:1979; $NH_4$, $NO_2$, $NO_3$ ($mg.kg^{-1}$) – ISO 14256-2:2005, $PO_4$, Total P ($mg.kg^{-1}$) – ISO 11263:1994 As reference data for heavy metals we used those appointed by Förstner and Salomons [7]. Sediment samples were collected and preserved according to BSS ISO 5667-12:2017 and BSS EN ISO 5667-15:2009.

Benthic samples were taken in correspondence to standard EN ISO 10870:2012 using a hand-net (frame 30x30 cm, mesh size 500 μm) and were preserved according to BSS EN ISO 5667-3: 2012. The density of chironomid larvae was calculated per 1 $m^2$.

**4. External morphology of larva**

The keys by Wiedreholm [8] and Bitušik and Hamerlik [9] were used for determining the chironomid genera.

**5. Cytotaxonomical analysis**

We identified six species cytotaxonomically [10, 11], collected from Marten (2014). For cytogenetic analysis was used $IV^{th}$ larval stage, fixed in alcohol (96%) – acetic acid (3:1). The chromosomes were stained by conventional acet – o rcein staining [10]. The detailed cytogenetic analysis of somatic and inherited aberrations was performed in salivary gland chromosome of *Chironomus piger* Strenzke, 1956 [2]. On the basis of somatic aberrations, the somatic index of this species has been calculated [2].

**6. Statistical analysis**

The transformation of the data and graphics was done in MS Office Excel 2016 and the Spearman's rs correlation coefficient was applied in PAST Software package v2.17c [12].

**Results and Discussion**

A total of 14 genera were determined at the sampled sites, belonging to 3 subfamilies: Chironominae (tribes Chironomini and Tanytarsini), Orthocladiinae and Tanypodinae. (Table 1).

**Table 1. Abundance of the Chironomid genera at the sampling sites. The number of every genus is presented by a Square-root transformation**

| Subfamily | Genus | RusLom 2013 | RusLom 2014 | Marten 2013 | Marten 2014 | Silistra 2014 |
|---|---|---|---|---|---|---|
| Orthocladiinae | *Cricotopus* (*Cricotopus*) van der Wulp, 1874 | 1.721326 | 7.45356 | 1 | 2.108185 | 1 |
| | *Cricotopus* (*Isocladius*) Kieffer, 1909 | 0 | 5.773503 | 0 | 9.545214 | 1 |
| | *Nanocladius* Kieffer, 1913 | 0.860663 | 0 | 0 | 0 | 0 |
| | *Orthocladius* van der Wulp, 1874 | 0 | 0 | 0 | 3.944053 | 0 |
| | *Rheocricotopus* Brundin, 1956 | 0.860663 | 0 | 0 | 2.108185 | 0 |
| Chironominae tribe Tanytarsini | *Paratanytarsus* Thienemann & Bause, 1913 | 0 | 7.45356 | 0 | 6.324555 | 0 |
| | *Tanytarsus* van der Wulp, 1874 | 2.854496 | 0 | 0 | 5.374838 | 1 |
| | *Virgatanytarsus* Pinder, 1982 | 1.721326 | 0 | 1.732051 | 0 | 0 |
| | *Tanytarsini* | 1.217161 | 0 | 1 | 0 | 0 |
| Chironominae tribe, Chironomini | *Chironomus* Meigen, 1803 | 0 | 0 | 1 | 2.108185 | 0 |
| | *Dicrotendipes* Kieffer, 1913 | 0 | 0 | 1 | 0 | 0 |
| | *Parachironomus* Lenz, 1921 | 0.860663 | 0 | 0 | 0 | 0 |
| | *Polypedilum* Kieffer, 1912 | 1.721326 | 6.666667 | 2 | 3.59011 | 1.732051 |
| Tanypodinae | *Conchapelopia* Fittkau, 1957 | 1 | 0 | 0 | 0 | 0 |

The genus *Cricotopus* van der Wulp, 1874 was presented by two subgenera*: Cricotopus* van der Wulp, 1874 and *Isocladius* Kieffer, 1909. The two genera: *Paratanytarsus* Thienemann & Bause, 1913 and *Polypedilum* Kieffer, 1912 were the most abundant in the studied stations. Moreover, the genus *Cricotopus* (*Cricotopus*) and *Polypedilum* were found in all studied stations. The values of Shannon-Wiener diversity index varied between 1.476 and 2.095 – indicating a disturbed community as the highest values of index could be 5, pointing a balanced one (Fig. 2).

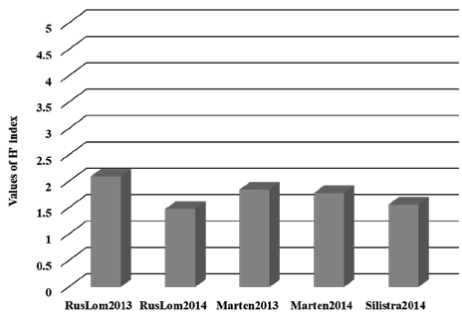


**Fig. 2. Values of Shannon-Wiener diversity index**

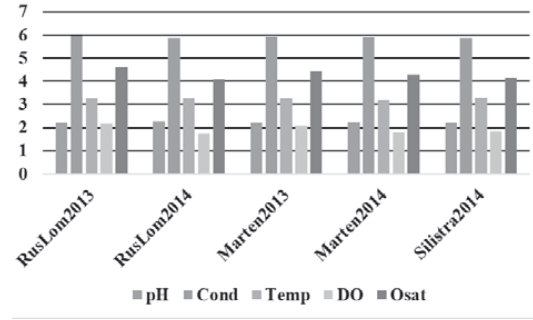


**Fig. 3. Log-transformed values of the *in situ* measured physical and chemical variables**

***Physical and chemical analysis of the water***

The sampling sites of RusLom and Marten (2014) were characterized with higher values of pH and lower values of oxygen concentrations, conductivity and temperature (Fig. 3). The values of pH at the site RusLom (2014) and dissolved oxygen at the sites RusLom, Marten and Silistra (2014) were characterized in "Moderate" ecological state/assessment according to Regulation N-4/23.09.2014. The values of the measurements in 2013 and pH-Marten and Silistra (2014) showed a "Good" ecological state/assessment.

Nitrate nitrogen and orthophosphates were higher at the sites of RusLom and Marten (2014) and RusLom (2013) (only for $NO_3$-N) (Fig. 4a). Those values determined a "Moderate" ecological state/assessment. The other values of the nutrients equally fell into the classes of a "Good" and a "High" ecological state/assessment as the nitrate nitrogen didn't indicate a "High" state/assessment at none of the sites.

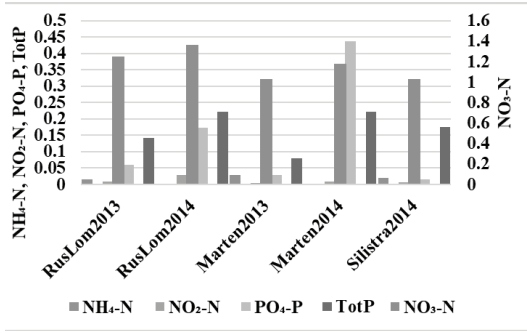


**Fig. 4a. Log-transformed values of nutrients in water**

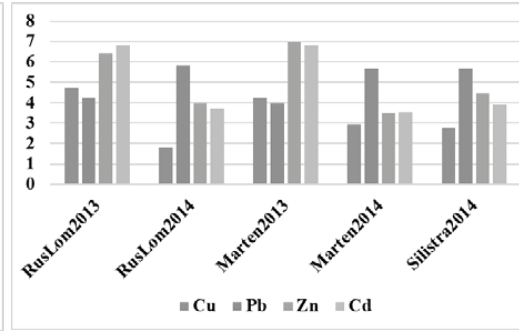


**Fig. 4b. Log-transformed values of heavy metals in water**

The heavy metals in water samples exceeded the average annual values according to Regulation N-4/23.09.2014, Directive 2013/39/EU with the exception of Cu at RusLom (2014) and Zn at sites RusLom and Marten in 2014 (Fig. 4b).

***Chemical analysis of the sediment***

Contrariwise, the heavy metal concentrations in the sediments in most cases didn`t exceed the reference data according to Förstner and Salomons [7]. Only at the site of Marten (2014) Cu, Pb, Zn and Cd exceeded the reference data. The Cd ion concentrations exceeded also the reference data at the sites of Marten and RusLom (2013) and Zn and Pb – at RusLom (2014) (Fig. 5a).

All nutrients in the sediment were higher at station Marten (2013) in comparison with the values at other sampling sites, except for the total phosphorus, whose concentration was higher at site Silistra (Fig. 5b).

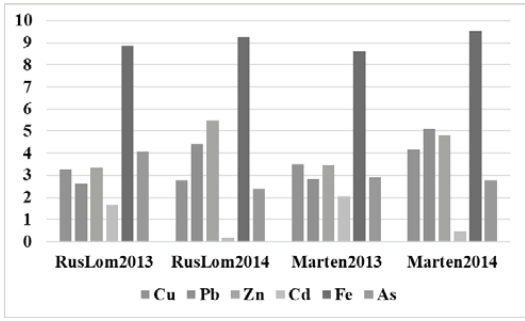


**Fig. 5a. Log-transformed values of heavy metals in sediments**

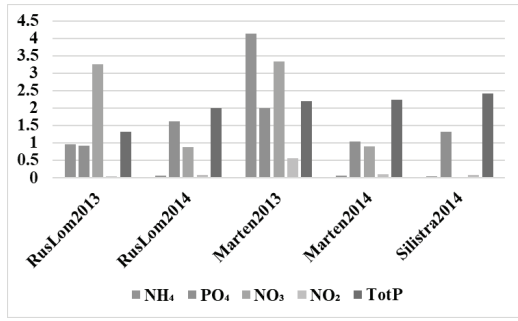


**Fig. 5b. Log-transformed values of nutrients in sediments**

***Relationships between environmental variables and genera's occurrence***

A positive correlation was found: 1. between the frequencies of the genera *Cricotopus* (*Cricotopus*) and *Cricotopus* (*Isocladius*) and alkalinity; 2. between *Cricotopus* (*Cricotopus*) and nitrate nitrogen in water. On the other hand, the frequency of genus *Cricotopus* (*Isocladius*) correlated negatively with Cu in water and nitrates in the sediment (Table 2).

**Table 2. Spearman's rs correlation coefficients among the chironomid genera and measured variables (bolded values are significant, p<0.05)**

| | Variable | | | | |
|---|---|---|---|---|---|
| Genus | pH | Cu | $NO_3$-N | $PO_4$Sed | $NO_3$Sed |
| *Cricotopus* (*Cricotopus*) sp. | **0.84407** | -0.47708 | **0.96296** | 0.66058 | -0.32407 |
| *Cricotopus* (*Isocladius*) sp. | **0.91453** | **-0.87477** | 0.77248 | 0.45726 | **-0.8427** |
| *Polypedilum* sp. | **0.89912** | -0.53213 | **0.87963** | **0.88077** | -0.26852 |

Genus *Polypedilum* also correlated positively with pH and nitrate nitrogen in water and phosphates in the sediment (Table 2). Both genera were more abundant at the sites of RusLom and Marten (2014) which were with elevated concentrations of heavy metals in the sediment and nutrients in water. Also, these genera (from subfamilies Orthocladiinae

and Chironominae) were found to be more abundant at sites receiving mine drainage [13], however, the same authors underlined the lower tolerance of Tanytarsini. In our study the genus *Paratanytarsus* (Tanytarsini) occurred in higher numbers. Other authors [14] showed that Orthocladiinae prefer the metal-tolerant blue-green algae as a food resource and therefore the Orthocladiinae was more tolerant to metal-contaminated sites.

***Malformations of mentum and mandibuls***

In the studied genera malformations in mentum and mandibles were established in subgenus *Isocladius* (1.72%), subgenus *Cricotopus* (12.36%), genus *Polypedilum* (16.66% - 25%) and genus *Chironomus* (30.77%) (Fig. 6a, b). However, the response at phenotype level should be considered very carefully. Some authors found the influence of the temperature and season on the appearance of the deformities [15]. Therefor in addition, the genome of larvae was studied for occurrence of aberrations.

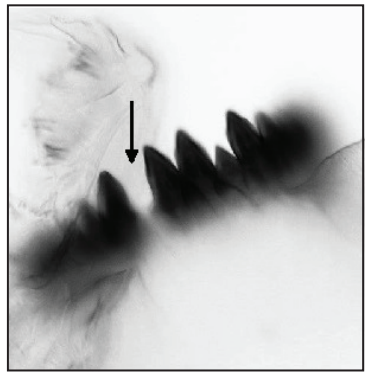

**Fig. 6a. Malformation of mentum of *Polypedilum* sp. (original)**

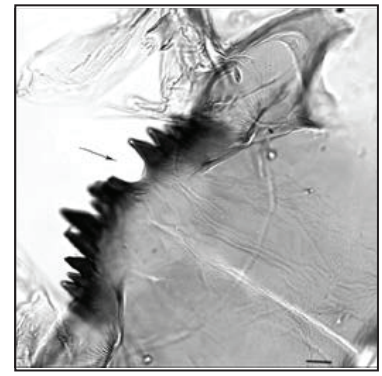

**Fig. 6b. Malformation of mentum of *Chironomus* sp. (original)**


**Fig. 7a. Somatic heterozygous inversion in *C. piger* (original)**


**Fig. 7b. Polytene chromosomes of *C. piger* (2n=8). NOR Nucleolar Organizer; BR- Balbiani Ring; arrow indicates the localization of the centromere region (original)**

***Genome instability***

Five species were identified cytotaxonomically: *Cricotopus gr. flavocinctus, Chironomus parathummi* Keyl, 1961, *Cricotopus gr. sylvestris*, *Chironomus piger* Strenzke, 1956, *Cricotopus bicinctus* (Meigen, 1818). Detailed cytogenetic analysis was performed with *C. piger.* The species belongs cytogenetically to the thummi cytocomplex with chromosome arm combinations: AB CD EF G (Fig. 7b).

Only somatic heterozygous inversions were established in the polytene chromosomes of the species (Fig. 7a). Their localization and frequency can be seen in Table 3.

These aberrations occurred in low frequency, affected a very small region of the polytene chromosomes and appeared in few cells of the salivary gland. The somatic index (S) was 3.66. The highest degree of chromosome damage to the polytene chromosomes of *C. piger* was found in populations of other Bulgarian and UK Rivers [6]. It is known that the somatic aberrations and somatic (S) index are a very good biomarker for evaluation the degree of environmental pollution [5].

**Table 3. Somatic heterozygous inversions in polytene chromosomes of *Chironomus piger* Strenzke from sampling site Marten**

| **Arm – aberration, localization/%** | **Arm – aberration, localization/%** | **Arm – aberration, localization/%** | **Arm – aberration, localization/%** | **№ of studied individuals /cells** | **№ of cells with aberr./%** | **Somatic index** |
|---|---|---|---|---|---|---|
| Chromosome AB | Chromosome CD | Chromosome EF | Chromosome G | 3/33 | 8/24.24 | 3.66 |
| **A-** B1g/3.03 | **C-**B3d/3.03 | **F-**B2k/3.03 | **G-**A1b-c/3.03 | | | |
| **A-**B2d-e/6.06 | **D-**D1d-e/3.03 | **F-**D2d-e/3.03 | | | | |
| **A-**B3i/3.03 | | **F-**D3b/3.03 | | | | |
| **A-**B4d-g/3.03 | | **F-**E2a/3.03 | | | | |

Our results showed variability in chironomid diversity in different anthropogenically impacted sites, as well as changes at phenotype and genome level. A further deeper research is needed to explain the exact impact of environmental variables on chironomid community and the response at individual level in studied sites.

**Conclusions**

The pollution by heavy metals and nutrients in the studied sites reach up to 600 times the reference data. The sampling sites of RusLom and Marten (2014) were more loaded with nutrients in water and heavy metals in sediments, while in the year 2013 the concentrations of nutrients in sediments and heavy metals in water were higher.

The most abundant and very sensitive to the environmental variables were the genera *Cricotopus (Cricotopus, Isocladius)* and *Polypedilum,* where the malformations of the mentum and mandibles of the head capsule were found.

The somatic chromosome aberrations and somatic index (S) established in salivary gland chromosomes of *C. piger* confirmed the occurrence of an anthropogenic impact on the site Marten in the Danube River. The application of polytene chromosomes due to

their great resolution is very useful in applied investigations on environmental quality. The analysis of cytogenetic responses is therefore potentially a powerful tool in preventing the long-term effects of anthropogenic stress at the populations and community level.

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# DYNAMICS OF THE HEALTH STATUS OF COPPICE OAK FORESTS IN SOUTHWESTERN BULGARIA

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**Abstract**

**Aim:** The aim of this study is to analyze the dynamics of the health status of coppice oak forests in Southwestern Bulgaria and to identify the main stress factors that affect it.

**Material and Methods:** The research is carried out in oak forest stands located in the Bulgarian Southwestern State Forestry Enterprise. Dendrochronological analysis is used predominantly, which is combined with defoliation assessment, macroscopic and microscopic phytopathological analyzes.

**Results:** Representative radial increment chronologies for the main oak species in the studied area are developed, which reflect the dynamics of their health status. The obtained coefficients of determination by multifactor regression analysis for the influence of temperature and precipitation regimes on the condition of the stands are high ($R^2 > 50\%$).

**Conclusion:** The health status of the studied coppice oak forests was the most deteriorated in the mid-20$^{th}$ century and after the early 1980s. The main predisposing stress factor that causes this condition is the unfavorable temperature-precipitation regime.

**Keywords:** dendrochronology, oak decline, coppice forests, *Quercus petraea* (Matt.) Liebl., *Quercus frainetto* Ten.

## Introduction

Climate anomalies, air pollution and biotic stress factors have caused several periods of dieback of different forest types in Bulgaria and in Europe as a whole over the last decades. The progressive deterioration of the forests' health and the high level of tree mortality raised the necessity of applying new approaches in studying the etiology of forest diseases. This has led to the development of new concepts of their causes. Nowadays, these diseases are mostly considered as a polyetiological processes, i.e. caused by successive or simultaneous influence of several factors. Significant contributions in this direction were made by Houston [1] and Manion [2], who formulated a new type of forest disease – 'forest decline'.

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It was described as a complex disorder, resulting from simultaneous or successive action of factors, belonging to three hierarchically ordered groups – **predisposing, inciting and contributing**. This conceptual novelty made easier the determination and understanding the role of the factors in the disease etiology. The advantages of the concept of forest decline made possible its wider application in studies of pathological dieback in a number of tree species [3, 4].

The same concept was followed also in studying the decline of oak trees in the Mediterranean region [5]. The scientists adapted Manion's disease spiral for the oak decline by specifying as predisposing factors soil characteristics and *Phytophthora cinnamomi*, both affecting root expansion and water retention, draught and inadequate cultural practices as inciting factors and opportunistic pathogens as contributing stressors. A number of other authors also consider unfavorable growth conditions, which are mainly linked with abiotic (climatic anomalies) and biotic (pathogens and insects) stressors, as the main causes of the oak decline in Southern Europe [4]. The majority of the authors point out the unfavorable temperature-precipitation regime as the key factor for originating of this disease [3, 4, 6].

Predicted droughts, higher air temperatures and strong winds will inevitably affect the phytosanitary status of oak forests in Bulgaria, too. Over the last decades comprehensive research of such problems has been carried out in representative coppice and high oak stands in several state forestries and protected areas in the country [6, 7, 8, 9]. Several phytopathologists have performed also surveys of the occurrence of *Phytophthora* pathogens in oak forests in Bulgaria. Although such species were isolated from oak trees, there are no data that the most dangerous ones (*Ph. cinnamomi* and *Ph. ramorum*) are present in our country [10]. The results of these studies, however, are not directly applicable to the coppice forests throughout the country, which requires additional research to be carried out.

The problems of the coppice oak forests has long attracted the attention of a wide range of people, working in the forest sector in Bulgaria. This has led to holding of a National meeting on the prospects and guidelines for the management of these forests in 2016 [11], where the possibilities for their transformation into high stands were discussed. Kostov and Aleksanrov [12] pay attention also to the importance of the history of coppice oak stands management and stress the need of its analysis in studies of their health status and Tonchev, Aleksandrov and Molle [13] determine the optimal rotation ages of such forests.

The most likely way to precisely investigate the influence of the variety of environmental factors on trees' health status is by considering the dimension of time. The past environmental situation could be shown in true light by analysis of the tree rings, which makes dendrochronological methods the most suitable ones for studying the impact of the stressors, affecting tree health [14].

In order to obtain up-to-date information on the development of the decline of coppice oak forests, **the aim** of this study is to analyze the dynamics of their health status in Southwestern Bulgaria and to identify the main stress factors that affect it. For this purpose, several **research tasks** had to be settled: 1) to analyze the dynamics of the radial increment of the studied trees in the past; 2) to assess the effect of both the temperature and precipitation regimes on the health status of the coppice oak stands and 3) to determine the species composition of important biotic stressors for these tree species in the research area.

## Material and Methods

The research is carried out in oak forest stands located in two state forestries (Zemen and Blagoevgrad) in the Bulgarian Southwestern State Forest Enterprise (Fig. 1). The dominant natural tree species are: *Quercus petraea* (Matt.) Liebl., *Quercus frainetto* Ten., *Quercus pubescens* Willd. and *Quercus cerris* L.

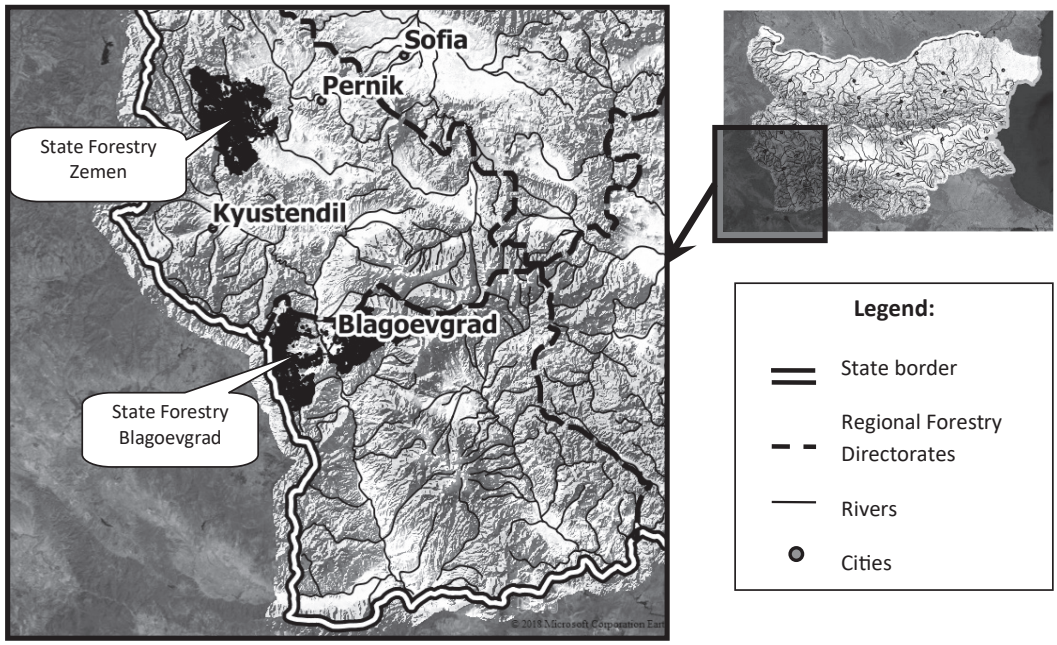


**Fig. 1. Location of the studied coppice oak stands**

The territories of both forestries fall into the water catchment area of Struma River. Most of the forests in them are located in the low and middle mountain ranges. Due to the large differences in altitudes and the intersection of the relief, the slopes are varied, but the inclined and steep terrains prevail. From north towards south the climate in the studied region changes from Moderate Continental (in Zemen) to Transition Continental (in Blagoevgrad). The higher parts of the mountains fall into the mountainous climatic area.

Dendrochronological analysis is used predominantly, which is combined with defoliation assessment, macroscopic and microscopic phytopathological analyzes [3, 14, 15]. The general phytosanitary status of the coppice oak forests is determined by route investigations and data from selected sample plots. For this purpose the European system for assessment of the defoliation of tree crowns, applied in our country, is used.

The choice of one or another method of phytopatological diagnosis is based on the health status of a particular tree species and the nature of the stress factors. In the **macroscopic method**, the diagnosis is most often performed on the terrain by means of characteristic external signs of the damaged plant (symptoms). The **microscopic method** is used when the previous one does not allow precise and unambiguous diagnostic results to be obtained.

This method makes it possible to determine the species or genus of the relevant biotic stressors in laboratory conditions.

The main method, used in the study, is the **dendrochronological analysis**. Core samples were taken by means of increment borer from all of the oak species in the research area. The sampling was done by increment borer at 1.0–1.3 m height above the tree base. Two cores per tree were taken. The number of the collected samples is presented in Table 1.

**Table 1. Locations of the sample plots (divisions in the state forestries) and number of the collected samples from the different tree species**

| State Forestry | Division | Lat. (N) | Long. (E) | Tree species | Altitude, m | Number of samples |
|---|---|---|---|---|---|---|
| Zemen | 199 в1 | 42.556262 | 22.795458 | *Q. petraea* | 1000–1050 | 16 |
| | 199 д1 | 42.554744 | 22.796605 | | | |
| | 199 а2 | 42.557223 | 22.794580 | | | |
| | 211 у | 42.575173 | 22.766213 | *Q. frainetto* | 750–800 | 18 |
| | 212 в | 42.578643 | 22.762975 | | | |
| | 214 г | 42.579735 | 22.779095 | | | |
| | 214 е | 42.576852 | 22.783632 | *Q. pubescens* | 950–1100 | 18 |
| | 235 б1 | 42.586479 | 22.774050 | | | |
| Blagoevgrad | 255 а | 41.968192 | 22.926653 | *Q. petraea* | 950–1200 | 30 |
| | 264 г | 41.968268 | 22.881352 | *Q. frainetto* | 950 | 12 |
| | 265 а | 41.949379 | 22.877411 | | | |
| | 269 б | 41.934446 | 22.879290 | *Q. cerris* | 950–1050 | 12 |

Standard dendrochronological methods were used for processing of the ring-width series (Fig. 2) [15, 16, 17]. The samples were polished with sandpaper, scanned (A3, 1800 dpi scanner) and the tree-ring widths were measured by the use of the CooRecorder computer program in the University of Forestry, Sofia. Both visual and statistical techniques (COFECHA computer program) were applied for cross-dating of the tree-ring series. The standardization of the obtained ring-width chronologies was performed by ARSTAN and DendroStat application software [19]. Modified exponential, Weibull and Hugershoff functions were used for detrending the individual increment series. The mean index chronologies for the forest stands were computed as arithmetic means of the individual standardized series from the different oak species. The mean index chronologies were smoothed by 11-year moving averages.

The mean index chronologies for the radial increment range around a mean value of 1, with higher indices than this value ($I_t > 1$) being indicative of good health condition of the trees, and with lower index values ($I_t < 1$) – for impact of stress factors and poor health condition of the trees [4, 6, 14, 18]. The prolonged periods of their retention below the mean value are defined as stress periods [6, 14, 18].

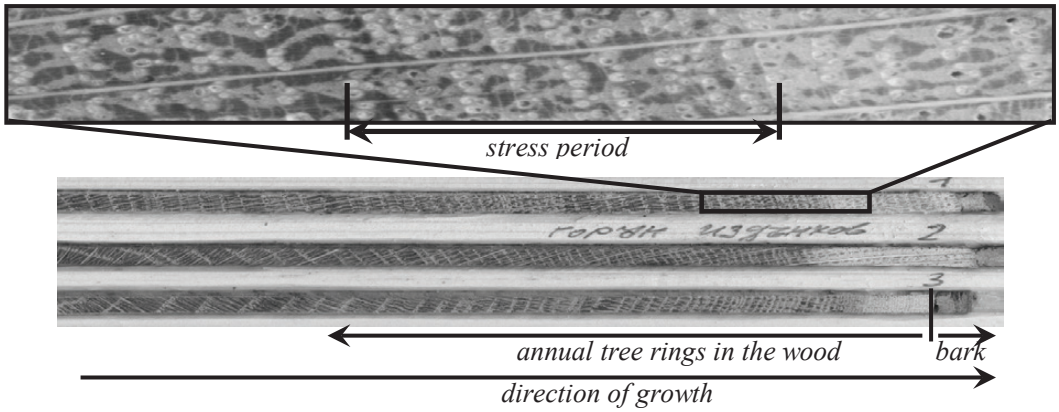


**Fig. 2. Three of the processed tree-ring samples and a magnified section with narrow annular rings (stress period)**

For assessment of the climate impact on oak growth in the study area data were provided for the Sofia and Blagoevgrad hydro-meteorological stations (HMS), which are the nearest available for the studied forest stands (Fig. 3). HMS Sofia is located in the northern part of the region with Moderate Continental climate (550 m a.s.l., mean annual temperature of 10.3°C and mean annual rainfall of 603 mm) and HMS Blagoevgrad is located in its southern part with Transitional Continental climate (110 m a.s.l., mean annual temperature of 12.2°C and mean annual rainfall of 543 mm). The period from 1940 to 2016 was used since this is the common time span of the mean index chronologies for the radial increment of the main oak species. The indices for the mean air temperatures and precipitation sums for the growing season were computed as ratios of their values to the average temperature and precipitation sum for the whole analyzed period, respectively [18]. A multiple linear regression analysis was performed for further assessment of the climate influence on the dynamics of oak growth. The following variables were used as predictors in the models: mean monthly temperatures and monthly precipitation over a period of twelve months – from October of the previous year to September of the current year.

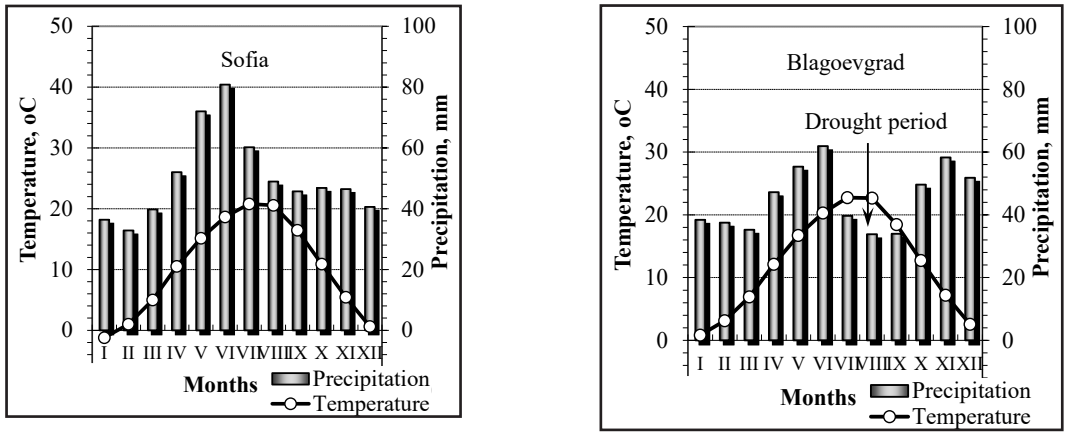


**Fig. 3. Climate diagrams for the northern part (Sofia – Moderate Continental climate) and the southern part of the studied region (Blagoevgrad – Transition Continental climate)**

## Results and Discussion

Representative radial increment chronologies for the main oak species in the studied area are developed, which indicate the dynamics of their health status. No missing tree rings were found during the cross-dating of the core samples, which shows that the environmental conditions in the studied sites had not been unfavorable enough to cease the radial growth of the oak trees. The calculated chronology signal (Expressed Population Signal) for all of the forest stands has values above 0.85, which shows that the number of sampled trees was sufficient for creating the mean chronologies.

The mean increment of *Q. petraea* is 1.32 mm/year in Zemen and 1.28 mm/year in Blagoevgrad. The time span of the developed mean index chronologies cover the period from 1945 until 2017 for the first forestry and from 1937 until 2017 for the second one. The dynamics of the radial increment of this oak species and 11-year smoothing lines are presented in Fig. 4.

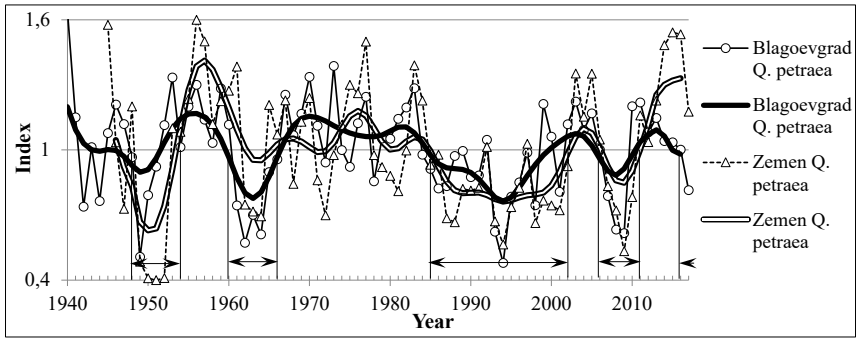


**Fig. 4. Dynamics of the *Q. petraea* chronologies in Zemen and Blagoevgrad and stress periods**

Both increment chronologies from *Q. petraea* had similar dynamics probably because of the similar age of the trees. They had started with radial increment around the mean value ($I_t$ varies around 1). After several years, however, this species had faced two consecutive stress periods: from 1948 to 1954 and from 1960 to 1966. An increase of the increment had followed, but in 1985, the longest stress period for this species had started. It had ended in 2002 and after only four years of increased growth, the increment had dropped below its mean value again. This period of decreased growth had ended on 2011. During the last years of the growth period the trees in Zemen have high radial increment, but the increment in Blagoevgrad in 2017 is below the average value again. This shows that the health status of this tree species in Zemen is better than that in Blagoevgrad.

The mean increment of *Q. frainetto* is 1.84 mm/year in Zemen and 1.04 mm/year in Blagoevgrad. This high difference between the two forestries probably is a result from different age of the trees from this species. The time span of the developed mean index chronologies cover the period from 1961 until 2017 for the first forestry and from 1925 until 2017 for the second one. The dynamics of the radial increment of this oak species and 11-year smoothing lines are presented in Fig. 5.

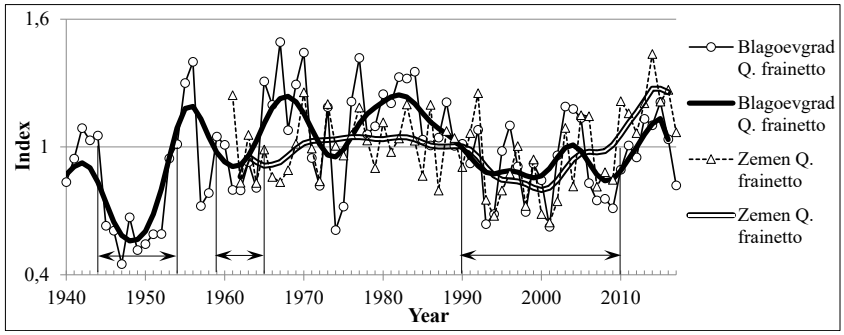


**Fig. 5. Dynamics of the *Q. frainetto* chronologies in Zemen and Blagoevgrad and stress periods**

The chronology from this tree species had started with increment below the average value ($I_t < 1$). It had raised above it in 1954, but after 5 years of increased growth, a new stress period had begun. It had lasted from 1959 until 1965. After that, this tree species had been in good health status until the end of 1980s in both state forestries. At that time, a prolonged stress period had started in this tree species, too. It had lasted until 2010 and after that the increment of *Q. frainetto* had raised above the average value. In 2017 (the last year of the growth period), the increment of this species in Blagoevgrad dropped below the average value again, but no clear stress period can be defined.

The parallel graphical analysis of the smoothed mean index chronologies for the two oak species show some similarities. They have common stress periods in the middle of the 20$^{-th}$ century and after 1980s. It can be noticed that in 1960s the health status of the trees in Zemen was relatively better compared to that in Blagoevgrad. The dynamics of the radial increment and the duration of the stress periods of the other two oak species (*Q. pubescens* and *Q. cerris*) is similar to that of the previous ones.

These similarities in the changes of the health status of all tree species implies that the reasons for them are similar. Graphical analysis of the temperature-precipitation regime during the growing season in the studied area (Fig. 6 and 7) showed that within the analyzed period there had been two periods with low precipitation in the northern part of the area and three in the southern one. The common drought periods had lasted from mid ’60s until mid ’70s of the last century and after the beginning of the ’80s until the beginning of the new 21-st century. There is another one prolonged drought period in the ‘60s in Blagoevgrad.

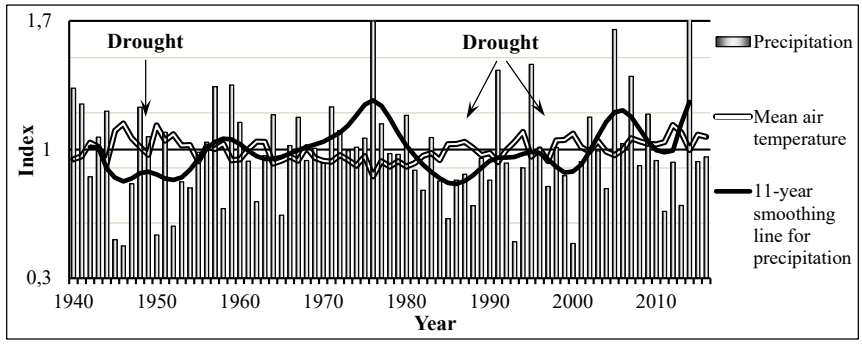


**Fig. 6. Dynamics of the temperature and precipitation in the northern part of the study area (Sofia)**

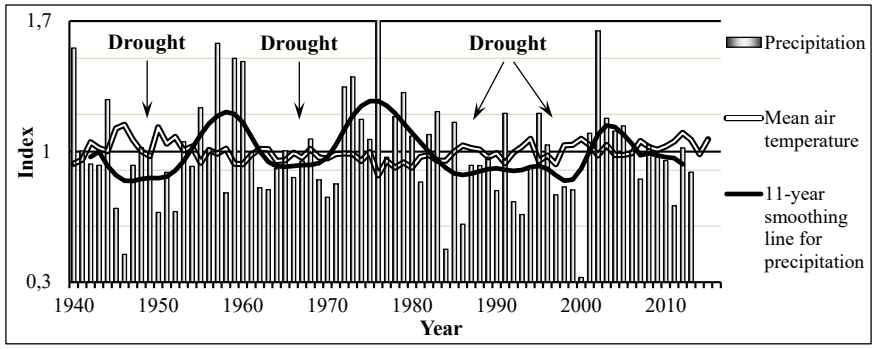


**Fig. 7. Dynamics of the temperature and precipitation in the southern part of the area (Blagoevgrad)**

It can be noted that in most of the years during both common droughts the air temperature had been above the average values more frequently.

The revealed stress periods for the coppice oak stands corresponded almost exactly to these droughts combined with high temperatures. An additionally performed multifactor linear regression analysis, in which monthly precipitations and mean monthly air temperatures were included as predictors, confirmed that there is a strong climatic signal in the chronologies for the studied coppice oak forest stands. The obtained coefficients of determination of the models ($R^2$) are above 50% and the level of significance ($p$) is below 0.05 for all tree species.

The coppice origin of the oak forests is also a very important factor for their health status. It had been found out that after the second timber harvest rotation a surface root system develops and this leads to significant increase of their vulnerability during periods of prolonged droughts [6].

The results of the pathological survey, which was carried out through routing and sample sites, show that there are no mass outbreaks of fungal diseases and insect pests in the coppice oak forests in the studied state forestries. The list of species composition and the occurrence of important biotic stressors in the research area are given in Table 2.

**Table 2. Species composition and abundance of the more important diseases and insect pests in the oak forests in the research area**

| № | Pathogens and insect pests | Occurance |
|---|---|---|
| | **A. Pathogens** | |
| **1** | *Armillaria mellea* | + |
| **2** | *Ganoderma applanatum* | + |
| **3** | *Microsphaera alphitoides* | + |
| **4** | *Gnomonia quercina* | ++ |
| **5** | *Nectria galligna* | + |
| **6** | *Stereum hirsutum* | + |
| **7** | *Bacterial slime flux* | + |
| | **B. Insect pests** | |
| **8** | *Attelabus nitens* | + |
| **9** | *Euproctis chrysorrhoea* | + |
| **10** | *Operophthera brumata* | + |
| **11** | *Dryomyia circinuans* | ++ |
| **12** | *Diplolepis quercus* | + |
| **13** | *Andricus quercustozae* | ++ |
| **14** | *Carpocapsa splendana* | + |
| **15** | *Curculio glandium* | + |

***Note:*** *With the sign "+" the following degrees of abundance for the biotic stressors are differentiated:*

*+ – occurs; + + – occurs often; + + + – occurs very often*

It can be seen from the table that, according to the phytopathological classification of Manion, there are representatives of both the inciting stress factors (insect defoliators and leaf pathogens) and the contributing stressors (xylotrophic insects, facultative parasites and saprophytes). However, the occurrence of unfavorable climatic conditions, that we found by the dendrochronological analysis to be a major growth factor for the oak species in the studied area, can cause sudden insect outbreaks and epiphytotia from dangerous pathogens.

## Conclusions

The health status of the coppice oak forests in Northwestern Bulgaria was the most deteriorated in the mid-20$^{th}$ century and after the early 1980s. The trees in the southern

part of the study area (State Forestry Blagoevgrad) passed through another stress period in the beginning of the 1960s, while those in State Forestry Zemen were in relatively better condition during this period. In most of the forest stands, the health status of the oaks is relatively good at the end of the analyzed period, but in some of them a new stress period may begin in the last year (2017).

The most unfavorable climatic conditions for the studied oak stands are associated with low precipitation combined with high air temperatures. They coincide with the determined stress periods, which shows that unsuitable temperature-precipitation regime is the main predisposing stress factor in these stands. This is confirmed by the performed multiple regression analysis for the influence of the climatic factors.

The coppice origin of the oak forests is a very important factor for their health status. After the second timber harvest rotation a surface root system develops and this leads to significant increase of their vulnerability to unfavorable climatic conditions and biotic stressors. Therefore, the transformation of these forests into high stands is a very important task, but this needs to be done in differentiated way according to the environmental conditions and the health status of the forest stands.

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# CHARACTERISTICS OF THE PHOTOSYNTHETIC APPARATUS OF *ERYNGIUM MARITIMUM* L. AND *POLYGONUM MARITIMUM* L. – HALOPHYTIC PLANTS FROM DUNES OF POMORIE LAKE (BULGARIA) WITH DIFFERENT ADAPTIVE STRATEGY AGAINST SOIL SALINITY

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**Abstract**

**The aim** of this study was to investigate the structural-functional peculiarities of chloroplast membranes of halophytic species with different adaptive strategy against soil salinity.

**Materials and Methods:** Leaves of the euhalophyte *Eryngium maritimum* L. (Apiaceae) and glycohalophyte *Polygonum maritimum* L. (Polygonaceae) were collected in August 2016 and 2017 from the sand dunes of the Pomorie Lake, (Bulgaria). The pigment content was determined spectrophotometrically. Thermoluminescence (TL) measurements from intact leaves and isolated chloroplast membranes were carried out using a computerized equipment. Thylakoid membranes were isolated by standard procedure. The initial oxygen burst was measured using a polarographic oxygen rate electrode. The analysis of fatty acids composition was performed by gas – chromatographic techniques.

**Results:** Comparative TL glow curves analysis show similar overall TL intensity and oscillation of the main TL B- and AG bands in both investigated plants. The oxygen induction curves exhibit biphasic exponential decay thus suggesting some decrease in the proportion of functionally active PSIIα centers in thylakoids which could be attributed to the reduced grana formation and dominant operation of stroma situated PSIIβ centers. The comparative analysis of fatty acids composition of thylakoid membranes confirmed the existence of specific quantitative and qualitative differences of euhalophyte and glycohalophyte species.

**Conclusion:** The results show that the investigated euhalophytic and glycohalophytic species have some specific structural characteristics of photosynthetic membranes, reflecting different adaptive strategies to soil salinity but similar capability to maintain efficient PSII function under environmental conditions in their natural habitat.

**Keywords:** *E. maritimum* L., *P. maritimum* L., fatty acid composition, PSII $O_2$-evolving activity, thermoluminescence.

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## Introduction

Halophyte plants grow in a wide variety of saline habitats, from coastal regions to inland deserts, but the physiological mechanisms underlying positive growth responses to salinity are not clear. The plants living in these extreme environments have to deal with frequent changes in salinity level, being therefore excellent models to study salt resistance and tolerance mechanisms. The situation related to salinity stress is frequently much more complex, since high light intensity and higher temperature stress factors co-occur. Halophytes are a heterogeneous group of plants that unites representatives of various taxa, living forms and ecological types. Adaptation to the existence in saline soils results from the implementation of various metabolic and physiological strategies and goes from simple morphological adjustments and production of specific osmolytes to efficient energy dissipation in order to prevent the accumulation of excessive energy within the chloroplasts and to avoid the production of ROS. Thus, euhalophytes accumulate salts in the middle of the plant, krinohalophytes, on the contrary, remove salts out while glycophytes are salt impermeable [1]. Existence in saline soils affected the halophyte phenotypic characteristics. Succulence is typical of euhalophytes whereas glycohalophyte leaves have a xeromorphic structure. Krinohalophytes excrete salts through special salt glands. Euhalophytes are characterized by a great size of photosynthesizing cells while in krino- and glycohalophytes chlorenchyma cells are much smaller [2].

Properties of photosynthetic apparatus may contribute to a great extent to plant successful adaptation to environmental factors, governing mechanisms for effective light energy utilization under unfavorable conditions. Perturbations by different stressful environments are first manifested in alterations in the structure of thylakoid membranes and the photochemical efficiency of photosystems, especially photosystem II (PSII). PSII oxygen-evolving enzyme complex of thylakoid membranes appears to be the main stress sensitive site in plants. On the other hand, the resistance of PSII could be an important strategy for halophytic plants living in saline soils.

The physiological properties of the membrane such as permeability, selectivity, etc., are changed in dependence of environment conditions, resulting in changes in the physical orientation of membrane lipids and functional activity. A widespread cation of the salt stress is $Na^+$ that is toxic to plants. Permeability of the plant membranes depends on the lipid bilayer state and the response of the cell in such case is a series of quantitative and qualitative changes in the lipid composition of the membrane in order to restore the initial orientation and the physical properties of the membrane [3, 4].

In the present study, oxygen-evolving reactions, thermoluminescence emission and lipid analysis have been used for selective monitoring of functional and structural peculiarities of photosynthetic apparatus in two halophytic plants with different adaptive strategies against soil salinity in their natural habitats. We studied glycohalophyte *Polygonum maritimum* L. and euhalophyte *Eryngium maritimum* L., typical perennial plants that grew on the dunes under natural conditions of Pomorie Lake (Bulgaria) whose high salinity (60-80%) contributed to the formation of a unique ecological environment.

## Materials and Methods

Samples of the euhalophyte *Eryngium maritimum* L. (Apiaceae) and the glycohalophyte *Polygonum maritimum* L. (Polygonaceae) were collected in August 2016 and 2017 from the sand dunes of Pomorie Lake, (Bulgaria).The leaves were maintained in a hydration condition by being wrapped in wet paper at room temperature in darkness. Thylakoid membranes were isolated and re-suspended in a medium containing: 40 mM HEPES (pH 7.6), 10 mM NaCl, 5 mM $MgCl_2$ and 400 mM sucrose. The pigment content of the leaves was determined spectrophotometrically [5].

The initial oxygen burst (induction curves) was measured using a polarographic oxygen rate electrode (Joliot-type), 100 µl sample volume, without any artificial electron acceptors, as described in [6]. Deconvolution of the oxygen burst decay was performed by fitting of the function with two exponential components: $A_1 e^{(tk1)} + A_2 e^{(tk2)}$, where $A_1$, $A_2$ are amplitudes of the fast and slow components.

Thermoluminescence (TL) measurements were carried out in darkness using computerized equipment, described in detail in [7]. The samples were kept in the dark for 2 h before measurements. The excised pieces of a leaf with equal size and appearance were used in order to obtain reproducible results. After dark incubation the leaf samples were cooled to 2°C, illuminated with a single turn-over flash and warmed up to 70°C at a heating rate of $0.6°C.s^{-1}$. Alternatively, samples of the isolated thylakoid membranes ware illuminated at 2-5°C to generate charge pairs within the PSII reaction centers and then rapidly cooled down in liquid nitrogen to trap those charge-separated states. Subsequent warming of the samples reveals several peaks of thermoluminescence emission [8, 9]. Decomposition analysis of the TL glow curves was carried out using OriginPro 8.

For lipid analysis small pieces from the aerial parts were extracted consecutively with chloroform-methanol (2:1) to obtain the total lipophylic extracts. The isolation of total fatty acids and the analysis of their fatty acid composition were performed using thin-layer and gas chromatographic techniques [10].

## Results and Discussion

Thermoluminescence was used as a probe of the behaviour of the PSII reaction centres, both in whole leaves and in isolated chloroplasts. Typical TL signals from the leaves are presented on Fig. 1. Upon illumination of leaf samples by 1 and 2 turn-over flashes at around 2°C, the leaves from the investigated species showed complex TL glow curves with nearly similar overall intensities. Decomposition analysis of the curves charged with two flashes showed the existence of two main TL components: TL B-band (from $S_2Q_{B^-}$ charge recombination) with $T_{max}$ at around 28-30°C and AG-band at around 49°C. When leaves were illuminated by a flash series, the intensity of TL B- and AG-band exhibited a typical for active PSII period four-oscillation pattern with maximum on the second flash (not presented).

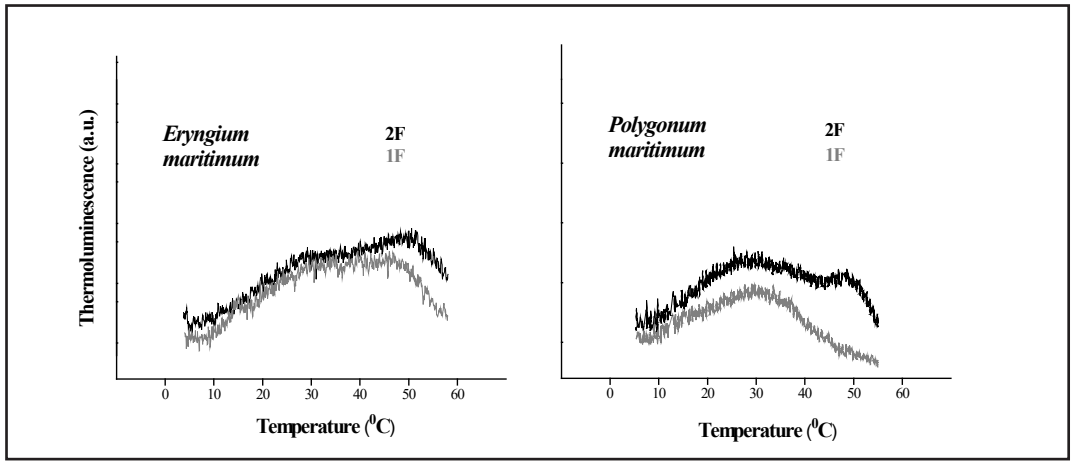


**Fig. 1. Thermoluminescence of *Eryngium maritimum* and *Polygonum maritimum* leaves after charging with single turn-over flashes (1F and 2F)**

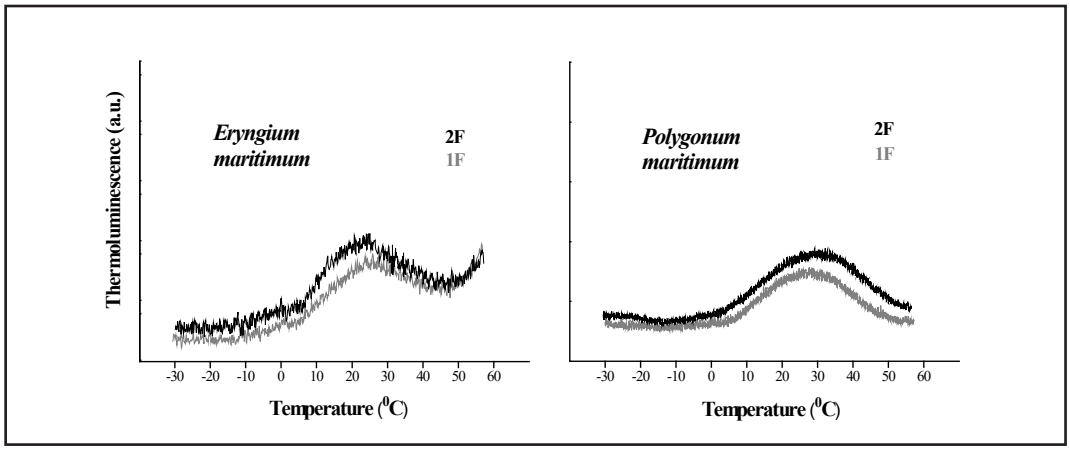


**Fig. 2. Thermoluminescence of *Eryngium maritimum* and *Polygonum maritimum* isolated chloroplasts after charging with single turn-over flashes (1F and 2F)**

The TL glow curves and oscillation pattern of isolated chloroplasts followed particularly well the TL parameters recorded with intact leaves (Fig. 2). The decline at $T_{max}$ in chloroplasts can be explained by the removal of certain protective substances from the stroma in the isolation procedure.

TL B- and AG- bands in intact unfrozen leaves are related to dark distribution of the $S_2$ and $S_3$-states of oxygen evolving complex and $Q_B/Q_{B^-}$ ratio. It is generally accepted that the amplitude of TL B-band is proportional to the number of centers having $S_{2(3)}Q_{B^-}$ charge pairs after flash illumination, while the maximal emission temperature of this band is a measure for the redox span between the separate charges [11]. The AG-band is related to

photosystem II centers initially in the $S_{2(3)}Q_{B^-}$ oxidation state, in which the electron acceptor $Q_B$ becomes reduced either by reverse electron flow or reduction of the plastoquinone pool via an NAD(P)H plastoquinone oxidoreductase and is thought to be an indicator of the metabolic state of the leaf [12]. The cycling of the PSII charge pairs was typical for higher plants, thus suggesting no peculiarities in this parameter of investigated halophytic species.

Another reliable approach used to study the properties of the PSII complex in the investigated halophytic plants species was analysis of the kinetics of oxygen-evolving reactions under continuous excitation of isolated chloroplasts [13].

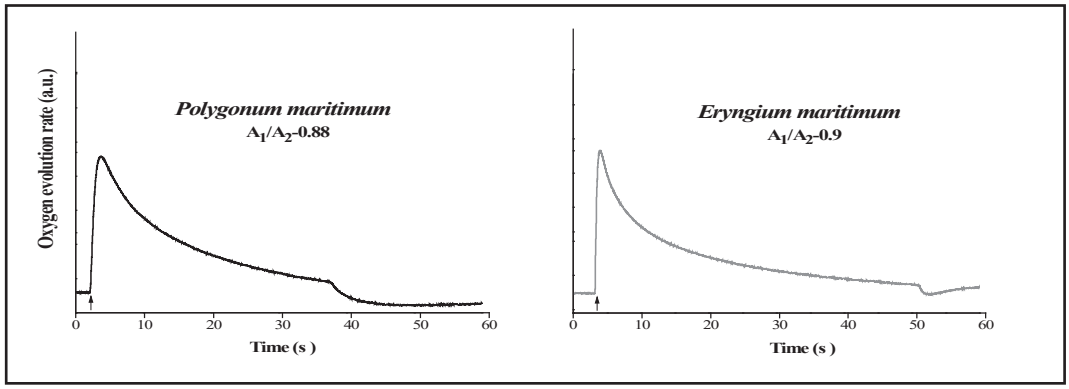


**Fig. 3. Oxygen induction curve recorded after irradiation of *Eryngium maritimum* and *Polygonum maritimum* isolated chloroplasts (chlorophyll concentration of 300 µg/ml) with continuous white light (450 µmol photons $m^{-2}$ $s^{-1}$)**

The amplitude (A) of the initial oxygen burst and the area under the curve (which is a measure of the oxygen volume evolved) are proportional to all functionally active oxygen-evolving centres (i.e. both PSIIα in the grana and PSIIβ in the stroma domains). The decay kinetics after the oxygen burst are fitted with two exponential decay functions and the ratio $A_1/A_2$ of the obtained amplitudes for the fast ($A_1$) and the slow ($A_2$) components corresponds to the ratio of functionally active PSIIα to PSIIβ centres. The results suggest some decrease in the proportion of functionally active PSIIα centres in thylakoids in both halophytic plants which could be attributed to the reduced grana formation and dominant operation of the cooperative mechanism of oxygen evolution in the stroma situated PSIIβ centres [14].

Differences in fatty acid composition may be species-specific or due to the varied environmental conditions. The plants respond to changes in the major environmental factors through lipid restructuring in the membranes, as well as changes in their fatty acid composition. Six common plant fatty acids (C16 and C18) are detected in the total lipophilic extracts from the leaves of investigated plants, including palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) (Fig. 4). The content of palmitoleic acid is very low. The membrane fluidity of the plants depends on the content of the unsaturated 18:2 and 18:3 acids. The 18:2/18:3 ratio is related to the salt resistance and it is reduced when the salt resistance of halophyte plants

decreases [3]. The content of polyunsaturated fatty acids in the membrane lipids is one of the major factors determining the increase in their fluidity, until the maintenance in optimal degrees affects the resistance of photosynthetic apparatus to environmental stress. It has been proved that polyunsaturated fatty acids in thylakoid lipids play important roles in the stability of oxygen-evolving machinery and acclimation of the photosynthetic apparatus to fluctuations in environmental factors, and various forms of environmental stresses [15]. The amount of unsaturated fatty acids is consistent with the oxygen-evolution activity of the studied species.

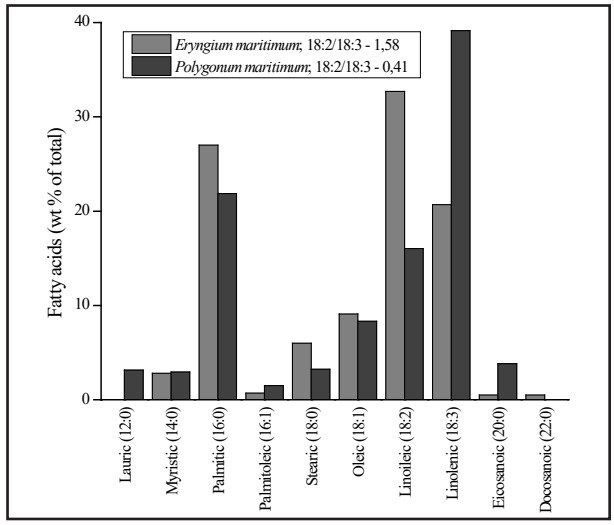


**Fig. 4. Fatty acid composition of leaves from *Eryngium maritimum* and *Polygonum maritimum***

During our studies of euhalophytic *Eryngium maritimum* and glycohalophytic *Polygonum maritimum*, using highly sensitive TL and polarographyc techniques were demonstrated similar capability to maintain efficient PSII function under environmental conditions in their natural habitat. In the same time some specific structural characteristics of photosynthetic membranes, reflecting different adaptive strategies to soil salinity are observed. The physical properties of membranes are largely determined by chain length, polarity, and the degree of unsaturation of fatty acids that comprise their lipids. The fact that each membrane in the cell consists of a characteristic set of lipid classes and that each class has a distinct fatty acid composition suggests that the lipid structure/composition is important for membrane function [6]. Increasing the ratio of 18:2/18:3 acids enhanced the tolerance of PSII to environmental stresses including salt stress. In our study, this ratio is higher at *Eryngium maritimum* compared to *Polygonum maritimum* (Fig. 4.**)**, indicating a better adaptation of this euhalophytic species to saline habitats.

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# NURELLE D® BIOACTIVITY DEPENDING ON THE TEST SYSTEM

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**Abstract**

**Aim:** to evaluate genotoxic, mutagenic and carcinogenic potential of the insecticide Nurelle D® on different test systems; to compare Nurelle D® effects with those of CPF as a main constituent of Nurelle D® on rats.

**Materials and Methods:** *Chlamydomonas reinhardtii* (WT) and *Saccharomyces cerevisiae* (strains 551 and D7ts1) at the end of exponential and the beginning of stationary phase were treated with various concentrations of Nurelle D® (12.5 – 200 ppm and respectively for *Saccharomyces* strains 100 – 6000 ppm) for 30 min: cell survival, "visible" mutations, gene conversion, reverse mutation, mitotic crossing-over and Ty1 test for carcinogenic potential were analyzed;

- non-tumorigenic epithelial cell line MCF-10A: cytotoxic/antiproliferative activity of Nurelle D® were investigated;
- *in vivo* experiments were conducted to reveal clastogenic potential of chlorpyrifos (CPF). Chromosomal aberrations, mitotic index and micronuclei (MN) in polychromatic erythrocytes (PCEs) of rat bone marrow were counted.

**Results:** Concentrations of Nurelle D® that induce 50% lethality were calculated: *C. reinhardtii* – $LD_{50}$ = 55 ppm, *S. cerevisiae* – $LD_{50}$ = 2000 ppm (haploid strain 551) and $LC_{50}$ = 100 ppm (diploid strain D7ts1). No mutagenic effect was found in *C. reinhardtii* in a concentration range used by us and an increase of gene conversion, reverse mutations and total aberrant in *S. cerevisiae* when concentrations equal or higher 1000 ppm were applied.

*In vitro* experiments show that $IC_{50}$ varies depending on the incubation time (24h-72 h) in the range of 26.5 ppm to 33 ppm, respectively. Concentrations higher than 8 ppm can reduce cell proliferation of MCF-10A significantly.

*In vivo* rat studies indicate that treatment with CPF can result in a reduction in the mitotic index of about 2-fold, an increase in chromosomal aberrations about 7-fold, and an increase in the total number of micronucleated polychromatic erythrocytes (MMPSE) about 4 fold.

**Conclusion:** Interspecies and genotype's variability was obtained comparing concentrations of Nurelle D® that determine 50% lethality. Well expressed cytotoxic and genotoxic effects of Nurelle D® were revealed

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even after the treatment with very low concentrations in both test systems *C. reinhardtii* and mammalian cell line MCF-10A. Concentrations equal to or higher than 1000 ppm could provoke different genetic events in *S. cerevisiae*. The clastogenic capacity of CPF was confirmed by *in vivo* experiments with rats. Further experiments should be performed to clarify the molecular mode of action of Nurelle D® and its main constituents as well as the mechanism of both cytotoxic and genotoxic interaction.

**Keywords:** *C. reinhardtii,* chlorpyrifos, Nurelle D®, *S. cerevisiae*, cell lines, rats

## Introduction

According to the European Agency of the Environment, pesticides water and food pollution are considered as one of the principal environmental problems that similarly to the action of air pollution, chemical emissions and the depletion of atmospheric ozone could influence human health. Today's 890 synthetic chemicals are approved as pesticides all over the world and the number of marketed products is estimated to be 20700.

Organophosphorus insecticides (OPIs) are one of the biggest group of insecticides on the market with 67 active ingredients [1]. The primary toxicological effect of OPIs is associated with the inhibition of acetylcholinesterase (AChE) in both central and peripheral nerve tissues [2]. OPIs are known to disturb the biochemical and physiological functions of the cell, thereby affecting membrane integrity [3, 4, 5] and resulting in oxidative tissue damage [6, 7, 8]. Several studies have demonstrated that OPIs could induce oxidative stress in animals and humans, both in acute and chronic poisoning [reviewed in 9] by evoking lipid peroxidation [10, 11], by inhibiting antioxidant enzymes as well as by inducing generation of reactive oxygen species [10, 12]. Also, oxidative stress has been implicated in insecticide-induced neurotoxicity based on its role in the cascade of biochemical changes that lead to dopaminergic neuronal cell death [13].

In short, human activity contributes for increased anthropogenic pressure on the biota, generating cell damages at different levels, inducing mutations, genome degradation and changes in population structure, serious diseases, including cancer etc.

One commonly applied insecticide is Nurelle D®. Nurelle D® (55% EC) is an insecticide containing two active substances – OPI insecticide chlorpyrifos, CPF ((0,0-diethyl-0-[3,5,6-trichloro-2-pirydinyl]phosphorotioate) and an pyrethroid insecticide cypermethrin (R-cyano-3-phenoxybenzyl (1R)-cis-3 (2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarb oxylate). Cypermethrin like other pyrethroid insecticides is known to stimulate nerves by disruption of the sodium channel function and causing pronounced repetitive acivity [14]. Although Nurelle D® is known as a suitable insecticide with recommended application rates of 1000 ppm in field, little is currently known about its activity on non-target organisms when very low doses are applied.

The aim of this study was to evaluate the genotoxic, mutagenic and carcinogenic potential of insecticide Nurelle D® on different test systems; to compare Nurelle D® effects with those of CPF as a main constituent of Nurelle D® on experimental animals (rats).

## Materials and Methods

Nurelle D® (55% EC) was purchased from the agricultural market, Sofia, Bulgaria. CPF (97%) was obtained from TaeGeuk Cop., South Korea. Concentrations used in the present study were calculated based on the recommended one for agriculture. Lower concentrations were also applied for our experimental tasks.

Cell cultures of *Chlamydomonas reinhardtii* (WT) and *S. cerevisiae* (strains 551 and D7ts1) in the end of the exponential and the beginning of stationary phase were treated with various concentrations of Nurelle D® (12.5 - 200 ppm and 100 - 6000 ppm) for 30 min.

The survival fraction (SF) of *C. reinhardtii* colonies was calculated according to [15]. The mutagenic potential of Nurelle D® on the unicellular algae was assayed as described by us previously [16]. The test of the "visible" mutant colonies was applied; changes in size, morphology and pigmentation of surviving colonies were analyzed [17].

Zimmermann's test [18] with the diploid strain D7ts1 (*MATa/α ade2-119/ade2-40 trp5-27/trp5-12 ilv1-92/ilv1-92 ts1/ts1*) was applied as described before [19]. The D7ts1 strain provides simultaneous detection of mitotic gene conversion at the *trp-5* locus, reversion mutations in *ilv1* locus and mitotic crossing-over between the centromere and *ade2* allele [20].

The Ty1 transposition assay was performed as described by [21]. Mean transposition rates were determined and results presented as "fold increase of Ty1 transposition rate" related to the control sample, taken as 1.00. A fold increase in treated cultures equal or higher than 2.00 is considered as a positive response of the Ty1 assay.

The *in vitro* cytotoxicity/antiproliferative activity testing was performed on cell cultures from the non-tumorigenic epithelial cell line MCF-10A using the standard MTT-dye reduction assay, as described by [22].

### *In vivo* study

#### *Animals and treatments*

Weanling male Wistar rats (average body weight of 55±5 g) were obtained from the Animal Breeding House of the National Research Centre (NRC), Dokki, Cairo, Egypt. Animals were given humane care, according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals." The Local Ethics Committee at the National Research Centre (NRC), Dokki, Cairo, Egypt approved the experimental protocols and procedures.

After acclimatization (seven days) to laboratory conditions, rats were randomly divided into two groups, each consisting of five rats. Group one – control, was given corn oil (1ml/kg b.wt) daily, via oral route for 28 consecutive days and adjusted weekly for body weight changes. Group 2 was given CPF at a dose 25.60 mg/kg b.wt. (1/25 $LD_{50}$) based on the published $LD_{50}$ (640 mg/kg b.wt.) [1].

#### *Chromosome aberrations (CA) assay*

Cytogenetic analysis was performed by the direct method of rinsing marrow of long bones, according to [23]. CA was identified based on the criteria established by the OECD Guideline 475, updated and adopted on 21 July 1997 (OECD, 1997) [24].

***Mitotic index determination***

The slides prepared for the chromosomal aberration assay were used to calculate the mitotic index as the ratio of the number of dividing cells to the total number of cells, multiplied by 100.

***The micronucleus assay***

The method described by [25] was used for the analysis of micronuclei (MN) in polychromatic erythrocytes (PCEs) of rat bone marrow. The study was done in accordance with the OECD Guideline 474, updated and adopted on 21 July 1997 (OECD, 1997) [26].

**Results and Discussion**

*Genotoxic activity*

The potential genotoxic effect of Nurelle D® was evaluated based on the cell survival of two test-systems – *C. reinhardtii* and *S. cerevisiae*.

Data obtained indicate that the genotoxic effect is strongly dependent on the genotype.

Treatment with Nurelle D® at 25 ppm or higher concentrations can decrease cell survival of *C. reinhardtii* significantly (Fig. 1).

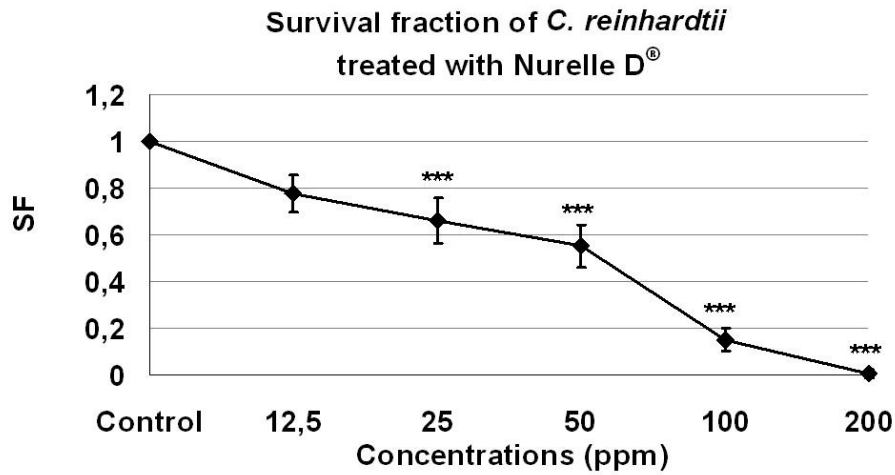


**Fig. 1. Survival fraction (SF) of *C. reinhardtii* treated with Nurelle D® at concentration range from 12.5 to 200 ppm. Where no error bars are evident, they are equal or less than the symbols**

Statistically significant reduction of cell survival of the diploid strain *S. cerevisiae* D7ts1 was observed after the treatment with all Nurelle D® concentrations used by us. The lowest tested concentration led to 75% cell survival, comparable with the one after the treatment with the known mutagen methyl methane sulfonate – 72.575% (Fig. 2).

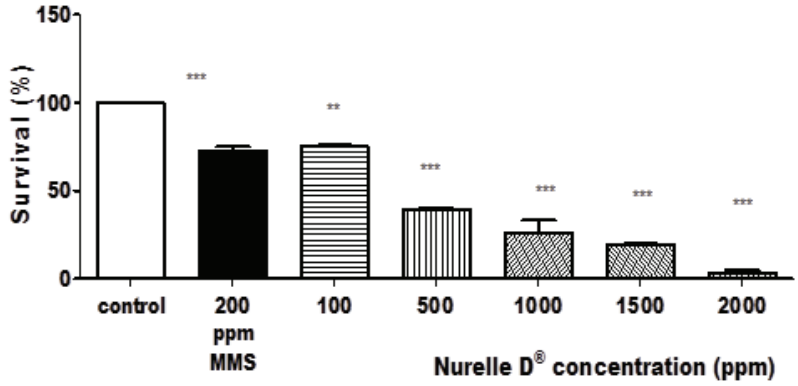


**Fig. 2. Cell survival of *S. cerevisiae* strain D7ts1 after treatment with Nurelle D® at concentration range from 100-2000 ppm. Where no error bars are evident, they are equal or less than the symbols**

Interestingly, when the haploid strain *S. cerevisiae* 551 was treated with concentrations in the range 100-1500 ppm no effect on cell survival was observed.

Statistically significant reduction of cell survival was obtained after the treatment with concentrations equal or higher than 2000 ppm (Fig. 3).

Such difference in the response to mutagens between diploid and haploid strains has also been observed in other studies. Lada et al. [27] reported that some diploid cells are more sensitive to a base analog or an APOBEC deaminase than the haploids. They explained this observation with a potential uneven distribution of the mutation rates. In our study, the difference in the response between the haploid and diploid strain could be attributed to their genotype characteristics. Although, the haploid strain is supposed to be more sensitive to Nurelle D® treatment, the diploid strain is designed with more mutations inserted.

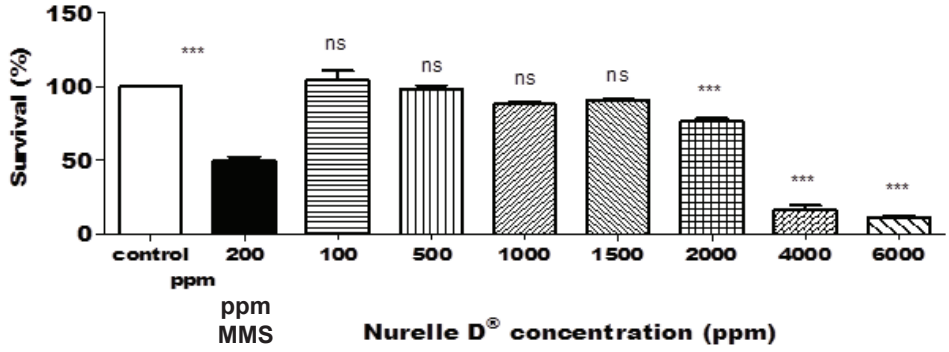


**Fig. 3. Cell survival of *S. cerevisiae* strain 551 after treatment with Nurelle D® at concentration range 100 – 6000 ppm. Where no error bars are evident, they are equal or less than the symbols**

Figure 4 shows a comparison among the concentrations of Nurelle D® that induce 50% lethality and the recommended for commercial use dose of Nurelle D® – 1000 ppm. *C. reinhardtii* – $LD_{50}$ = 55 ppm, *S. cerevisiae* – $LD_{50}$ = 2000 ppm (haploid strain 551) and $LD_{50}$ = 100 ppm (diploid strain D7ts1).

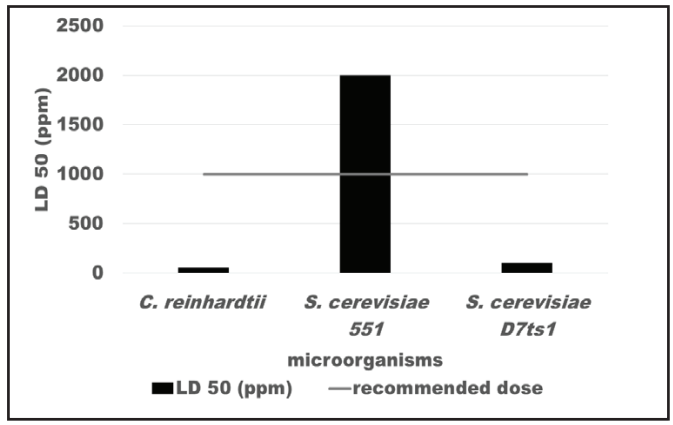


**Fig. 4. Concentrations of Nurelle D® that induce 50% lethality: *C. reinhardtii* – $LC_{50}$ = 55ppm, *S. cerevisiae* – $LC_{50}$ = 2000 ppm (haploid strain 551) and $LC_{50}$ = 100 ppm (diploid strain D7ts1) and the recommended for commercial use dose of Nurelle D® is 1000 ppm**

***Mutagenic activity***

Commonly the spectrum of spontaneous "visible" mutations in *Chlamydomonas reinhardtii* includes five main groups - small size mutations, pigmental, morphological, sectorial and mosaical. The similar types of "visible" mutations were obtained in treated samples. No statistically significant increased number of mutant colonies comparing with those in the control sample was counted. In this regards it could be concluded that Nurelle D® possess no mutagenic effect in *C. reinhardtii* in a concentration range used by us (data not shown).

Despite the low survival rate of *S. cerevisiae* D7ts1 after the treatment with 100 ppm and 500 ppm Nurelle D® (75.375 and 39.69% respectively) a lack of statistically significant differences in the induction of gene conversion was obtained. The recombinogenic effect presented as a 2-fold and a 13-fold increase in the gene conversion was observed only after the treatment with high concentrations Nurelle D® – 1000 and 2000 ppm, respectively (Fig. 5).

Similar results were obtained also for the other parameters – reverse mutation and mitotic crossingover. The statistically significant increase in both criteria was calculated after the treatment with concentrations higher than the recommended for commercial use concentration of 1000 ppm Nurelle D® (Fig. 5).

The available data in literature show that concentrations equal or higher than 1000 ppm Nurelle D® possess strong genotoxic and mutagenic effect on barley [28]. Our results on *S. cerevisiae* concerning the recombinogenic, mutagenic and carcinogenic potential at such high doses are in correspondence with these results.

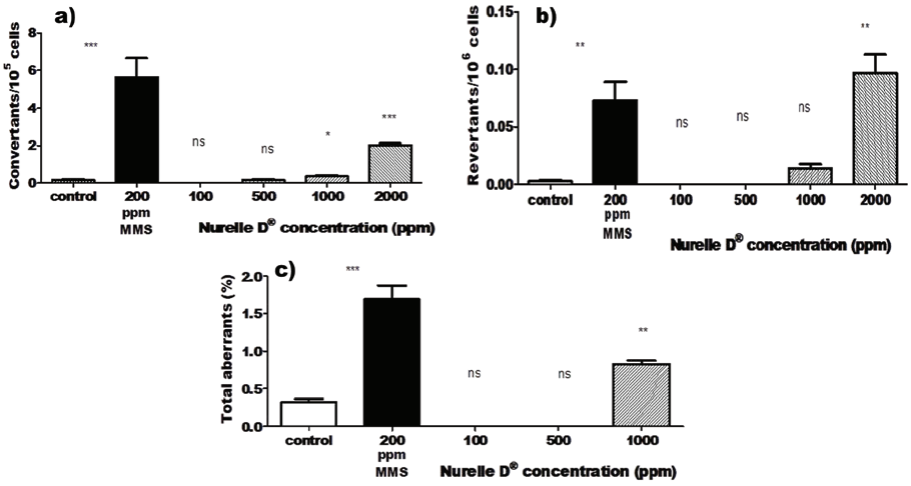


**Fig. 5. Mutagenic effect of Nurelle D® at concentration range 100- 2000 ppm measured as increase in gene conversion (a), reverse mutation (b) and mitotic crossingover (c) on *S. cerevisiae* strain D7ts1. Where no error bars are evident, they are equal or less than the symbols**

***Carcinogenic potential***

Lack of statistically significant difference in the transposition rate is observed after the treatment with Nurelle D® at concentrations' range 100- 2000 ppm. Data obtained is comparable with this of untreated cells (Fig. 6).

Around a 3-fold increase in the transposition rate is obtained after the treatment with Nurelle D® at concentrations 4000 and 6000 ppm.

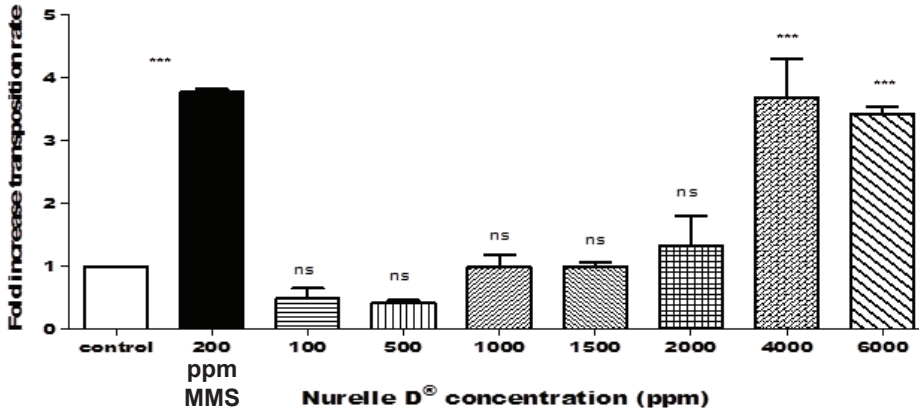


**Fig. 6. Carcinogenic effect of Nurelle D® at concentration range 100- 6000 ppm measured as fold increase transposition rate on *S. cerevisiae* strain 551. Where no error bars are evident, they are equal or less than the symbols**

***Cytotoxic and antiproliferative effect***

Dose dependent response to treatment with Nurelle D® at concentration range 4 – 2000 ppm was observed after the treatment of the cell line MCF-10A (Fig. 7).

The $IC_{50}$ cytotoxicity of Nurelle D® after 24 hours of incubation of the cell line was calculated to be 33.0± 4.0 ppm.

The strongest antiproliferative effect – around 80-90 % was obtained after the treatment with both concentrations of 4 and 8 ppm Nurelle D®.

The $IC_{50}$ antiproliferative effect of Nurelle D® after 72 hours of incubation of the cell line was calculated to be 26.5 – 1.062 ppm.

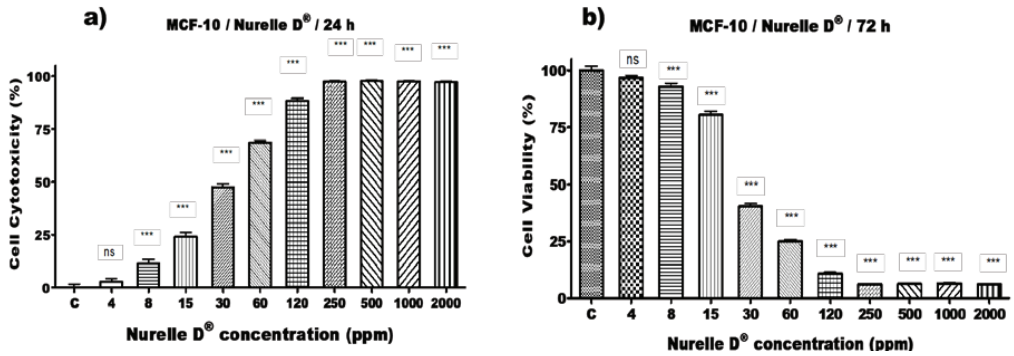


**Fig. 7. Cytotoxic and antiproliferative effect of Nurelle D® at concentration range 4- 2000 ppm on cell line MCF-10. Where no error bars are evident, they are equal or less than the symbols**

***In vivo experiments on rats***

***Mitotic index***

The mitotic index was used in order to determine the rate of cell division. The status of the mitotic index evaluated as a percentage of dividing cells was found to be 8.43±0.41 in control rats. The mitotic index was found to be declined (4.42±0.42 $P< 0.05$) in CPF-treated rats (Fig. 8). These data indicate the cytotoxic potential of CPF.

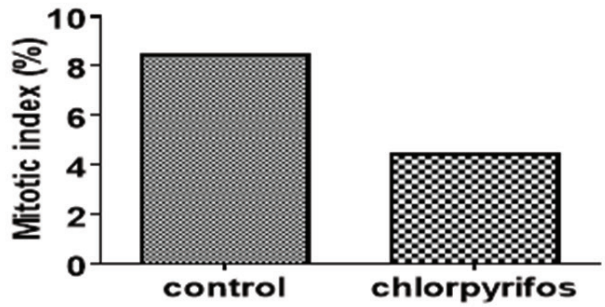


**Fig. 8. Mitotic index calculated after application of chlorpyrifos**

***Chromosomal aberrations in rat bone marrow***

Table 1 presents the chromosomal aberrations induced in rat bone marrow cells after the treatment with CPF. The results show that the tested dose (25.60 mg/kg. b.wt.) of CPF can induce a statistically significant increase in the percentage of CA in bone marrow cells. The percentage of CA is 1.85 ±0.014 in control rats and has increased to 13.42±0.04 in CPF-treated rats.

**Table 1. Effect of CPF on chromosomal aberrations in rat bone marrow cells**

| Group | Percent chromosome aberrations (Mean ± S.E.) | | | | Total number of aberrant cell (%) (Mean ± S.E.) | | No. of aberrations per cell (Mean ± S.E.) | |
|---|---|---|---|---|---|---|---|---|
| | Gaps | Breaks and/ or Fragment | Deletions | Multiple aberrations | Including gaps | Excluding gaps | Including gaps | Excluding gaps |
| Control | 1.02±0.01 | 1.46±0.06 | 0.18±0.001 | 0.21±0.002 | 2.87±0.08[b] | 1.85±0.014[b] | 0.0287 ±0.0001[b] | 0.0185 ±0.0001[b] |
| Chlorpyrifos | 4.32±0.06 | 5.87±0.08 | 0.97±0.005 | 6.58±0.008 | 17.74±0.53[a] | 13.42±0.04[a] | 0.1774 ±0.020[a] | 0.1342 ±0.010[a] |

Value is mean ± S.E.; n = 5 rats/group. Values are shared the same superscript letters not differ significantly at $p<0.05$.

***Induction of micronuclei in rat bone marrow PCE***

Data in Table 2 show that the tested insecticide CPF possesses clastogenic capacity. It is well evidenced by the significant increase (28.40±1.76) of the total number of bone-marrow micronucleated polychromatic erythrocytes (MnPCE), when compared with control rats (6.40± 0.14).

**Table 2. Clastogenic potential of CPF in the bone marrow measured as induction of micronuclei in bone-marrow micronucleated polychromatic erythrocytes**

| Group | No. of micronucleated polychromatic erythrocytes (MnPCE) (Mean ± S.E.) | | | |
|---|---|---|---|---|
| | PCE with one Mn | PCE with two Mn | PCE with more than two Mn | Total MnPCE |
| Control | 5.60±0.074 | 0.60±0.001 | 0.20±0.001 | 6.40± 0.14[b] |
| Chlorpyrifos | 17.4±0.18 | 7.80±0.19 | 3.20±0.11 | 28.40±1.76[a] |

Value is mean ± S.E.; n = 5 rats/group. The number of the scored cells was 2000 cells/animal. Values are shared the same superscripts letters not differ significantly at $p < 0.05$. Mn: micronucleus, MnPCE: micronucleated polychromatic erythrocytes, PCE: polychromatic erythrocytes.

## Conclusion

Our study provides original data about the effect of Nurelle D® on different test systems. The commonly recommended dose for agricultural application is 1000 ppm Nurelle D®. Our results demonstrate that doses lower than this one possess genotoxic effect on *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae.* Additionally, strong cytotoxic

and antiproliferative potential was observed after the treatment of the cell line MCF-10A with Nurelle D® with concentrations lower than 1000 ppm.

Based on the calculated $LD_{50}$, the most susceptible model system is the cell line MCF-10A, followed by *Chlamydomonas reinhardtii* and *S. cerevisiae* D7ts1.

Further experiments would provide information concerning the effect of the Cypermethrin and CPF at low concentrations and comparison of their effect with this of the mixture – Nurelle D®.

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# SHORT COMMUNICATIONS

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

# BIOLOGICAL AND STATISTICAL COMPARISON OF EXPERIMENTAL RESULTS-GOOD APPROACH FOR ANALYZING THE ADAPTIVE POTENTIAL OF GENETICALLY CLOSELY RELATED GENOTYPES

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**Introduction:** For better understanding of the mechanisms involved in the formation of adaptive potential of genotypes different approaches are needed. Modern biology requires mutual implementation of biological data, mathematical models, computational tools and statistical analysis.

**Aim:** to identify the most reliable marker/s for the evaluation of genotype's adaptive potential of three *Phaseolus vulgaris* L. genotypes to single and combined treatment with PEG and UV-B irradiation using two approaches (biological and statistical/ mathematical).

**Material and Methods:** Three *Phaseolus vulgaris* L. genotypes – Dobrudjanski 2, Dobrudjanski 7 and Dobrudjanski ran were studied. The seeds were kindly provided by Prof. Svetleva from Plovdiv Agricultural University. Previously with molecular analysis it was found that these genotypes are genetically closely related [1, 2]. Seeds were germinated 3-7 days in a growth chamber (GC 400) at standard conditions (t=23±0.2°C, moisture 70%, in continuously light). Seedlings were grown on autoclaved tap water at the same experimental conditions to the cotyledon phase. Mild drought stress was simulated by 16% PEG treatment for 24h. UV-B irradiation with 100, 250, and 500 J/m$^2$ was carried out in BLX 254 UV Cross-linker. Drought and three UV-B doses given on the 12 hour of PEG treatment were applied. Then plants were grown to the first leaf phase (ten days after the removal of the stress factors) in sterile tap water. After that measurements of four biochemical/molecular markers were performed – malondialdehyde (MDA) [3], total peroxides (Pox) [4], proline (Pro) [5] and heat shock protein content (HSP70B) [6].

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One-way ANOVA with Tukey multiple comparison posttest (Graph Pad Prism 6.04 software, San Diego, USA), ANOVA (EXCEL 2007) and Analysis ToolPak were calculated to assess differences among samples. To identify the most reliable marker/s statistical data were summarized with Boolean algebra. Further possible combinations were analyzed using Boolean operators AND, OR, XOR.

**Results:** No effect of genotype was shown using both Pox and MDA as markers. No statistically significant differences with controls were found ($p>0.5$), after the removal of single and combined treatments. Statistically data are confirmed with Analysis ToolPak (Excel 2007) and ANOVA (Excel 2007).

Ten days after drought recovery no statistically significant differences with controls in the measured amounts of Pro were found for genotypes Dobrudjanski 2 and Dobrudjanski 7 ($p<0.5$; One-way ANOVA analysis, Analysis ToolPak (Excel 2007), ANOVA (Excel 2007) and negligible lower Pro content ($p<0.001$) than that in the control for Dobrudjanski ran. These results are in agreement with those of other authors and demonstrate the importance of a recovery period to overcome the effects of drought. Similar tendency in Pro levels was observed after UV-B recovery for both genotypes Dobrudjanski 2 and Dobrudjanski ran ($p <0.5$). Interestingly, in same experimental conditions, dose dependent enhancement of Pro content was measured for genotype Dobrudjanski 7. Comparing Pro accumulation when 10 days period of recovery was given after both single treatments it could be suggested that UV-B irradiation is a more stressful factor than drought. Having in mind the modern understanding of the protective role of Pro against UV-B induced stress it could be assumed that Pro over accumulation could be one of the protective mechanisms of genotype Dobrudjanski 7. The effect of pretreatment with PEG on Pro content was observed ten days after combined treatment. Reduced Pro content ($p>0.5$) in comparison with UV-B irradiated samples, or lower values up to the control were obtained in all genotypes except in sample 16% PEG+500 J/m$^2$ ($p<0.1$) in Dobrudjanski 7. In the available literature, no studies for levels of Pro accumulation after combined treatment of various abiotic factors and a recovery period have been found. Some studies demonstrate the significance of the experimental scheme, type of the impact, presence/absence of recovery period, genotype, etc. Having in mind the mentioned above understanding that elevated levels of Pro could be considered as protective mechanism, it could be speculated that Pro accumulation is one of mechanisms involved in defense strategy of genotype Dobrudjanski 7.

Based on One-way ANOVA, Analysis ToolPak (Excel 2007) and ANOVA (Excel 2007) analyses no statistically significant differences with controls ($p>0.5$) were calculated for samples undergoing drought stress in all genotypes when HSP70B content was measured. In this case no effect of the genotype was revealed. No effect of the applied UV-B dose was found for all studied genotypes. Although not everywhere higher values of HSP70B were statistically proven compared with those in the controls for genotypes Dobrudjanski 2 and Dobrudjanski ran, enhanced levels of HSP70B were measured (Dobrudjanski 2-about 3.5-4 fold; Dobrudjanski ran-about 2.5-3 fold). It could be supposed that the more stressful factor is UV-B in comparison with drought for genotypes Dobrudjanski 2 and Dobrudjanski ran (based on levels of HSP70B). Interestingly, for genotype Dobrudjanski 7 levels of HSP70B remain close to those in the controls in

both single and combined variants. No statistically proven differences with the control were found ($p>0.5$) in combined variants (16% PEG+100 J/m$^2$ и 16% PEG+250 J/m$^2$) for the genotype Dobrudjanski 2, using One-way ANOVA analysis, Analysis ToolPak (Excel 2007) and ANOVA (Excel 2007). Higher HSP70B accumulation was measured than that in the control only in 16% PEG + 500 J/m$^2$ variant (about 3.5-4 fold), and approximately similar with those measured after single UV-B in Dobrudjanski 2. Ten days after the removal of combined treatment Dobrudjanski ran still respond with an increased HSP70B content in comparison with those in the controls and single UV-B treatments. Although genotypes are genetically closely related, there is a difference in HSP70B content even after ten days recovery time (higher HSP70B content than that in the controls for Dobrudjanski 2 and Dobrudjanski ran; lower than that in the control for Dobrudjanski 7). Higher levels of HSP70B could be an indication for the presence of protective mechanisms. Probably the studied genotypes activate different protective mechanisms ten days after the removal of the combined treatment. It could be considered that in combined variants, mild drought stress led to adaptation to a certain extent (about 3.5 - 4 fold) and in these variants, the mild drought stress increases the adaptive potential of Dobrudjanski 2 and Dobrudjanski ran (especially in 16% PEG + 500 J/m$^2$) compared to single drought. Probably, overproduction of HSP70B could be considered as one of the protective mechanisms of these genotypes.

Biochemical/molecular data were statistically confirmed with Analysis ToolPak (Excel 2007) and ANOVA (Excel 2007). The results of Analysis ToolPak (Excel 2007) and ANOVA (Excel 2007) were summarized by Boolean algebra with Boolean operators AND, OR, XOR. Based on mathematical methods it was found that both Pro and HSP70B could be considered as more reliable markers for the adaptive potential.

**Conclusions:** Our experimental data show that the most reliable marker/s for the evaluation of the genotype's adaptive potential of the three *Phaseolus vulgaris* L. genotypes to single and combined treatment with PEG and UV-B irradiation are Pro and HSP70B. Statistical data confirmed this observation. Mutual use of experimental data and statistical/mathematical (ANOVA/Excel/, Analysis ToolPak /Excel/, Boolean algebra) methods could be recommended as a good approach for the evaluation and comparison of the adaptive potential of genetically closely related genotypes.

Both biochemical and molecular markers-Pro and HSP70B in a combination with mathematical methods could be recommended in order to obtain reliable information concerning adaptive potential of genotypes.

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# STRESS HIGH-LIGHT-INDUCIBLE PROTEINS AND THEIR LOCALIZATION IN THE CHLOROPHYLL-PROTEIN COMPLEXES OF CYANOBACTERIA

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**Aim:** Oxygenic photosynthetic organisms synthesize stress-associated proteins during exposure to high light. These proteins are often important for the acclimation of cells to high light. A family of high light- inducible genes, called *hli*, encoding high light-inducible proteins (HLIPs), are critical for the survival of cyanobacteria under high light conditions [1, 2]. The association of high-light-inducible (HliA/HliB) small stress proteins with photosystem I (PSI) complexes of the cyanobacteria was studied to understand their function.

**Material and Methods:** Two cyanobacteria *Synechocystis* sp. PCC 6803 and *Arthrospira platensis* were studied. Thylakoid membranes from the cyanobacteria were isolated as described by Akulinkina et al. [3]. The cells were disrupted mechanically with quartz sand (3 min) in buffer for the isolation of thylakoid membranes (25 mM MES/NaOH, pH=6.5; 5 mM $CaCl_2$; 10 mM $MgCl_2$; 25% glycerol; 1mM ε-aminocaproic acid; 10 mM PMSF). The homogenate was centrifuged at 5000 g for 15 min to remove unbroken cells, cellular debris and quartz sand. Further the supernatant was centrifuged, at 18000 g during 1 h. The sediment of cyanobacterial thylakoid membranes was lysed in 1.0% n-dodecyl-β-maltoside. The lysate of the thylakoid membranes was applied to PAGE with the addition of sample buffer (100 mM BisTris/HCl, pH=7.0; 30% sucrose; 500 mM ε-aminocaproic acid).Then chlorophyll-protein complexes were fractionated by two-dimension polyacrylamide gel electrophoresis (2D PAGE). Clear Native PAGE was performed in 4 - 12% acrylamide gradient (50 mM BisTris/HCl, pH 7.0; 0,5 M ε-aminocaproic acid; ammonium persulphate; TEMED) for 3h at 100B. The gel tracks obtained after 1D electrophoresis were used for the following SDS-PAGE. Proteins identification was performed by MALDI-TOF mass spectrometry and by

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Western-blot analysis. Western blotting was performed in Tris-glycine buffer (25 mM Tris; 250 mM glycine; 20% ethanol; 0.2% SDS) for 1,5h at 30B. Then the membrane was placed in a blocking buffer TBST (50 mM Tris/HCl, pH=7.5; 200 mM NaCl; 0.1% Tween 20; 5% dried skim milk) for 1h at $4^0$. The membranes were incubated with the primary antibody against HliA/HliB at 1/4000 dilution (Abcam, CIIIA) overnight at 4°. Secondary antibodies goat anti-rabbit IgG conjugated to horseradish peroxidase (1:10 000) (AgriSera, Sweden) were used. Each step was accompanied by repeated washing with membrane buffer TBST. The immune complexes on the membrane were detected by fluorescence detection system ECL (GE Healthcare, Little Chalfont, Buckinghamshire, England) and the signals recorded on x-ray film (Retina, Germany). The x-ray film was scanned and the data were processed using Image J (http://rsbweb.nih.gov/ij/).

**Results:** The following protein complexes were revealed after electrophoresis of *Synechocystis* sp. thylakoid membranes: trimers and monomers of the PSI complex, dimers and monomers of the PS2 complex, cytochrome complex, ATPase complex, NADPH-quinone oxidoreductase complex, and also the free proteins zone. HliA/HliB proteins were identified by western blot analysis. Mass spectrometric analysis showed that Hlips are associated with photosystem I (PSI) trimers, complexes PSII and PSI monomers. In addition, they were found in the free protein zone. Chlorophyll-protein complexes of *Synechocystis* sp. and *Arthrospira platensis* were fractionated by clear native PAGE. It was shown that complexes distribution of these two cyanobacteria was similar (Fig. 1).

Detailed investigation of individual photosystems of *Synechocystis* sp. reveled that HliA/HliB proteins are associated not only with PSI trimers, but also with PSI monomers and with PSII complexes. Moreover HliA/HliB proteins are presented in the thylakoid membranes of mutant cells lacking PSI and PSII.

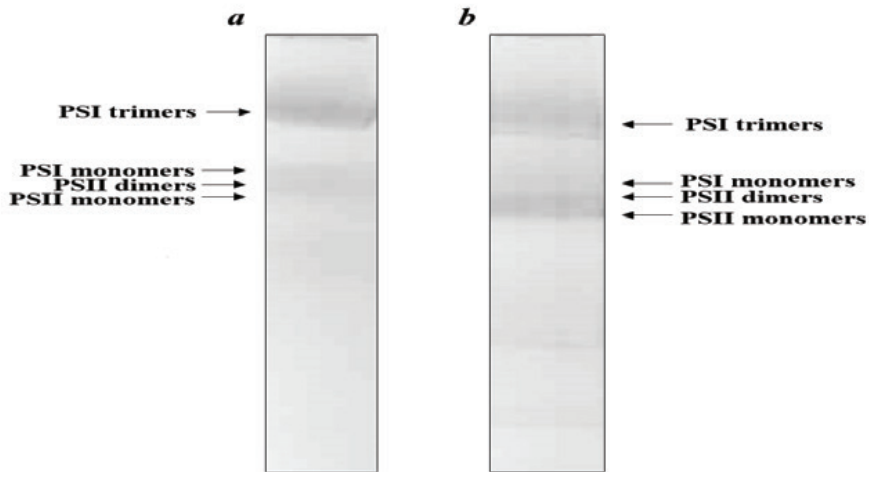


**Fig. 1. Clear native PAGE for resolution of thylakoid membrane protein complexes from *A. platensis* (a) and *Synechocystis* (b)**

**Conclusion:** The association of HliA/HliB proteins not only with PSI trimers, but also with PSI monomers and PSII complexes was shown. This fact suggests a universal role of these proteins in the protection of the photosynthetic apparatus from light stress.

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# IS THERE A RELATIONSHIP BETWEEN DNA REPAIR EFFICIENCY, HSPs AND GENOTYPE RESISTANCE?

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**Introduction:** Organisms respond to environmental impact by developing a series of physiological, biochemical and molecular strategies. Different mechanisms of adaptation to environmental stress have been developed by organisms during the evolution: efficient DNA repair systems, cell membrane stability, induction of heat shock proteins (HSPs), particularly chaperons etc. Heat shock proteins possess a special place among defense systems and could contribute to cellular homeostasis [1].

*Green algae* are cosmopolitan and could be found in a wide range of habitats such as soil, freshwater lakes, snow as well as hot springs. They are one of the best studied phototrophic eukaryotes and could be used as a robust model for several main reasons:

- Widely spread photosynthetic unicellular eukaryote;
- Cell structure and genome organization typical for plants so the results could be extrapolated to higher plants;

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- Short life cycle
- Haploid genome-recessive mutations could be revealed at the next generation;
- Routine inexpensive laboratory cultivation techniques;
- Suitable for molecular methods or techniques.

**The aim** of this study is to analyze the contribution of DSBs induction and repair efficiency and HSP70B for the formation of genotype resistance.

**Material and Methods:** Unicellular green algae are used:

*Chlamydomonas reinhardtii* strains – 137C WT (isolated from soil in Massachusetts, USA, by Smith in 1946), UVS10 *rec* and UVS14 *mismatch* repair deficient (from prof. Vlcek collection) have been cultivated in our lab for a long time.

*Chlorella species – Chlorella vulgaris* (Antarctic, isolated from soil in island Livingston, Antarctic), *Chlorella vulgaris* strain 8/1 (Thermophilic, isolated from thermal springs in the region of Rupite, Bulgaria) and *Chlorella kessleri* (Mesophilic from Trebon collection).

*Growth conditions* - The algae are cultivated on liquid TAP medium under standard conditions – continuous light 5000 – 5000 lx and temperature 23°C±0.1°C in a growth camera Phytotron GC 400. The cell suspensions at the end of the exponential/beginning of the stationary phase are used. *Chlorella* species are irradiated with UV-B (λ= 312nm) in BLX-254, Life Technology, UV crosslinker. The irradiation is in a dose range of 50, 100, 250, 500, 1000 J/m$^2$. In order to determine the role of photo-reactivation cells were kept in a continuous light (samples with photo-reactivation) and in the dark for 24h (samples without photo-reactivation). The genotype resistance is established on the basis of spot-test, microcolonies assay, photo-reactivation sectors and grow rate – the ability to restore the cell division after UV-B irradiation. Zeocin (InvivoGen/www.invivogen.com) is a radiomimetic – copper chelated glycopeptide antibiotic isolated from *Streptomyces*, a member of bleomycin family, causes double strand breaks (DSBs), oxidative stress and cell death.

***Chlamydomonas*** **strains** are treated with 2, 4, 8, 10, 50 and 100μg/ml zeocin. Induction and repair efficiency of double strand – breaks are measured by constant field gel electrophoresis (CFGE) as described by [2 and 3]. The levels of heat shock proteins (HSP70B) are determined by gel-electrophoresis and Western blotting [4].

**Data analysis:** The experiments are repeated at least three times using independently grown algal cultures. One-way ANOVA with Tukey multiple comparison test is performed to compare mean differences among genotypes (GraphPad Prism 6.04). Two-way ANOVA with Bonferroni post-test is applied to determine how a response is affected by genotype, concentration and interaction between them (GraphPad Prism 6.04).

**Results:** Genotoxic capacities of different UV - B doses are revealed via Spot - test data. The Intensity of spots illustrates that doses higher than 250 J/m$^2$ are bioactive for *Chlorella* species.

To test whether species are photo-reactivation proficient or not two experimental schemes were applied – with and without photo-reactivation. In samples without photo-reactivation a decrease in cell survival even after the irradiation with 100 J/m$^2$ is found. Irradiation with 500J/m$^2$ and 1000J/m$^2$ UV –B results to survival fraction lower than 0.3. In samples with photo - reactivation survival fractions (SF) are quite different. Significant decreases of SFs after the irradiation with doses 500J/m$^2$ and 1000J/m$^2$ UV-B are observed.

The next step of our experiments was to compare photo-reactivation capacity of *Chlorella* species. For such purpose photo-reactivation sectors (PRS) were calculated - for *Chl. vulgaris* Antarctic – 1; *Chl. vulgaris* 8/1 – 1; *Chl. kessleri* – 1.424. Based on the magnitude of PRS it could be said that *Chlorella* species are photo-reactivation proficient.

Commonly accepted approach for the evaluation of genotype's resistance to different mutagens and environmental stimuli is to compare doses that can induce three levels of lethality – $LD_{20}$, $LD_{50}$ and $LD_{80.}$ Our experimental data demonstrate well-expressed differences depending on the genotype and experimental conditions – with and without photo-reactivation. It was calculated that both $LD_{50}$ and $LD_{80}$ for *Chlorella vulgaris* Antarctic are induced by doses that are 25% - 40% higher than that causing similar effects in *Chlorella vulgaris* 8/1 and *Chlorella kessleri* (in samples with photo-reactivation).

Analyzing results concerning these levels of lethality in samples without PR significant differences depending on the genotype are observed. The doses inducing $LD_{50}$ and $LD_{80}$ in *Chlorella vulgaris* Antarctic are 50% and 40% higher than that for *Chlorella vulgaris* 8/1 and 70% and 100% higher than that for *Chlorella kessleri*.

*Chlorella* species are found to differ in their capacity to overcome harmful effect of UV-B measured as grow rate. In the samples with photo-reactivation grow rate is reduced after 500J/$m^2$ and 1000J/$m^2$ UV-B irradiation in *Chlorella vulgaris* Antarctic. It is found that the same doses of UV-B can inhibit cell division in *Chlorella* vulgaris 8/1 and *Chlorella kessleri*. The similar effects are observed in the samples without photo-reactivation after 250J/$m^2$ UV-B – grow rate are decreased in *Chlorella vulgaris* 8/1 and *Chlorella kessleri* and are inhibited after 500J/$m^2$ and 1000J/$m^2$ UV-B.

Differences in DNA susceptibility of *Chlamydomonas reinhardtii* genotypes to zeocin are found. About 2 – fold lower levels of DSBs in WT in comparison with both DNA repair deficient genotypes are obtained after high zeocin concentration (10 - 300 μg $mL^{-1}$) treatment. The most pronounced increasing of DSBs after low doses zeocin (2 - 10 μg $mL^{-1}$) treatment is measured for *rec*-repair deficient genotype UVS-10 – about 5-fold higher than the level of induced DBSs in *mismatch*-repair deficient genotype UVS- 14.

Increased levels of HSP70B content 1h after the treatment with 100μg $mL^{-1}$ zeocin are measured for both strains UVS-10 and UVS-14. Four hours later chaperone levels are similar to those in the control samples. Strains with WT resistance – 137C+ WT, CW15 respond to zeocin induced oxidative stress later – on the 4$^{th}$ hour after the treatment with 100μg $mL^{-1}$ zeocin. More pronouced effect is obtained for strain 137C.

**Conclusions:** Based on the results obtained it could be said that HSP70B and both repair systems - *rec* and *mismatch* contribute to the formation of genotype resistance of investigated by us strains.

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**Keywords:** unicellular green algae, DNA repair efficiency, HSPs, genotype resistance

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# GENOTOXIC AND MUTAGENIC EFFECTS OF *NARCISSUS TRIANDRUS* L. TOTAL LEAF EXTRACT ON *CHLAMYDOMONAS REINHARDTII*

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It is known that Amaryllidaceae plants can produce specific alkaloids with antimicrobial, antiparasitic, antifungal, cytotoxic, some pharmacological activities and in many cases could be used for medicinal purposes. Recently, the revealing of the potential bio-toxicity of alkaloids is of great interest related to further application as natural biocides. The application of biocides in agriculture has many advantages compared with synthetic pesticides such as the rapid environmental biodegradation and low toxicity to non-target organisms.

**Aim:** to evaluate the genotoxic and mutagenic potential of total leaf extract of *Narcissus triandrus* L. on a test-system of *Chlamydomonas reinhardtii*.

**Materials and Methods:** *N. triandrus* methanol extract was derived and prepared by prof. Berkov's team. *Chl. reinhardtii* strain 137 C+ (WT) was used as a test system. The strain 137 C+ was cultivated on 30ml TAP medium and grown under standard conditions (t = 23°C ± 0.2°C and continuous light of 5000–5500 lx) in a Phytotron GC 400. Cultures at the end of the exponential and the beginning of stationary phases were treated for 2 hours with several concentrations of *N. triandrus* leaf extract – 250 μg/ml, 500 μg/ml, 750 μg/ml and 1000 μg/ml. The range of these concentrations and exposure time were chosen based on preliminary obtained results by Spot-test. Survival fraction (SF) was analysed on the basis of colony forming ability [1]. Test of visible mutations was performed to reveal mutagenic capacity of the extract [2]. The percent of mutations was calculated as the

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number of mutant colonies/total number colonies x 100. The mutagenic index (MI) was calculated by following the formula – MI = % of induced „visible“ in treatment variants/% spontaneous „visible“ mutations in control. When MI < 2.5 – no mutagenic action is obtained; MI = 2.5 – 10 a weak mutagenic action; MI > 10 – a strong mutagenic action [2].

Results in treated samples were compared with those in control samples – Sager-Granick liquid medium (SG), 0.1% DMSO as negative controls and 5 μM Paraquat (PQ) as a positive control.

Three levels of lethality were calculated - $LD_{20}$; $LD_{37}$ and $LD_{50}$ [3] following the formula:

$$\lg LD_{20} = \lg A + (\lg B - \lg A) \times \frac{20-A}{B-A}, \text{ where}$$

A – percent lethality close to but lower than 20%; lg A – logarithm of the concentration corresponding to A; B – percent lethality close to but higher than 20%; lg B - logarithm of the concentration corresponding to B. Other levels of lethality were calculated analogously.

One-way ANOVA with Tukey's Multiple Comparison Test (GraphPad Prism) was used to evaluate the effect of concentrations.

**Results:** Our data show no statistically significant genotoxic effect of the lowest concentration of the extract – 250 μg/ml. The treatments with concentrations 500 μg/ml ($p<0.001$), 750 μg/ml ($p<0.0001$) and 1000 μg/ml ($p<0.0001$) resulted in a decreased colony forming ability in a statistically significant way compared with both negative controls – SG and DMSO. Approximately similar colony forming ability was found after the treatment with both concentrations 750 μg/ml and 1000 μg/ml (SF=29%, $p<0.001$ and SF=33%, respectively, $p<0.05$). Survival fraction curves show the presence of a plateau of the obtained SF values, forming one group of action irrespectively of the two different extract concentrations applied. Here it could be possible to speculate that this concentration range possess similar genotoxic capacity. Further experiments should be done to clarify this statement.

Comparing the genotoxic effect obtained after the treatment with 500 μg/ml leaf extract (SF=66%) and those in PQ positive control (SF=75%) it could be speculated that both stimuli express similar genotoxic potential because no statistically significant difference was calculated between them ($p>0.05$).

Both concentrations 750 μg/ml (SF=29.4%) and 1000 μg/ml (SF=32.5%) leaf extract were found to have higher expressed genotoxic capacity comparing with those of the positive control (SF=74.5%).

The calculation of the 3 levels of lethality – $LD_{30}$, $LD_{50}$ and $LD_{90}$, and especially $LD_{50}$ is one of the basic criteria in revealing the genotoxic capacity of different substances. In this regards using the Reed and Muench [3] method three levels of lethality $LD_{20}$, $LD_{37}$ and $LD_{50}$ were calculated after the treatment with *N. triandrus* L. total leaf extract. Based on our experimental results the following lethal doses were calculated: $LD_{20}$=352 μg/ml, $LD_{37}$=482 μg/ml and $LD_{50}$=598 μg/ml. Comparing the genotoxic capacity of *N. triandrus* L. total leaf extract and PQ on the basis of $LD_{20\text{-}25\%}$ (13 μg/ml) it could be said that the effect of PQ is approximately 30 fold stronger than the one of the extract. It is known that PQ demonstrates toxic effect in plants and animals. It is known that PQ

possess deleterious capacity, affecting DNA and different cell compartments and in such way genotoxic, clastogenic, cytotoxic, mutagenic effects are available [4, 5]. Additionally effects can vary depending on the test system, experimental design, concentration, endpoints and etc. [4, 5].

Many alkaloids have been isolated from Amaryllidaceae plants [6]. Alkaloids are not toxic for organisms producing them, but they can be very toxic for other organisms and cells – mammalian cells, humans [6]. Alcaloids toxicity varies depending on test-system, concentrations, exposition and etc. [6].

Here we focus our attention on genotoxic and mutagenic potential of *N. triandrus* L. total leaf extract that could be related to NCS (natural complex substances) – biological materials, with unknown or variable composition [7].

The spectrum of induced „visible" mutant colonies is considered as a very informative data and can help us to clarify the type of induced and realized genetic events. In unicellular green algae "visible" mutations are grouped in five basic types – small size mutations, pigmental, morphological, sectorial and mosaical. The five basic mutation types were detailed described by Shevchenko [2]. Concerning the origin of small size mutations it has been proposed that the inhibition of cell division is responsible. Pigmental mutations are usually associated with nuclear and/or chloroplasts genes controlling different pathways of photosynthesis. Morphological mutations are believed to arise as a result of a mixed group of hereditary changes. This type of mutations may also be due to the presence of biochemical changes in the composition of the cell wall. It is considered that induction of sectorial mutants is due to mutations occurring in cells having several nuclei at the time of treatment. The sectorial mutations are also indicative of the heterogeneity of the cell population. The ratio between the whole and sectorial colonies reveals the degree of synchronization. The mosaical mutations are informative for the presence of long term living microchromosomal aberrations during a number of cellular generations, resulting in partial cell death. Mosaicism in colonies is associated with lethally segmentation and lysis of non-viable cells.

In our experiment various types of spontaneous and induced „visible" mutations – growth, morphological, pigments and sectoral were observed. Mutation frequency at negative controls and treatment variants was 0.074% and 0.078%, respectively. Based on these results mutagenic index was 1.05. In this regard we concluded that *N. triandrus* L. total extract possess no mutagenic effect.

**Conclusion:** Statistically significant genotoxic effect was found after the treatment with concentrations of *N. triandrus* leaves extract higher than 250 μg/ml for 2 h without any mutagenic capacity. To analyze the complex effect of such NCS the contribution of each constituent should be analyzed. Additionally to go inside to the mode of action of *N. triandrus* L. total leaf extract, strains that differ in repair pathway could be used in future.

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**Keywords:** *Chlamydomonas reinhardtii*, extract, genotixity, mutagenic, *Narcissus triandrus* L.

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# EFFECTS OF *IN VITRO* MORPHOGENESIS AND DEVELOPMENTAL PATTERNS OF *ARTEMISIA ALBA* TURRA ON POLYPHENOLICS PRODUCTION AND ENDOGENOUS STRESS HORMONES

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*Artemisia alba* Turra is a fragrant shrub, whose chemical composition and pharmacological properties of the essential oil and non-volatile extractable constituents have been widely studied in literature, based on their utilization in traditional medicine. The essential oil of the plant has been characterized by great variability related to certain climatic, ecological and genetic factors [1 and references cited within]. Less information is available on the polyphenolic production of this species.

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**Aims:** The present work aims at the elucidation of the effect of the morphological development of *Artemisia alba* Turra on the production of polyphenolics and the content of endogenous stress hormones *in vitro.*

**Materials and Methods:** Plant growth regulators treatments were applied as follows: GAIP_0 – PGR-free control; GAIP_1 – 0.5 mg/l IBA; GAIP_2 – 1.0 mg/l IBA; GAIP_3 – 0.2 mg/l BA + 0.5 mg/L IBA and GAIP_4 – 0.2 mg/L BA + 1.0 mg/L IBA. Total phenolic and flavonoid contents were assayed colorimetrically. The endogenous levels of abscisic acid (ABA) and its metabolites (dihydrophaseic acid, phaseic acid, neophaseic acid, abscisic acid-glucose ester and 9-hydroxy-absicic acid), jasmonic acid (JA) and jasmonic acid-isoleucine, as well as salicylic acid (SA) were analyzed by HPLC-ESI-MS/MS as described in [1 and references of previous development of the analytical method, cited within].

**Results and Discussion:** Irrespectively of the different concentrations, PGR treatments of *A. alba in vitro* resulted in the development of two main morphotypes. While PGR-free control and IBA treated plants were characterized with the development of both aerial and root tissue, the combined IBA and BA treatments resulted in root inhibition and callogenesis at the explant base.

The root inhibited morphotype displayed elevated polyphenolic levels as compared with the plants with normally developing root system (about 18 % for phenolics and 21 % for flavonoids when comparing GAIP_3 with GAIP_0 treatment). In addition, root inhibited plants demonstrated also a 2.5 times elevation of JA and double elevation of the total pool of jasmonates (upon the same comparison as above). On the contrary, a double drop of SA and 3.5-fold drop of ABA was observed in the root-inhibited morphotype.

A great part of phenolics have structural roles (for example in plant cell wall), others are related to growth and survival of the plant organism. The phenolic biosynthesis encompasses numerous overlapping regulatory signals. They are related to both internal developmental processes (lignification during new growth, anthocyanin biosynthesis during blossoming and fruiting), as well as to reactions of the plant organism to external stimuli (biotic and abiotic stress protection). For some key compounds (such as flavonoids) the chemical nature of signaling molecules, as well as signal transduction leading to gene activation of phenolic biosynthetic genes are well known [2]. Most flavonoid synthesizing enzymes are situated in the soluble cellular fractions, most probably bound to the endoplasmic reticulum in the cytosol, and are accumulated in the vacuole (anthocyanidins, procyanidins) and the cell wall. A number of phenylpropanoids have vital roles in the structural integrity, UV protection, reproduction, physiological regulation and signalling in the plant organism. Phenylpropanoids are also involved in the chemical modulation of the interaction of the plant with insects and microorganisms (attractants, repellents), as e.g. phytoalexins against pathogens and herbivores, and play vital role in plant pollination by insects. Other vital roles also include root nodulation in symbiosis with nitrogen fixation microorganisms [3 and references cited within]. The result of the present work indicate a tendency of elevation of JA as a stress signal, leading to a rise of polyphenolics levels in the root inhibited morphotypes. As a result, the levels of the stress hormones ABA and SA remain low.

In our previous work [1] we have established that the plant growth regulators (PGRs) *in vitro* strongly affect the monoterpenoid/sesquiterpenoid ratio of the essential oil. The application of different combinations of auxin indole-3-butyric acid (IBA) and cytokinin $N^6$-benzyladenine (BA) led to the formation of two main morphotypes *in vitro*. While the formation of a well-developed root system led to a higher monoterpenoid/sesquiterpenoid profile of the oil, root inhibition was related to a significant drop of this parameter, related to enhanced sesquiterpenoid production in the latter *in vitro* system [1]. The root suppressed plants also expressed a drop of endogenous isoprenoid cytokinins in the aerial parts. In addition, impairment of the photosynthetic apparatus and chloroplast architecture were also observed [1]. These findings led to the hypothesis that the interplay between terpenoids and cytokinin biogenesis and chloroplast architecture might be indicative of the differential involvement of the mevalonic acid (MVA) and methylerythritol phosphate (MEP) biogenetic pathways in the two *in vitro* models [1]. The results of the present work indicate that the content of secondary metabolites of different chemical character could be successfully influenced through modification of culture conditions.

**Conclusion:** Secondary metabolites production was shown to be affected by the developmental patterns and morphogenesis of *A. alba in vitro*. The in-depth understanding of the fundamental basis of these dependencies could be used as a tool for the targeted biotechnological secondary metabolite production of medicinal and aromatic plants without performing genetic transformations.

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# REPORTS

## Topic:
## ECOSYSTEM RESEARCH, SERVICES AND ECOLOGICAL AGRICULTURE

# WEED SPECIES DIVERSITY AND COMMUNITY COMPOSITION IN ORGANIC POTATO FIELD

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**Abstract:**

Weeds are the component of biological diversity in the agricultural systems (agrobiodiversity) and one of the greatest limiting factors to efficient organic crop production.

**The goal** of the experiment is to define the influence of organic agricultural practices on the dynamics of weed infestation, weed species diversity and community composition in organic potato field included in three-field crop rotation.

**The results show** that all analyzed weed parameters are characterized by high dynamics during potato grown seasons. The biological development of weeds in the cover crop before incorporation to the potato field was strongly limited. According to the data obtained, cover crop for green manure and mechanical soil tillage in the organic potato field were effective practices for controlling the weed infestation. The effect of organic practices on the weed community parameters were assessed by ecological indexes (Shannon index (H'), evenness (J'), index of dominance (D) and similarity indexes.

**Keywords:** organic potato, green manure, weed community, ecological indexes

## Introduction

The weeds are one of the greatest limiting factors to the efficient organic crop production. The sustainable weed management requires applying of agricultural practices which not only influence the weed density and community but also keep their positive impact on the ecology. The published results show that the organic fields are usually characterized by high weed density and biomass compared to the conventional ones [1, 2, 3] because the organic practices have lower weed control effects than that of the herbicides.

The crop rotation is a fundamental part of the organic farming systems. The diversification of species in the farming systems is one of the strategies for regulation of the weed density and community structure but the effect depends on many biotic and abiotic factors [4].

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The aim of the present study is to analyze the influence of different organic practices on the weed species diversity and community composition in an organic potato field which is included in a biological crop rotation.

## Material and Methods

The study was conducted at the Suhodol Experimental Station (near Sofia) of the Institute of Soil Science, Agro-Technology and Plant Protection „N. Pushkarov". The potato field was a part of a three-field crop rotation: 1) winter wheat (*Thriticum aestivum* L.)*;* 2) green bean (*Phaseolus vulgaris* L.) with organic manure, 3) cover crop (mixture of *Secale sereale* + *Pisum sativum*) for green manure – potato (*Solanum tuberosum* L.). The cover-crops mixture was sown in autumn and incorporated as a green manure in spring before potato planting. All crops have been grown according to the organic standards since 2004. In this paper data for 2012-2013 are presented. The weed infestation of crop area was determined by weigh-counting method on 1 $m^2$ area (3 samples) in each 4 replications. The assessment of weed infestation included weed species composition as well as the number of weeds and their green and dry matter. The weed species composition was measured in cover crop mixture before incorporation into the potato field and three times during the potato vegetation period. Different ecological indexes (Shannon' index of biodiversity (H')), evenness (J'), Simpson dominance index were applied for weed community assessment. Sørensen similarity indexes (qualitative and quantitative) were used for comparison of the weed communities similarity in the potato field between 2012 and 2013.

Weed biodiversity within the communities was estimated and compared based on: species richness (S) - number of species in the community; Shannon-Wiener diversity index (H): $H' = - \Sigma p_i (\text{Ln } p_i)$, where Ln-natural logarithm and $p_i$ –relative abundance of the $i$-$^{th}$ species in the community, Shannon-Wiener evenness index (Pielou's index, J'): J'= H'/ LnS, Simpson dominance index: $D = \Sigma p_i^2$. The qualitative, accordance to weed species, and quantitative similarities between weed communities compared using the Sørensen similarity indexes: 1) Sørensen qualitative index: $C=[2C(A+B)^{-1}]*100$, where C - the number of the common to each community species and A + B - the sum of total number of species in each community; and 2) Sørensen quantitative index: $C_N=[2jN(Na + Nb)^{-1}]*100$, where jN - the sum of lower of the two abundances of each species in the community; Na – all number of individuals in population A; and Nb - all number of individuals in population B. All indexes were described by Magurran [5].

## Results

The first assessment of weed infestation in 2012 and 2013 was done in a grass-leguminous mixture field before incorporation as a green manure into the potato field. The weed infestation level in 2012 was not very high – the weed density reached 33 plants/$m^2$ with green biomass of 16.56 g/$m^2$. We have to note that at this moment the grass-leguminous mixture has accumulated a high amount of fresh biomass, which suppressed the weeds. The data of weed density are presented in Table 1, and fresh and dry weed biomass on the Fig.1.

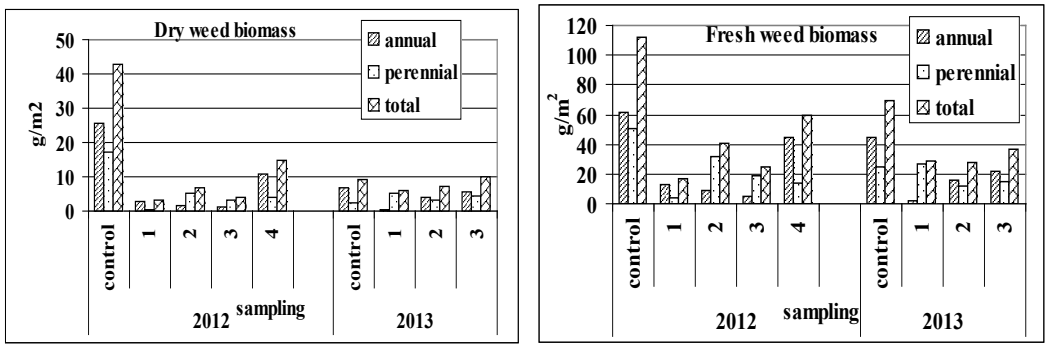
**Table 1. Weed species density (plants/m$^2$) during the potato vegetation**

| Weed species | 2012 | | | | 2013 | | |
|---|---|---|---|---|---|---|---|
| | Weed sampling | | | | | | |
| | 1st 22.04. | 2nd 20.06. | 3rd 6. 07. | 4th 12 .08. | 1st 28.04. | 2nd 18.06. | 3rd 14.08. |
| *Lamium amplexicaule* L. | 1.5 | - | - | - | - | - | - |
| *Veronica hederifolia* L. | 13.0 | - | - | - | - | - | - |
| *Anthemis arvensis* L. | 6.5 | 1.0 | - | - | - | - | 1.0 |
| *Ranunculus arvensis* L. | 5.0 | - | - | - | 1.0 | - | - |
| *Setaria viridis* L. | - | 27.5 | 2.5 | 15.7 | | 5.3 | 4.0 |
| *Amaranthus retroflexus* L. | - | - | - | 2.6 | - | - | |
| *Anagallis arvensis* L. | 1.0 | - | - | - | - | - | - |
| *Polygonum aviculare* L. | - | - | - | - | 2.5 | - | - |
| *Taraxacum officinale* L. | 1.0 | - | - | - | - | - | - |
| *Portulaca oleracea* L. | - | - | - | - | - | 2.3 | 2.0 |
| *Echinochloa crus-galli* L. | - | - | 1.0 | 0.7 | - | 1.7 | 1.5 |
| *Plantago major* L. | - | - | - | - | - | 1.0 | 4.0 |
| *Mentha arvensis* L. | 1.0 | - | - | - | - | - | - |
| *Cirsium arvense* L. | - | 8.5 | 8.5 | 2.3 | 3.0 | 2.0 | 3.0 |
| *Convolvulus arvensis* L. | - | - | 5.0 | 4.0 | 25.5 | 1.3 | - |
| *Lithospermum arvense* L. | - | - | - | - | - | - | 1.5 |
| *Hibiscus trionum* L. | - | - | 13 | 17.3 | - | 3.7 | - |
| Euphorbia sp. | 4.0 | - | - | - | 1.5 | - | - |
| *Sonchus arvensis* L. | - | - | - | - | - | 2.7 | 1.0 |
| *Chenopodium album* L. | - | 1.0 | - | 1.0 | - | - | - |
| *Xanthium strumarium* L. | - | - | - | - | 3.0 | - | - |
| *Hibiscus trionum* L. | - | 34.0 | - | - | - | - | 1.5 |
| *Mentha arvensis* L. | - | - | 1.5 | 1.0 | - | - | - |
| **Total weeds** | **33.0** | **72.0** | **31.5** | **44.6** | **36.5** | **20.0** | **19.5** |
| The statistical significance using one-way ANOVA | $GD_{5\%}$=16.2; $GD_{1\%}$=18.0; $GD_{0.1\%}$=24.8 | | | | $GD_{5\%}$=7.2; $GD_{1\%}$=12.4; $GD_{0.1\%}$=16.8 | | |

The weed infestation in the control plot (bare fallow) increased considerably (192.6 pl/m$^2$ and biomass of 112.5 g/m$^2$) in comparison to the field of cover crops. These results highlight the role of the cover crops for green manure for controlling the weed density. The observed results in early spring of 2013 were similar – the weed density was 36.5 pl./m$^2$ with 28.65 g/m$^2$ fresh biomass. The weed community structure in 2012 was dominated by annual weeds. The weed spaces were presented mainly by weeds with spring and winter-spring biological life cycle. *Veronica hederifolia* L. was the prevalent weed species. The population density of perennial weeds in the cover crops phytocoenosis was not very high (6 plants/m$^2$). The perennial weeds were dominated by *Euphorbia* sp. L. which was the main weed species in this group, but the weed plants were not well developed and the fresh weed biomass was only 2 g/m$^2$. The small abundance level of perennial weeds could be explained by the crop rotation design, where the potato planted after the other row crops (green bean), which technology was also based on the inter-row mechanical cultivations. In addition, the soil tillage operations for the seed bed preparation of the cover crops destroyed

the emerged weeds in late autumn. During spring weed plants were suppressed by the dense stand of the cover crops and they did not accumulate high amount of biomass. In the next experimental year (2013) the weed community in the cover crops was characterized by higher proportion of perennial weeds than in the previous measurement. *Convolvulus arvensis* L. was totally predominant in the community of perennial weeds (70%). The weed density was not an adequate parameter to characterize completely the infestation. At this moment low fresh weed biomass was measured – only 18.44 g/m$^2$.

The potato was planted immediately after the cover crops incorporation. The next weed sampling was done before the first inter-row mechanical cultivation of the potato in June. According to the received data, the weed community composition changed as a result of soil tillage before the potato planting, which eliminated the weeds of the early and spring-winter group have observed in the cover crop phytocoenosis. In this time of vegetation, the species of the late spring biological group dominated in the weed community. The total numbers of annual and perennial weeds were 72 pl/m$^2$ with higher fresh biomass compared to the previous assessment. The meteorological conditions were more suitable and stimulated the crop and weeds development. The weed community was dominated by *Hibiscus trionum* L. with 34 pl/m$^2$ and proportion of 50% from weeds abundance. Opposite to the high density at the beginning of the vegetation, the weed species formed a very small amount of green biomass (3.51 g/m$^2$) without any practical influence. The species *Setaria viridis* L., which is a mono-cotyledonous weed had higher density than the other species presented in the community. The group of perennial weeds was presented in a small proportion and included only *Cirsium arvense* L. with 8.5 pl/m$^2$.




A) B)

**Fig. 1. Fresh (A) and dry weed biomass (B) during the potato growing season (1, 2, 3, 4 – weed samplings)**

The observation shows that the arable weed vegetation was a very dynamical system. Weed flora changed over the growing season. It responds to the agronomic factors associated with crop cultivation and, at the same time, with the climatic conditions. During the potato vegetation period the new germinated weeds were destroyed by timely applied mechanical cultivations and in the tuber formations stage the number of weed plants was lower than in

the previous sampling. It is well known that soil tillage operations considerably influence the weed germination, weed seeds bank in soil and also their distribution within the soil profile. In the third weed sampling before the other soil cultivation in July the weed density was twice as lower as compared to the first measurements and the differences are statistically significant (GD=0.01%). The number of weeds was 32 individuals per 1 $m^2$ with of 24 g/$m^2$ green biomass and very small dry biomass of 4 g/$m^2$. This means that the weeds formed insufficient weed biomass which could not influence the plant growth. Both annual and perennial weeds were presented in the community composition by equal proportion (52% and 48%). The weeds were mainly of the late-spring biological group. The *Hibiscus trionum* L. species was dominant with a share of 40% but the weed density was 60% lower compared to the previous measurement. *Setaria viridis* L. and *Echinochloa crus-galli* L. were practically eliminated to the non-significant number of 1-2 individuals per 1 $m^2$. The weed infestation with perennial weeds also was decreased substantially. The perennial weeds were represented mainly by *Mentha arvensis* L., *Cirsium arvense* L., *Convolvulus arvensis* L., which reached 79% of the total weed biomass in the samples. The control of the weed density with species of this group usually is more difficult for biological management because several mechanical and cultural operations are to be applied. These figures emphasize on the effectiveness of two mechanical cultivations applied during the potato vegetation period for substantial decreasing of the weed density. The effective weed control during the previous crop in the crop rotation also had important role for this result.

In the next experimental year (2013) the weed density accounted during the potato flowering and tuber formation was not very high too, despite of the heavy rains in June and July (131.6 mm and 59.7 mm) and the fact that after the first cultivation new weeds were provoked to germinate. The annual and perennial weeds were identified with total weeds number of 20 individuals per 1 $m^2$, green biomass of 27.41 g/$m^2$ and dry weight of 7 g/$m^2$. The proportions of both weed groups in the weed community were 65% to 35%. The annual weeds were identified by different mono- and dicotyledonous weeds, presented by a few numbers in diapason of 1 to 5 individuals per 1 $m^2$. The weed community consisted mainly of *Setaria viridis* L., *Hibiscus trionum* L., *Echinochloa crus-galli* L. and *Portulaca oleracea* L.

In general, the potato field at harvesting was characterized by a significantly lower number of weed species than at the beginning. In 2012, late-spring annual weeds *Setaria viridis* L., *Hibiscus trionum* L, and *Amaranthus retroflexus* L., dominated. In the weed community, few numbers of *Echinochloa crus-galli* L. and *Chenopodium album* L. species were also identified. They accounted 84% of the total weeds abundance. The weeds of these biological groups typically occurred in the area of the spring-summer crops and usually germinate after the last mechanical tillage operation. At harvesting, when part of the potato biomass was dead and good conditions for secondary infestations were created, new weeds germinated. This was the reason for establishing an increased number of annual weeds in the last vegetation stage of the potato, about twice higher, compared to their number at the beginning of vegetation. Weed density remained at a low level. The density of the perennial weeds substantially decreased as a results of mechanical soil tillage operations during the summer period when the weed roots and belowground stems were cut off and buried in the

soil or exposed to the climatic factors. The successful control on perennial weeds *Mentha arvensis* L., *Cirsium arvense* L., *and Convolvulus arvensis* L. was measured.

The observed tendency at potato harvesting in 2013 was similar and accounted 45.3 % lower weed numbers than the beginning of potato vegetation. It is obvious that timely soil tillage operations during potato vegetation are very effective for weed control against both annual and perennial weeds.

The summarized results show that the weed density values change substantially during the period of investigation. The coefficient of variation (CV) was higher than 30% which indicated that the weed communities were strong in heterogeneity and the infestation level depended on many factors and their interactions.

The results indicated that the weed species richness in the potato field varied substantially during the study period – from 5 to 10 species. The greatest weed richness (10 species) was counted in the cover-crops field in 2012 which was higher than the measured species in 2013 (6 species).

In the study of the weed community structure in a potato field it was found that the weed species composition was significantly affected by the agronomy management. The measured weed species in an organically managed potato field were from 5 to 9 species. The weed flora changed over the grown season under the influence of the cover crop for green manure and the mechanical soil cultivation in the potato field.

Fig. 2 demonstrates the reflected by the Shannon-Wiener index of diversity (H'), evenness (J') and Simpson index of domination (D) weed flora diversity.

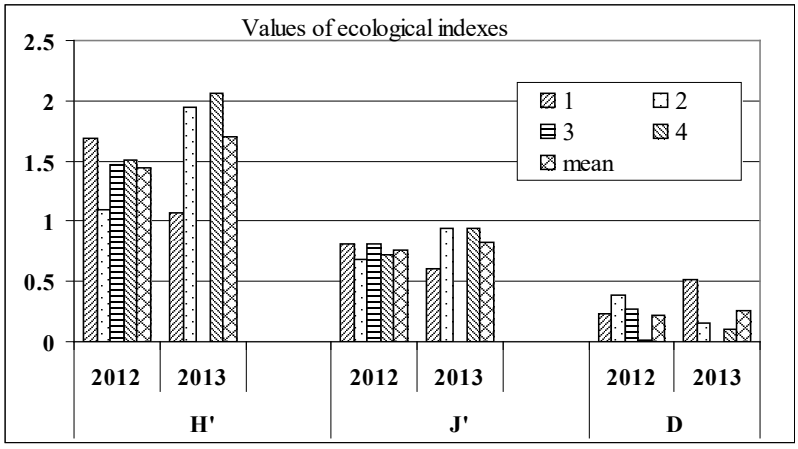



**Fig. 2. Values of ecological indexes for assessment of the weed community structure during the potato grown season (1, 2, 3 and 4 are the weed sampling)**

The Shannon-Wiener index of diversity (H'), from the beginning of the growing season up to harvesting of potato, increased from 1.09 to 1.50 in 2012 and from 1.95 to 2.06 in 2013 showing increased biodiversity, irrespective of the intensive soil tillage. The weed species diversity was unsatisfactory which indicated for a low level of complexity

of the weed communities. The evenness (J') is the measure of abundance heterogeneity among the species in a community. According to the average values of species evenness (J' =0.75-0.82), the weed communities were relatively even. The values of the Simpson index (D) in the potato harvesting indicated that none of the weed species dominated in this community. On the contrary, the highest value of this index was observed for the flora community in the cover crop in 2013 because of the dominance of one species *Convolvulus arvensis* L. The comparison of similarity of weed communities in the potato field between 2012 and 2013 by using Sørensen indices show that the similarity was higher on the base of the qualitative index (35.3%) compared to the quantitative ones (20.8%).

**Discussion**

The role of weeds in the agroecosystems has been largely debated because of both their potential delivery of ecosystem services and the competition between weeds and crops. Weeds of arable land are a component of the biological diversity in the agricultural ecosystems, and they play a vital role in supporting the diversity within the crop fields and also offer different ecological and agronomical services [3, 6]. The organic fields are usually characterized by higher levels of weed infestation than the conventional ones, probably because of mechanical weed control and due to the fact that the other cultural methods commonly achieve lower control effect than the herbicides [2, 6, 7].

Organic farming system is based on the use of environmentally friendly production methods that include crop rotations with a large share of legumes, organic fertilizers, and non-chemical methods of plant protection. The use of cover crops in crop rotations is usually recognized as an important management method in the organic production systems [8]. Cover crops can affect weed populations in the short and long terms with different mechanisms. They suppress the weeds by competing for the use of light, nutrients and moisture, by effects of allelopathy and in the organic farming are an alternative to the chemical weed control [9, 10]. The results of the present study indicated that the cover crops suppressed the weeds and the weed infestation level in early spring before incorporation to the soil was twice lower than in the bare fallow field. The mechanical soil tillage for preparing the soil bed for the cover crop sowing also influenced the weed infestation through destroying the germinated weeds in the late autumn.

Mechanical cultivation is a common method of weed managing in the organically managed systems. According to Barberi (2002), direct (physical) weed control can be successful only where preventive and cultural weed management is applied to reduce weed emergence (e.g. through an appropriate choice of crop sequence, tillage, smother/cover crops) and improve the crop competitive ability (e.g. through appropriate choice of the crop genotype, sowing/planting pattern and fertilization strategy). Potatoes were traditionally regarded as a cleaning crop. We observed that weed density and weed biomass changed over the potato growing season depending on the inter row mechanical cultivation and the other factors. The low number of accounted weeds could be explained also with the crop rotation design where potato was grown after the other spring row crop (green bean).

The studied three-field crop rotation with winter wheat, green bean, peas and rye mixture as cover crops and potato are a strategic scheme for achieving good weed control. This is similar to previous studies that reported that by rotating crops with different planting dates and growing periods, contrasting competitive characteristics and dissimilar management practices, weeds can be under control [11]. Weed dynamics are disrupted when seasonal crops are arranged in a sequence of two cool season crops followed by two warm season crops [12] like in the studied crop rotation. In the experiment discussed here, the density of the perennial weeds at the end of the potato vegetation period substantially decreased.

Organic management changed the weed community structure and, as consequence, the agroecosystem functioning. The values of Shannon weed biodiversity index, evenness (J') and richness (S) were influenced by the crop rotation complexity, crop characteristics and kind of organic fertilization [7, 13]. According to Jastrzębska [6], the organic farming contributed for higher weed species diversity, observed in root crops, and the values of Shannon-Wiener index was statistically higher in organic systems, including cover crops for green manure (H'=1.70) [14]. Nikolić et al. [15] found small differences in the weed flora structure between conventionally and organically grown potato crops, most likely due to agrotechnical practices. The comparison between the weed communities by using Sørensen indices manifest that qualitative changes are slower than the quantitative ones [2]. Our data also supported this conclusion because the comparison between the similarity of the weed communities in a potato field between 2012 and 2013 by using Sørensen indices show that the similarity was higher on the base of the qualitative index (35.3%) than the quantitative one (20.8%). We agree with the opinion of the researchers who indicated that the weed infestation is the major crop protection problem in the organic farming systems, and the development of weed management strategies requires detailed information on the weed population dynamics.

**Conclusion**

The arable weed vegetation was a very dynamical system. All analyzed weed parameters (weed density and weed biomass) and weed community structure (species diversity, richness, evenness and dominance) were influenced by the cover crop for green manure and mechanical soil cultivation and other factors in the potato field. The studied three-field crop rotation that included winter wheat, green bean, peas and rye mixture as cover crops and potato is a strategic scheme for achieving good weed control at the end of the rotation. The comparison of similarity of weed communities in a potato field between 2012 and 2013 using Sørensen indices shows that the similarity was higher on the base of the qualitative index (35.3%) than of the quantitative ones (20.8%).

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# APHID PEST SPECIES ON *CHRYSANTHEMUM MORIFOLIUM* RAMAT. IN BULGARIA

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**Abstract**

**Aim:** The objective of the present study was to investigate the aphid pest species on *Chrysanthemum morifolium* Ramat. and the effect of the aphid infestations on this host plant.

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**Material and Methods:** Investigations on the species composition of aphids on *C. morifolium* in more than 20 greenhouses and garden centers in Bulgaria were observed. The present study is conducted over a period of 10 years, from 2008 to 2018. The observed greenhouses and garden centers are located in the regions of Sofia, Plovdiv, Smolyan, Pavlikeni, Ruse, Varna and Burgas.

**Results and Discussion**: Six different aphid species were detected during the observations. They are *Aphis fabae* Scopoli, 1763, *A. gossypii* Glover, 1877, *Aulacorthum circumflexum* (Buckton, 1876), *A. solani* (Kaltenbach, 1843), *Macrosiphoniella sanborni* (Gillette, 1908) and *Myzus persicae* (Sulzer, 1776). All the identified species are polyphagous, except *M. sanborni*. The most important, widespread and harmful aphid pest species on *C. morifolium* was *M. sanborni* detected in greenhouses all year round. The damages reveal in deformations of stems, leaves, and flowers. Due to the heavy aphid infestations the flowers cannot open normally and have low trade quality.

**Conclusions:** The most important aphid pest on *C. morifolium* is *M. sanborni.* It causes damages and decreases the trade quality of the grown plants and cut flowers. It was found all year round in the investigated greenhouses. Less importance in *Chrysanthemum* cultivation, have all other detected species, which were sporadically founded.

**Keywords:** Aphididae, aphids, *Chrysanthemum*, mums, pests

## Introduction

*Chrysanthemum* is a genus of flowering plants that were have been cultivated in China as early as the 15th Century B.C. Currently there are many hybrids of *Chrysanthemum*. The most common species used as cut flowers and in landscaping is *Chrysanthemum morifolium* Ramat. This species is widely grown for its beautiful and rich variety of colorful flowers. It is used as long-lasting cut flower and in certain conditions can be forced to flower all year round. When grown in parks and gardens it provides a long-lasting Fall blooming. Some cultivars can be trained into different forms of its habitus.

*C. morifolium* is infested by several pests – white flies, thrips, leaf miners, European mole cricket, May bug, nematodes, spider mites etc., but one of the most important among all them are aphids. They cause serious damage on infested plants. That may lead to deterioration of the ornamental qualities and sometimes even death of the plants. One of the most important and serious consequences is virus transmission.

Twenty-eight aphid species on *Crysanthemum* plants are reported worldwide [1], 14 of them are found in the USA [2]. Surveys on aphids feeding on Chrysanthemums in Bulgaria was conducted by Tashev [3] and Yovkova et al. [4], given as a check list without any further information. Currently, there is no extensive research on *Chrysanthemum morifolium* Ramat. in Bulgaria, which prompted the current study.

The purpose of this investigation was to identify the species composition of aphids on *C. morifolium* and the effect of the aphid infestations on host plants in Bulgaria. The results of our survey contribute to the scientific knowledge in the investigated field, but also have a practical application, benefitting producers of mums in Bulgaria.

## Material and Methods

Investigations on the species composition of aphids on *C. morifolium* in more than 25 greenhouses and garden centers in Bulgaria were performed. The present study is conducted in over a period of 10 years, from 2008 to 2018. The observed greenhouses and garden centers are mainly located in the regions of Sofia, Plovdiv, Krichim, Smolyan, Pavlikeni, Ruse, Varna and Burgas. Aphids were collected in plastic bags together with the infested plant parts. Larvae were reared in laboratory conditions to the stage of adults. The species identification was carried out using permanent microscope slides, after the traditional method of Hille Ris Lambers [5]. Identification keys included Blackman and Eastop [6, 7].

## Results

Six different aphid species were detected during the observations. They are *Aphis fabae* Scopoli, 1763, *A. gossypii* Glover, 1877, *Aulacorthum circumflexum* (Buckton, 1876), *A. solani* (Kaltenbach, 1843), *Macrosiphoniella sanborni* (Gillette, 1908) and *Myzus persicae* (Sulzer, 1776).

***Aphis (Aphis) fabae* Scopoli, 1763**

Host plants: Polyphagous species [6].
World distribution: Cosmopolitan [8].
Plant parts infested: Aphid feeds on the hind surface of the leaves, and the apex of the stems.
Malformations: not noticeable.
Damages: not noticeable.
Virus vector of: more than 30 plant viruses [9].
Comment: During the current observations this aphid was found indoors in 2 of the observed objects in Sofia and nearby areas, and it didn't have a high impact on the grown plants. After the applied phytosanitary measures, the plants were grown normally.

***Aphis* (*Aphis*) *gossypii* Glover, 1877**

Host plants: Polyphagous species [9].
World distribution: Cosmopolitan [8].
Plant parts infested: This aphid infests stems, leaves, buds, and flowers of mums. Characteristic for that aphid is that it has a higher density of its colony on the hind side of infested leaves and on the down side of the open flowers, even in between ligulae.
Malformations: not noticeable.
Damages: not noticeable.
Virus vector of: more than 50 plant viruses [9].
Comment: Aphid was detected indoors in 3 of the observed objects in Sofia. Infestations of that aphid reduces the quality and prise of mums' cut flowers.

***Aulacorthum* (*Aulacorthum*) *solani* (Kaltenbach, 1843)**

Host plants: Extremely polyphagous species [7].
World distribution: Cosmopolitan [8].
Plant parts infested: Aphid is found on the stems and hind side of the leaves.

Malformations: not noticeable.
Damages: not noticeable.
Virus vector of: around 40 plant viruses [10], and Tomato aspermy cucumovirus (TAV) and chrysanthemum B carlavirus (CHVB) on mums [11, 12].
Comment: Aphid was registered indoor in 2 of the observed objects in Sofia and nearby areas. This aphid species has not significant impact in mums growing in Bulgaria.

***Aulacorthum* (*Neomyzus*) *circumflexum* (Buckton, 1876)**

Host plants: Extremely polyphagous species [7].
World distribution: Cosmopolitan [7].
Plant parts infested: Aphid is found on the stems and hind side of the leaves.
Malformations: not noticeable.
Damages: not noticeable.
Virus vector of: more than 30 plant viruses [10].
Comment: Aphid was registered indoor in 1 of the observed objects in Sofia. This aphid species has not significant impact in mums growing in Bulgaria.

***Macrosiphoniella* (*Macrosiphoniella*) *sanborni* (Gillette, 1908)**

Host plants: mostly species form Asteraceae family and especially *Chrysanthemum* [1, 6].
World distribution: Cosmopolitan [13].
Plant parts infested: That aphid can be found on the top stem parts, on the hind side of the young leaves and when buds and flowers are presented, they can be found mostly on that parts of the plant.
Malformations: noticeable
Damages: The damages reveal in malformations of stems, leaves, and flowers. Due to the heavy aphid infestations the flowers cannot open normally and have low trade quality.
Virus vector of: *Chrysanthemum* viruses [10], and Tomato aspermy cucumovirus (TAV) and chrysanthemum B carlavirus (CHVB) on mums [11, 12, 14].
Comment: Aphid was registered indoor (6 objects in Sofia and nearby areas, Ravda) and outdoor (1 object in Sofia, Evksinograd, Krichim and Pavlikeni). In greenhouses this species was detected all year round. Most harmful aphid species on Chrysantemums established during the current investigations.

***Myzus* (*Nectarosiphon*) *persicae* (Sulzer, 1776)**

Host plants: Extremely polyphagous species [7].
World distribution: Cosmopolitan [8].
Plant parts infested: detected on the mums' stems and on hind side of leaves troughout the whole plants.
Malformations: not noticeable
Damages: not noticeable
Virus vector of: more than 100 plant viruses [10], and Tomato aspermy cucumovirus (TAV) and chrysanthemum B carlavirus (CHVB) on mums [11, 12].
Comment: Aphid was registered indoor in 1 of the observed objects in Sofia. In the current investigations this aphid species has not significant impact in mums growing in Bulgaria.

All the identified aphid species are polyphagous, except *M. sanborni*. They all can be found on indoor and outdoor grown plants and are virus vectors. *M. persicae* was

detected on the mums' plant parts throughout the whole plants as a contrast of the all other detected aphids, that were found mostly on the youngest parts of the infested plants. The most important, widespread and harmful aphid pest species on *C. morifolium* was *M. sanborni* detected in greenhouses all year round. The lower impact of the other detected species is most probably due to the lower density of aphid colonies. Mixed colony of *Aulacorthum circumflexum* and *Macrosiphoniella sanborni* was registered in one of the observed objects in Sofia. From the current investigations we could say that *M. sanborni* is a dominant species and when presented it displaces the other detected aphid species.

**Conclusions**

The most important aphid pest on *C. morifolium* is *M. sanborni.* It causes damages and decreases the trade quality of the throughout grown plants and cut flowers. It was found all year round in the throughout investigated greenhouses. Less importance in *Chrysanthemum* cultivation, have all other detected species, which were sporadically found.

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# SHORT COMMUNICATIONS

## Topic: ECOSYSTEM RESEARCH, SERVICES AND ECOLOGICAL AGRICULTURE

## MAPPING AND ASSESSMENT OF THE CONDITION AND ECOSYSTEM SERVICES OF INLAND WETLANDS IN BULGARIA OUTSIDE THE NATURA 2000 ECOLOGICAL NETWORK

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The **aim** of the project is to map and assess the condition of „inland wetland" ecosystems outside NATURA 2000 ecological network and their services in Bulgaria and to present the major abiotic and biotic characteristics of the target ecosystems. The project is funded by the BG03 Program Biodiversity and Ecosystems under Call BG03.02: Mapping and Assessment of Ecosystem Services, and is implemented by a team of scientists from the Institute of Biodiversity and Ecosystem Research at the Bulgarian Academy of Sciences.

**Material and Methods:** The object of mapping and assessment of the condition and services provided are the following subtypes of "inland wetland" ecosystems: 1 /701 Valley mires, poor fens and transition mires (EUNIS code D2); 2/702 Base-rich fens and calcareous spring mires (EUNIS code D4); 3/703 Sedge and reedbeds, normally without free-standing water (EUNIS code D5) [1]. The inland wetland ecosystems have met the following criteria: 1/have natural vegetation; 2/have a level of water at or above the soil level at least about half of the year; 3/are dominated by grass or peat vegetation, or dominated by acid grasses from families Sedge (Cyperaceae), Rush (Juncaceae), Cattails (Typhaceae) and/or Reed (*Phragmites australis*); 4/are not associated with open waters and 5/are outside NATURA 2000 network.

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The work on the project is based on the *National Methodology for assessment of ecosystems and services provided by them* developed under the project "Methodological support for assessment of ecosystems and their biophysical valuation" (*MetEcosMap*). It is developed in English and has the following title in the section on Inland Wetland Ecosystems: *Methodology for Assessment and Mapping of WETLAND ecosystems condition and their services in Bulgaria, Part B7*[2].

**Results:**

**1. Mapping of inland wetland ecosystems**

Ecosystems mapping is a complex activity on gathering spatial information on ecosystems and services provided by them, organizing it in databases and generating maps for the areas of their provision and the need of ecosystem services.

As a result of the analysis of the available data on ecosystem mapping in Bulgaria for the purposes of the current project the following sources of digital information are used: 1/Geospatial database for ecosystems in Bulgaria, developed in the project "National priority action framework for NATURA 2000"; 2/Borders of NATURA 2000 zones in Bulgaria in shape files from MOEW website (http://natura2000.moew.government.bg/Home/Documents); 3/High resolution layers (HRL) for Bulgaria, derived from the GIO land project of the European Environment Agency (EEA); 4/Topographic maps of Bulgaria in scale 1:25000; 5/Raster orthophoto images of the territory of Bulgaria from 2011.

An initial layer of spatial objects polygons has been made as a result of GIS analysis of the data mentioned above. The polygons have been visited during the field surveys on data gathering of the exact location on spot, the ecosystems condition and data for the provided ecosystem services. As a result of the fieldwork, corrections of attributes and polygon geometry, as well as removing and adding new polygons have been made. Thus the final layer with polygons of wetland ecosystems has been formed.

The total number of mapped polygons is 285, from which one is subtype 701 – *Valley mires, poor fens and transition mires* (EUNIS code D2), six are subtype 702 – *Base rich fens and calcareous spring mires* (EUNIS code D4) and 278 (97.5%) are subtype 703 – *Sedge and reedbeds, normally without free standing water* (EUNIS code D5). For each ecosystem subtype, digital maps have been drawn at a scale of 1:125 000. Each map contains one cell of the 50-kilometer EEA Grid for Bulgaria. Similar maps have also been prepared for the condition and for the services provided by ecosystems. A geospatial database of ecosystems subtypes, their condition and the ecosystem services provided by them has been created.

**2. Assessment of the condition of inland wetland ecosystems**

Assessment of the condition of inland wetland ecosystems is performed by using 10 indicators and their 19 parameters in 285 mapped polygons. The assessment of the separate indicator parameters is done on a scale basis. Each indicator is evaluated on a scale of 1 to 5 and presents the condition of ecosystems. Each polygon of terrestrial wetlands has attribute information about the indicators and parameters stored in a geospatial database. The *Index of Ecosystem Performance* (IP), presenting the state of ecosystems, is calculated and represents the ratio of the sum of the estimates of the indicators to the maximum possible sum of the indicators. The results show that the condition of the inland wetlands is good and only 3%

of the polygons are moderate. For both subtypes – 701 (D2) and 702 (D4) all polygons are in good condition, while for 703 (D5) most of the polygons (97%) are in good condition and only 3% are in moderate condition.

The target ecosystems occupy small areas between fields, along roads, canals, settlements, but create conditions for preserving biodiversity that is attached to wetland habitats. The studied wetland polygons are characterized by a specific interaction between the soil-water components that form the intermediate/eco-zone. This is a qualitatively new type of environment that differs from both land and water. The characteristics of shallow wetlands are the result of complementary action and interrelations of environmental factors, soil characteristics (pH, content of nutrient), nature and dynamics of water supplying and the specificity of ongoing biological processes in the analyzed ecosystems.

**3. Assessment of ecosystem services of inland wetlands**

Ecosystem services are natural assets. They are the processes through which the environment produces resources used by people like food, clean air and water, materials, medical supplies and contribute to social and cultural wellbeing of humankind.

The assessment of ecosystem services (ES) of inland wetland ecosystems has been performed using 16 indicators and their 16 parameters in 285 mapped polygons. The assessment of the separate parameters of the indicators has been carried out on the basis of capacity assessment scales. Each indicator is estimated in a scale of 1 to 5 plus 0 and presents the relevant ecosystem capacity to provide the relevant service. Each polygon of all sub-types of inland wetlands - 701 (D2), 702 (D4) and 703 (D5) has an attribute data base with indicator information. The analysis of the ES shows that the most relevant capacity (high and very high) is the service "*Maintenance of populations and habitats for reproduction* (2312)". High capacities to provide have also the ESs "*Flood protection* (2222)" and "*Scientific interest* (3121)". Nine of the ecosystem services are rated as irrelevant. With a low relevant capacity to provide is the service "*Stabilization of soil and erosion control* (2211)". Based on the results of the ecosystem services assessment project, the services provided by inland wetlands will be valued at a later stage and integrated into accounting and reporting systems at national and European level.

**Conclusion (main results of the project):** 285 inland wetland ecosystems have been identified and mapped through GIS and field studies. The condition of the identified ecosystems has been assessed by direct measurement or analysis of available data on plant and animal diversity, soils, waters, fires, dumping-sites and invasive species. The provisioning, regulating/maintenance and cultural ecosystem services have been assessed. Individual maps of subtypes of ecosystems, their condition and the ecosystem services they provided have been prepared. The information gathered and the assessments made have been inserted into a database. Information on the condition of inland wetland ecosystems and the services they provide for future inclusion of results in the legal framework of national and European sectoral policies has been provided.

The results of the project will benefit the Ministry of Environment and Waters, the Environmental Executive Agency, the RIEW, the Basin Directorates, regional and local authorities, water and sewage companies, non-governmental organizations, business, researchers, students and wetland users.

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# REPORTS

## Topic:
## LANDSCAPE ECOLOGY

# ACADEMICIAN ANASTAS ISHIRKOV'S CONTRIBUTION TO THE DEVELOPMENT OF BIOGEOGRAPHY IN BULGARIA (DEDICATED TO THE 150TH ANNIVERSARY OF HIS BIRTH)

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**Abstract**

**Aim:** The main purpose of the paper is to analyze the scientific work of academician Anastas Ishirkov and to highlight his importance as a founder of biogeography in Bulgaria.

**Academic Biography and Research:** Academician Ishirkov, as a teacher in Varna, in 1892 was sent to specialize in Leipzig where he attended Friedrich Ratzel's lectures. He was undoubtedly influenced by Ratzel's geographic determinism, which he further developed following the ideas of French philosophers of the 18th century. In his two-volume anthropogeography, under the influence of Darwin's evolutionary theory, Ratzel (1882) considers anthropogeography as part of biogeography. In August 1895, Ishirkov defends his doctoral dissertation on "South Bulgaria" with Professor Ratzel. Ishirkov has laid the foundations of many of the geographic disciplines at Sofia University, including biogeography. Using the concept of classical German geography, which includes three main sections – physical geography, anthropogeography and biogeography, an analysis of the biogeographic component of academician Anastas Ishirkov's scientific work is made.

**Conclusion:** When Ishirkov developed his scientific work, anthropogeography was seen as part of biogeography and, despite the later separation of anthropogeography, it remains a human science.

**Keywords:** biogeography, anthropogeography, evolutionary theory

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## Introduction

This year, on the fifth of April, the 150th anniversary of the birth of academician Anastas Ishirkov, who laid the foundations of university geography in Bulgaria, was celebrated. Various events, organized by scientific societies, faculties and institutes were devoted to that anniversary. Academician Ishirkov's work is a reflection of the historic period, in which he lives and works – in Europe, scientific geographic intellection develops the geographic determinism, and on the Balkans the different nations are striving to achieve their own national ideal. When the contributions of academician Ishirkov to Bulgarian geography are considered, the emphasis is usually on historical geography and ethnography, political geography and geopolitics, and geography of population and settlements. In fact, they all could be regarded as part of the anthropogeographic branch of German classical geography. Academician Ishirkov's analysts place second his merits to natural geography (then general physical geography), while the third major section of classical German geography – biogeography, is forgotten or mentioned among others as not a significant part of geographical knowledge.This fact prompted the author to examine the biogeographical merits of academician Ishirkov as the founder of geographic science in Bulgaria.

The main aim of the paper is to analyze the scientific work of academician Anastas Ishirkov and to highlight his importance as a founder of biogeography in Bulgaria.

## Academic Biography

The academic biography of Ishirkov and the evaluation of his publishing activity by his contemporaries and his followers in science make up the bulk of the material that draws inspiration for his founding tremendous scientific work, containing more than 300 scientific papers. Anastas Ishirkov (Fig. 1) was born on April 5th, 1868, in Lovech and is the only son in the family of Todor Ishirkov and Maria Hitrova (Ishirkova). His father comes from a wealthy family, which is engaged in kaftan production, but passes away in 1869. Ishirkov was raised by his mother Maria and his grandmother Nedka, his father's mother. During the Russo-Turkish War of 1877-1878 his mother died, followed by his grandmother Nedka soon after that. His raising was undertaken by his grandparenrs on the mother's line. Anastas Ishirkov lived with them until 1884, then he left to study at the Aprilov High School in Gabrovo and in 1888 moved to Tarnovo High School, where he graduated from high school.

After graduating from high school he attended the newly opened Higher Pedagogical Course at the Higher School of Sofia, at the History and Philology Department, where he listened to lectures by Lyubomir Miletic, Alexander Teodorov – Balan and Ivan Shishmanov. He graduated the first class of the Higher School of Sofia in 1891 and was appointed a teacher at the Varna Male Gymnasium where he taudht for one year and then left to Leipzig for a specialization in Slavic Philology.

The end of the 19th century was a period in which Bulgaria needed the formation of an academically educated leading elite. This required providing scholarships of students and graduates for various European universities, and Anastas Ishirkov was one of those who received a scholarship. One of the European university centers at that time, with great

importance for Bulgaria, was Leipzig. The role of the University of Leipzig is undoubtedly illustrated in the speech of one of the most prominent contemporary German Bulgarianists, Prof. Dr. Hilmar Walter (2013) on his election as a foreign member of the Bulgarian Academy of Sciences: "In the Leipzig Alma Mater there were at least 100 Bulgarian youths (with a population of about 3 154 000 people in 1888), distributed into 13 scientific fields, among which natural sciences, law, philosophy, philology and economics were preferred. From these 100 people, later 5 become ministers in their homeland, 20 become professors at Sofia University, 35 are active in the sphere of public education and municipal cultural activity, 15 occupy leading positions in community cultural centers and museums, as well as in the field of economy. In 1929, there are already 34 Bulgarian professors, graduated in Leipzig. In this connection, the following names are usually pointed out – the thracologist Gavril Katsarov, the researcher of antiquity Dimitar Dechev, the classical philologist Alexander Balabanov, the geographer Anastas Ishirkov, the philosopher and literary critic Krastyu Krastev, the botanist Stefan Georgiev, the chemist Pencho Raikov, the agronomist Petar Kozarov, the medics Vatev, Chavov, Karakashev, Sarafov and the dentist Davidov. Alexander Teodorov – Balan, Ivan Shishmanov, Benyo Tsonev, Stoyan Romansky and Mihail Arnaudov played an outstanding role in native philology. The most famous student of those years is undoubtedly the great poet Pencho Slaveikov" [1].

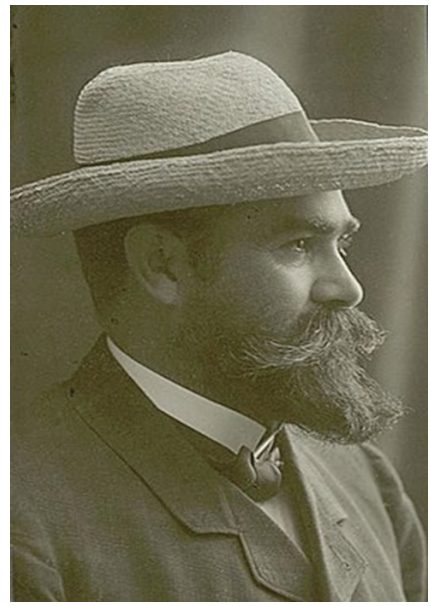

**Fig. 1. Photography of Anastas Ishirkov in Berlin**
(Source: Atelier Wertheim/Sofia University Library)

Anastas Ishirkov got the chance to study in Leipzig along with a plethora of Bulgarians who set the beginning of modern science in Bulgaria. Fate sent him to the lecture hall to attend the geography lectures of the famous scholar Friedrich Ratzel, who from 1866 until

his death is a professor in geography at Leipzig University. It is often said that students resemble their teachers, and in this context it should be noted that Ratzel graduated in Zoology at the University of Heidelberg, but his journeys redirected his research interests into geography. Ishirkov's meeting with Friedrich Ratzel, who for the first time used the term biogeography in 1888 as a theoretical contribution to geographic science [2], played an important role in his scientific bias, as he began to work in the field of geography. Under the leadership of professor Friedrich Ratzel in the period 1894-1895, Anastas Ishirkov elaborated a dissertation on South Bulgaria (Südbulgarien), which he successfully defended in the summer of 1895. Ishirkov showed a certain interest in Bulgarian history and literature as well, and during his studies in Leipzig he wrote 22 stories, tales and poems, some translated into German and French.

Ishirkov returned to Bulgaria and from September 1st, 1895, he was appointed a geography teacher at the Sofia Male High School. To obtain an additional qualification as a lecturer in the newly opened Department of Geography at the Higher School, in 1896 he got a scholarship from the Ministry of National Enlightenment for specialization with Ferdinand von Richthofen at the University of Berlin. Professor Richthofen is also a follower of geographic determinism and was considered to be the originator of geomorphology. He reveals and analyzes the internal connections between rock construction, relief, climate, plant and animal life, population density, human economic activity and its culture at large.

On February 1st, 1898, through a competition, he was appointed a regular associate professor at the Department of Geography and General Ethnography. His introductory lecture on March 19th, 1898, was entitled "Task and content of today's geographic science". From February 1st, 1903, he was appointed as an extraordinary professor and in 1906 he was elected as an active member (academician) of the Bulgarian Academy of Sciences. On June 10th, 1909, Anastas Ishirkov became a regular professor and head of the Department of General Geography and Cultural and Political Geography. Since 1909, in the Faculty of History and Philology, a History and Geography profile was established, where until 1924 geography and history were studied together, and after 1924 geography was separated as an independent specialty. In the academic years 1910-1911, 1918-1919 and 1920-1921 academician Anastas Ishirkov was the Dean of the Faculty of History and Philology. In 1915 - 1916 he was elected the 17th Rector of Sofia University. Since the 1924/1925 academic year, geography was established as an independent specialty at the Faculty of History and Philosophy and the Department of Geography and General Ethnography, headed by Anastas Ishirkov, was renamed into "General Geography and Cultural and Political Geography". At the initiative of academician Anastas Ishirkov, in 1918 the Bulgarian Geographical Society was established, whose chairman he was until his death on 06.04.1937.

Academician Anastas Ishirkov was involved in many political, cultural and scientific missions abroad, including the participation in the Bulgarian delegation for the signing of the peace treaty in Bucharest after the Second Balkan War of 1913 and the participation in the first composition of the delegation for the peace talks in Brest – Litovsk in 1917-1918. He is the author of memoranda to the Paris Peace Conference (1919). He speaks excellent

German, French and Russian and uses Czech, Hungarian, Serbian and English. In 1899 Anastas Ishirkov was the representative of Sofia University at the 100th anniversary of the University of Berlin, and in 1928 he represented the Bulgarian Geographical Society at the 100th anniversary of the Berlin Geographical Society. He undertook different geographical studies in the Balkan and European countries, among which of greatest significance wer those in Serbia, Bosnia, Herzegovina, Montenegro, Greece, Romania, Hungary, Austria, Czechoslovakia, Germany, Poland, Denmark, Norway, Sweden, England, Scotland, The Netherlands, Belgium, France, Switzerland, Northern and Central Italy, the Aegean islands and Smyrna in Asia Minor.

**Review of Scientific Research**

The biographical data on the life and work of academician Anastas Ishirkov are published for the first time in 1933 in a book collection dedicated to the 35th Anniversary of his professorship and appearing as volume №1 of the Bulgarian Geographical Society [3]. Besides these data, the same edition contains a full list of books and papers, written by academician Ishirkov. The main part of his work is arranged in the section "Geography, Ethnography and History", by first mentioning 10 books in Bulgarian, chronologically beginning with "Contribution to the Ethnography of Macedonian Slavs" [4]. The book is a criticism of Cvijić's views on Macedonia's ethnography and is first published in 1906, having a second edition in 1907. Any analysis of these books will diminish the effect of their perception, so it is worth each reader to make himself the relevant analysis and conclusions.

The list contains also books in foreign languages, including 10 in French, 4 in German, and one book per each of the following languages – Russian, Hungarian and Czech.

Altogether 143 articles in Bulgarian language follow in the same section. Brief review of the list of these articles shows that 5 of them refer to climate, 10 articles are devoted to the relief, and 4 are hydrological. Seven papers are published in the Natural Science Journal, of which 3 are biogeographical. Among the latter, there is a classical country study about Norway [5], in which Ishirkov makes an explanatory and comparative geographical analysis of the whole country based on the personal impressions of his journey. Together with the emphasis on the relief forms, which determined the development of the country, Ishirkov also does not miss to mention the biogeographical features such as "the beautiful coniferous forest, which covers large spaces in the low places, rapidly reduces and diminishes at medium altitudes; above 800 m only low birches grow...", comparing the snowbanks in Vitosha in summer with those located just a few tens of meters above the sea in Norway.

Among the articles published in foreing languages most numerous are those in French. The first of the 10 articles in French, which is about 20 pages long, considers the geographical principles based on the natural components that include the vegetation and the animal world. The list follows with 7 articles in German, 2 articles in Russian, 2 in Polish, 4 in English, and one per each of the following languages – Italian, Serbian and Swedish.

The "Criticism and Reviews" section contains 20 publications in Bulgarian and foreign languages. Finally, there are 22 literary articles, arranged in the “Literary Works” section.

This exhaustive list does not include the articles published in Bulgarian and foreign newspapers as well as the elaborated reviews in Bulgarian and other languages, with which the total number of Ishirkov's works exceeds 300.

The introductory lecture on March 19th, 1898, shortly after Ishirkov is appointed an associate professor, entitled "Task and content of today's geographic science" [6], which has already been mentioned, deserves particular attention. Referring to Richthofen, Ishirkov sets out three basic tasks in geography: 1) To explore the solid earth's surface in relation to the hydrosphere and the atmosphere; 2) To explore the earth's vegetation cover and the animal world in their relation to the earth's surface; 3) To explore the man and individual components of his material and spiritual culture in connection with the subject of the first two tasks. Continuing to analyze these three main tasks, Ishirkov writes about the second one: "The ultimate goal of this study is the relationship with botany, zoology and paleontology – to understand and explain causally the present distribution of the entire plant and animal world on the globe".

Ishirkov sets out four principles in geography: 1. The principle of form; 2. The principle of composition; 3. The principle of impact, and 4. The genetic principle. Following the concepts of the classical German geography and in line with the afore-mentioned tasks, he divides the geographic science into three major parts: 1. General physical geography; 2. General biological geography, and 3. General anthropogeography. Shortly thereafter, in his exposition, Ishirkov uses the term biogeography and says: "The link to physical geography is carried out by general biological geography or biogeography".

Analyzing the work of Academician Ishirkov is a laborious task that goes beyond this paper. It is necessary to point out that Ishirkov's numerous researhers consider, above all, the anthropogeographical essence of his work, covering historical geography and ethnography, political geography and geopolitics, geography of the population and settlements, school geography [7], to a much lesser extent the general physical geography, today perceived as natural geography and virtually nothing from the third part of geography, biogeography. We can highlight several objective reasons for this result. Although Ishirkov is a student of Friedrich Ratzel, who introduces biogeography into science, his perceptions are largely related to man or the anthropogeographic essence of biogeography. Here is the point to note that Ratzel's name in the second half of the last century is either not mentioned or interpreted in the extremely negative context of fascist ideology. The reason for this is the introduction by Ratzel of the term "living space" in science. Among the main ways to increase the power of the organism, according to Ratzel, is territorial expansion or the expansion of living space (Lebensraum). The idea is later developed by other scholars and followers of Hitler's ideology, who use this notion to justify the Third Reich's expansive conquest policy. Ratzel dies suddenly in 1904, and hardly imagined that the concept he introduced would gain such unhappy publicity during the Second World War.

Academician Ishirkov does not use the notion of living space in the context of fascist ideology, but the time in which he works is related to the unfulfilled national ideal of the Bulgarians. For this reason, the great part of his work is devoted to the ethno-geographic peculiarities of the lands inhabited by Bulgarians, remaining outside of our state borders. Even in these articles he pays deserved attention to the natural and biogeographical features

of Macedonia, Thrace and Dobrudzha. Academician Ishirkov (Fig. 2) publishes much of his works in foreign languages to promote the unfulfilled national ideal of the Bulgarians and even enters into controversy with the famous Serbian geomorphologist Jovan Cvijić about the ethno-geographical characteristics of the Macedonian population. At the same time, academician Ishirkov is fairly correct, recognizing and quoting Cvijić's views on a number of geomorphological questions regarding the Balkan Peninsula.

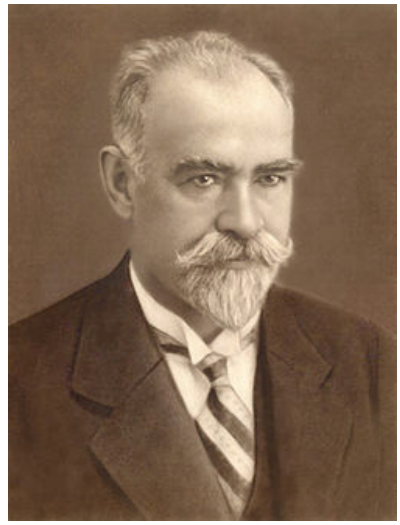

**Fig. 2. Photography of Anastas Ishirkov**
(Source: Honorary Citizens of Lovech Album)

**Conclusion**

Academician Ishirkov's significant scientific work before, during, and after the First World War is the result of his genius insights and a set of circumstances related to his qualities, skills and scientific realization in this period. At the height of the First World War (1915-1916) academician Ishirkov was the Rector of Sofia University "St. Kliment Ohridski", and this is probably the reason not to participate directly in the theater of warfare. In contrast, other ingenious Bulgarians believed they must necessarily fulfill their patriotic duty as participants in the Bulgarian army. This is the case with Dr. Stayko Dimitrov Staykov (1882-1915) [8] – chief assistant at the Central Meteorological Station, one of the most prominent Bulgarian climatologists and seismologists with great reputation abroad. Since 1915 he had been a member of the German and Austrian Meteorological Society. His most important climatologic work is his doctoral dissertation, which he defended and published in Berlin in 1914. In his dissertation, after a thorough analysis of the data from the still small number of meteorological stations, Staykov presented the first isothermal maps for the distribution of the average monthly air temperature at sea level. Of particularly important scientific value was the introduction by Staykov

of the instrumental determination of earthquakes in Bulgaria. He also established the connection between the epicenter's distance and the time of arrival of the first two types of volumetric waves. He was the first scientist in the world to create a formula to determine the depth of the hypocenter of earthquakes. After defending his doctoral thesis, he was invited as assistant professor at Potsdam Institute of Meteorology, but he refuses to take the position because he has to fulfill his patriotic duty as a mobilized lieutenant in the Bulgarian army and went to the front. Despite the special effort of the Supreme German Command, which send a dedicated courier to recruit Staykov from the front line, the courier arrives after a battle, just for the funeral of the lieutenant who died in the village of Kosturino near Strumica.

In the series of facts about ingenious Bulgarians who have given their lives for the fulfillment of the national ideal, we have to mention the poets Dimcho Debelyanov and Peyo Yavorov [9], who also worked at the Central Meteorological Station. Debelyanov served as a "calculator", and for 3 years (26.09.1906 - 10.09.1909) – as a "compiler", checking the registration of the rainfall, temperature and other climatic elements. Second lieutenant Debelyanov fell killed on October 2nd, 1916, and was buried in the courtyard of the Bulgarian church in Demirhisar (or Valovishte, today Sidirokastro). Peyo Yavorov, as a permanent telegraph-post officer in Chirpan, had to record weather and climate, and he also served as a voluntary meteorologist at the Chirpan rainfall station (01.1896 - 03.1897).

Academician Ishirkov is the first professor to lecture on biogeography at Sofia University “St. Kliment Ohridski”, a fact that is not mentioned frequently, and regardless of his anthropogeographic bias, he has given enough attention to the biogeographic research in his work. Despite the modest number of biogeographic articles he has published, in his overwhelming scientific work he adheres to the complexity of each geographic study and in each of his geographic articles he devotes attention to the biogeographical features of the site he is studying.

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# LAZARUS TAXA IN ANIMALIA KINGDOM

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**Abstract**

**Aim:** The main aim of the current research is to investigate the presence of Lazarus taxa in the animal kingdom during the last ten years, following the official information, published by the IUCN Red List.

**Materials and Methods:** The overview study is based on cameral research and the main focus is the Lazarus effect, occurring in the biological kingdom Animalia. The comparative method is applied in order to gain a broader view of the problem. The difference between the Lazarus taxa and the Zombie taxa, Elvis taxa and Ghost taxa is clarified for more explicitness. Some main discoveries of animal taxa are discussed, which is used as a basis for the present investigation.

**Results:** The category of 53 species in the Red List was changed for the last ten years from EX – Extinct to any other category, proving their rediscovery. More than a half of the taxa are fish (53%), followed by Mollusca (15%) and Amphibia (10%). Most of the species are moved to the CR – Critically Endangered catergory, followed by those, included in the DD – Data Deficient category. A more comprehensive information is provided for some of the Lazarus taxa, aiming for more in-depth understanding.

**Conclusion:** The Lazarus effect, leading to the rediscovery of species that were thought to be lost forever, is occuring even today. It is a major scientific achievement to find again a taxon, considered dead, however, we should focus our immediate efforts on the conservation of those species that are already a part of the biosphere, rather than hoping for the miraculous resurrection of the Lazarus taxa.

**Keywords:** Lazarus effect, Lazarus taxa, Animalia, IUCN Red List

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## Introduction

There are processes in the Biosphere that are well studied and clearly defined. At the same time, other occurencies are too vague for scientists to understand and time must pass by if they are willing to become more familiar with the specificity of the processes. And there are the cycles, which lead us to consider the fact that certain organismic taxa are extinct, but unexpectedly we discover them again. The term "Lazarus effect" was introduced in 1983 by two paleontologists: Karl Flessa and David Jablonski [1], it was developed a few years later in 1986 by Jablonski [2] and according to him the "Lazarus effect" should be described as the "disappearance and apparent extinction of taxa that later appeared unscathed" in the fossil record. It is well a established term and widely used for the "new discoveries" of taxa, no matter if they are included in Animalia or any other kingdom. Lazarus taxa are species lineages which have come back from the dead. The whole process of discovering a Lazarus taxon may go through the description of fossil relatives and then it moves to finding the living ones, which often are cryptic species. It is obvious that the extinct species were missing only in scientists' perception, but in fact they have never stopped being an indivisible part of the whole ecosystem. The "effect" is called "Lazarus", because it refers to the biblical character of Lazarus who, according to the Biblical Gospel of John, was raised from the dead by Jesus Christ. The term can be applied to one taxon or to an entire group, as well. Its indisputable paleontological origin is associated with an organismic disappearance that can be possibly be connected to any of the five major extinctions or to a minor one. Sometimes mass extinctions are followed by a period of a lack of certain taxa in the fossil record, which happen to appear again in more recent stages. Many species may become extinct for real, but other may only temporarily disappear when a major biotic crisis occurs. However, the "Lazarus effect" is not only applied in the field of paleontology, where fossils and fossil record are in the center of the studies, but it is also applied in conservation biogeography, as a subdiscipline of biogeography, and ecology. Organisms tend to seek a hideout and group in refugia when ecological conditions are not favourable and the essence of refugium is that it represents a restricted territory where a taxon can remain hidden for a long time. Lazarus taxa may flee to such areas and as Wignall & Benton state [3], the „Lazarus effect" can be related to a poor-quality fossil record or migration to refuges.

According to Fara [4] the fossil record holds the key for those who are willing to make an interpretation of the Lazarus pattern and there are two alternatives at present: a stratigraphic and a biological one. In the first alternative Lazarus taxa are a reminder of the incompleteness of the fossil record, while in the second one they reflect genuine phenomenon that is linked with species' extinction, but most importantly - the biological alternative is at present only when the documentation of the stratigrapic one is not possible. The fossil record is not only incomplete, but it is not well studied also and it is expected that many more taxa could be revived from the dead, when strata are investigated in more depth. An applied synonym of a Lazarus taxon is "living fossil", despite the fact that the latter is provoking a conundrum.

Lazarus species are gaining more and more recognition in scientific literature, as well as in popular science publications and they can be considered as a clear demonstration

that some applied measures are giving positive results. One of the most recent scientific works, mentioning the "Lazarus effect" in Bulgarian literature, is the book of P. Beron that is skillfully dealing with puzzles of zoogeography [5]. Following the statement of Meijaard & Nijman [6] if the reappearance of any taxon gains enough publicity, it may lead to the generation of financial and political support, which can be used for a prevention of the possibility the species to become "extinct" again. However, these authors also stress on the fact that publicity may have a negative impact and they provide an example – the famous case of the Sumatran rhinoceros *Dicerorhinus sumatrensis*. In 2013 evidence, proving that the species still exists in Kalimantan, surprised the world. According to the authors, this led to concentration of an unwanted attention on the species that revived the poaching threat by local hunters.

The study of Lazarus taxa is a perfect example of a field, suitable for application of interdisciplinarity. Moreover, the horizontal connections between paleontologists and biologists are required and strongly advised in order to move forward and gather sufficient data. Another good connection is with the scientific field of biogeography, especially when it comes to the geographic area where a certain taxon is discovered again. The current investigation, concerning rediscovered taxa is of a present importance due to the fact that it deals with natural capital even being done in an indirect way. Organisms are providing a great deal of ecosystem services and by discovering them once again, we may be able to receive certain benefits in a sustainable way. An organismic group may become extinct in a random area, but it is possible that is still survives in another territory. Taking in mind the fact that, even though we have studied a major proportion of the Earth's surface, there are still places, which have not been investigated completely, there is a quite real chance that certain taxa may still persist at similar ecological conditions, providing an important ecosystem service for us, neglected until their rediscovery.

It is essential to differentiate the term "Lazarus taxa" from "Elvis taxa", "Zombie taxa" and "Ghost taxa". The term Elvis taxon was introduced by Erwin & Droser in 1993 [7] and it was used to describe brachiopod, bivalve and gastropod taxa. It stands for an organism, whose development is marked by convergent evolution. The species has a morphological similarity to an older lineage, but it is not the same as it and it has been misidentified to reemerge in the fossil record. The term Zombie taxa was defined by D. Archibald in 1996 [8]. They are also called dead clade walking and are famous for being eroded from their original sediment strata and redeposited in a fossil record that may be millions of years younger. This can cause a confusion among scientists, which may lead to making a mistake, concerning the evolution of an organism. A Ghost taxon represents a phylogenetic lineage that is thought to have existed, but there is not any fossil record, proving this statement, while the molecular testimony for its existence emerges indirectly among fossilized organismic remains.

## Materials and Methods

The current study represents an overview article, concerning the presence of Lazarus species in the biological kingdom Animalia. The object of the research is studied by the investigation of the officially published data in the Red List by the International Union

for Conservation of Nature (IUCN) [9] in the chapter "Red List category changes". Several tables are included in this chapter, displaying any changes in a Red List's category and their particular differentiation, which is of central importance for the current investigation – from the EX – Extinct category to any other one, representing the newly discovered presence of a certain taxon. The authors of the present study regard this change to be a signal for the "Lazarus effect". The comparative method is applied in order to gain more comprehension of the problem and the investigated period is ten years – between the 2006-2007 and 2016-2017. But before moving to this section, it is important to focus on some of the most striking examples of the "Lazarus effect" in the animal kingdom.

When it comes to examples of Lazarus taxa, possibly among the best known is the case of the coelecanths – ancient forms of fish. Their lineage was thought to be extinct for 66 millions of years, but unexpectedly a *Latimeria* specimen was discovered in 1938 near the coastline of the Republic of South Africa (RSA). The importance of this discovery is enormous, considering the fact that coelecanths are regarded as an evolutionary transitional form, leading from fishes to tetrapods. But why there is not present a good fossil record for the living species? A possible explanation is that the taxon may have remained hidden in the ocean depths and didn't fossilized, because of the low number of individuals or a lack of suitable conditions for fossilization. Moreover, fishes are the most sensitive group from the faunistic natural capital, therefore every rediscovery of a fish species adds more value to it.

*Balaenoptera omurai* or Omura's whale is a small fin whale, discovered near the island of Madagascar in 2013. It was named just ten years earlier only from dead organisms. It was considered to be extinct, but the recent finding proved that scientists were wrong.

Another example of the "Lazarus effect" is the living rodent *Laonastes aenigmamus* that was described for the first time in 2005. Dawson et al. [10] proved that the initial interpretation of it as a sole member of the new Laonastidae family is incorrect and the rodent is an alive descendant of the extinct rodent family Diatomyidae.

Australian's Lord Howe Island was the home of a peculiar and enormous invertebrate – *Dryococelus australis* that was thought to be extinct since 1920s. However, in 2001 a living population was found on the erosional remnant of a shield volcano, named Ball's Pyramid, which is situated near Lord Howe Island.

Marking these several examples was important for representing the unbrokable chain of discovering Lazarus taxa in the not so distant past, which is essential for the better scientific understanding of the surrounding nature.

**Results**

The Lazarus taxa in the kingdom Animalia for the last ten years, included in the International Union of Conservation of Nature's Red List, are displayed in Table 1 and Figure 1.

**Table 1. Lazarus taxa**

IUCN Red List Categories: EX – Extinct, EW – Extinct in the Wild, CR – Critically Endangered, EN – Endangered, VU – Vulnerable, DD – Data Deficient, LC – Least Concern

| Period | Scientific classification | Scientific name | Common name | Previous IUCN Red List Category | Changed IUCN Red List Category |
|---|---|---|---|---|---|
| 2006-2007 | - | - | - | - | - |
| 2007-2008 | - | - | - | - | - |
| 2008-2009 | Invertebrates | *Partula affinis* | Polynesian Tree Snail | EX | CR |
| | | *Partula nodosa* | Polynesian Tree Snail | EX | EW |
| | Amphibia | *Craugastor milesi* | Miles' Robber Frog | EX | CR |
| 2009-2010 | Fish | 23 species of *Haplochromis* genus | | EX | DD, CR |
| | | *Hoplotilapia retrodens* | | EX | VU |
| | | *Pyxichromis parorthostoma* | | EX | DD |
| | Reptilia | *Cuora yunnanensis* | Yunnan Box Turtle | EX | CR |
| 2010-2011 | Odonata | *Sympetrum dilatatum* | St. Helena Darter | EX | DD |
| | Mollusca | *Beddomeia tumida* | | EX | CR |
| 2011-2012 | Mollusca | *Elimia varians* | Puzzle Elimia | EX | VU |
| | | *Elliptio nigella* | Recovery Pearly Mussel | EX | CR |
| | | *Pleurobema hanleyianum* | Georgia Pigtoe | EX | CR |
| | | *Pleurobema rubellum* | Warrior Pigtoe | EX | CR |
| | | *Pleurobema taitianum* | Heavy Pigtoe | EX | EN |
| | | *Rhodacmea filosa* | Wicker Ancylid | EX | CR |
| | Amphibia | *Adenomus kandianus* | | EX | CR |
| | | *Discoglossus nigriventer* | Hula Painted Frog | EX | CR |
| | | *Incilius holdridgei* | Holdridge's Toad | EX | CR |
| | Aves | *Pomarea mira* | Ua Pou Monarch | EX | CR |
| 2012-2013 | - | - | - | - | - |
| 2013-2014 | Coleoptera | *Allopentarthrum elumbe* | | EX | LC |
| | | *Dryotribus mimeticus* | | EX | LC |
| | | *Hadramphus tuberculatus* | Cantebury Knobbled Weevil | EX | CR |
| | | *Macrancylus linearis* | | EX | LC |
| | Crustacea | *Austrogammarus australis* | Dandenong Freshwater Amphipod | EX | CR |
| 2014-2015 | Amphibia | *Raorchestes travancoricus* | | EX | EN |
| 2015-2016 | Fish | *Ptychochromoides itasy* | | EX | CR |
| | | *Rhizosomichthys totae* | Pez Graso | EX | CR |
| | Reptilia | *Ameiva major* | Martinique Ameiva | EX | DD |
| | | *Borikenophis sanctaecrucis* | Saint Croix Racer | EX | CR |
| | Mammalia | *Cuscomys oblativa* | Machu Picchu Arboreal Chinchilla Rat | EX | DD |
| 2016-2017 | Reptilia | *Celestus occiduus* | Jamaica Giant Galliwasp | EX | CR |

Source: IUCN Red List™ (http://www.iucnredlist.org/about/summary-statistics)

There are some periods (2006-2007, 2007-2008, 2012-2013) when no Lazarus taxa were discovered, but this isn't the situation during the rest. After a quick view at Table 1, it

becomes obvious that the total number of Lazarus taxa for the last ten years is 53. The highest number can be investigated in 2009-2010 when a total number of 26 species were excluded from the EX – Extinct category and the majority of them (23 species) are representatives of the *Haplochromis* genus. Overall, the largest proportion of organisms are moved to the CR – Critically Endangered category, which is logical, due to the fact that if a taxon was considered to be extinct, when it is found again, the possibility that it will possess a vast and healthy population, is not high at all. The second mostly used category is the DD – Data Deficient, which follows a similar logic – information about a newly discovered taxon is not comprehensive enough for making stable and accurate assumptions.

More than a half of all Lazarus taxa that are included in the Red List during the last ten years, is belonging to the fish – 53% (Figure 1). This can be explained by the mobility of water taxa and the problems, which taxonomists face, when it comes to differentiating them. Mollusca (15%) and Amphibia (10%) come to the second and third place respectively, while Mammalia, Aves and Crustacea are represented only by 2% each. Several of the Lazarus taxa, presented in Table 1 and Fig. 1, are of a particular interest and the following lines will present a short description of them.

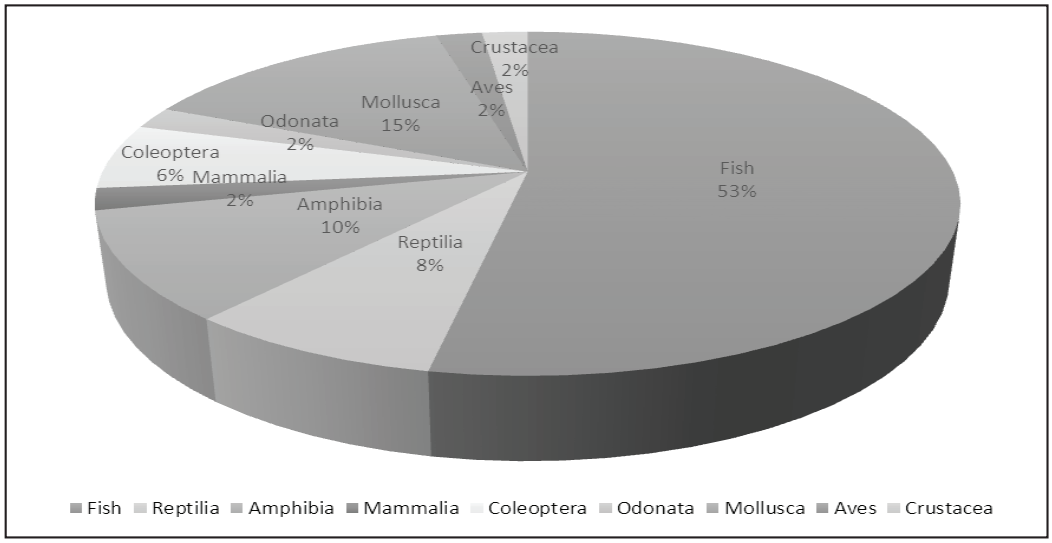


**Fig. 1. Distribution of different taxonomic groups**

*Cuscomys oblativa* Eaton, 1916 or the Machu Picchu Arboreal Chinchilla Rat is a mammal, assessed by Roach [11]. It is known only from two 400 years old skulls that have been collected at an Inca Burial site and then, unexpectedly, in 2014 a living rat specimen was captured in a photo about 3 km south of Machu Picchu. Species' habitat and ecology is unknown, as well as its current population size.

The fish *Ptychochromoides itasy* Sparks, 2004, assessed by Ravelomanana & Sparks [12] is a species, native to Madagascar that was considered to be extinct for half a century. The last specimen was found in the lake Lac Itasy and that water body is situated in the central

part of the island of Madagascar. A recent discovery of a small population, consisting of few individuals in the Sakay River resurrected this taxon back from the dead. The species is not famous for being a migrant and well oxygenated water is a perfect example for its natural habitat. The biggest threats for the taxon are habitat loss and the introduction of invasive fish species.

*Raorchestes travancoricus* Boulenger, 1891 is an amphibian species that is known to have three synonyms: *Ixalus travancoricus* Boulenger, 1891, *Philautus travancoricus* Boulenger, 1891 and *Pseudophilautus travancoricus* Boulenger, 1891. The taxon is assessed by IUCN SSC Amphibian Specialist Group in 2015 [13]. In 2004 it was rediscovered after being regarded as an extinct species. It occurs in three locations in India's Western Ghats Mountains, while its forest habitat is in continuous decline. The exact localities are Bodanaikanur in Tamil Nadu state, Vandiperiyar and Vagaman in Kerala state and Periyar Tiger Reserve. Isolated shrubs, surrounded by plantations are its habitat and when the breeding season is over it can be found under leaf litter.

The crustacean *Austrogammarus australis* Sayce, 1901 is also known as *Gammarus australis* Sayce, 1901. It is assessed by Preston [14]. The taxon was thought to be extinct before small populations, consisting of few specimens, were rediscovered in Dandenong Mountain Range, Australia (Victoria). It prefers cool freshwater streams with riparian vegetation that is in an intact state and a canopy cover more than 75%. Urbanization and run-off from roads lead to the destruction of the vegetation, as well as the introduction of invasive species.

The avian species *Pomarea mira* Murphy & Mathews, 1928, known by its common name, as Ua Pou Monarch is a part of the Monarchidae family. It was assessed by BirdLife International in 2016 [15]. The species was last recorded in 1985 when two birds were spotted in the Hakahetau valley on the island of Ua Pou in the Marquesas, French Polynesia. Then in 2010 an adult male was observed by a walker. It is an insectivorous species, preferring high elevations. However, the Marquesas Islands have been devastated by fire and grazing, leading to the reduction of the dry forest, forming a perfect habitat for the taxon. The black rat *Rattus rattus* is another factor that stays behind the decline of the species' population.

**Discussion**

The overview article sheds light to a problem that is gaining more and more popularity among human society. The "Lazarus effect" is a phenomenon, which is being well recognized by the scientific world and it is adding a little bit of peculiarity to the work of scholars. Lazarus taxa are bound to the fossil record and to the field of paleonthology, but they are also a part of studies in ecology and conservation biogeography. It is quite essential to distinguish them from Elvis taxa, which are similar to them, but not the same, from Zombie taxa and from Ghost taxa, as well. Even today new Lazarus taxa are being discovered and the prove for this is the total count of 53 species, that are moved from the EX – Extinct category to EW – Extinct in the Wild, CR – Critically Endangered, EN – Endangered, VU – Vulnerable, LC – Least Concern or DD – Data Deficient in the IUCN

Red List during the last ten years. This is a testimony that nowadays the addition of new information in science is not an uncommon event at all. The majority of the animal species are moved to the CR – Critically Endangered category, which is a backing up of the common logic and they are strictly followed by taxa, included in the DD – Data Deficient category. The highest share of the species is included in the group of the fish – more than a half of all organisms, while mammals, cructaceans and birds are at the bottom of this classification.

But what can be the overall thoughts when it comes to Lazarus taxa? Can their rediscovery be regarded as a symbol of hope, providing optimistic data that conservation measures work, even from an abstract point of view? Every effort from ecologists, who are trying all their best to save the existing taxa, may be pointed in another direction and provide help for finding once again and protect species, they weren't focused on in the very beginning. The pure fact that a species, regarded as an extinct, comes back from the dead, is fulfilling us with optimism. However, if a taxon, considered forever lost, is found again, the possibility that it has a very restricted population, consisting of few individuals, is extremely high. And this is only one side of the coin. We should strive for protecting these species, in order to keep them for the future generations. The fact that a new Lazarus taxon is discovered is inspiring, but in a broader perspective, its importance for the ecosystem and its significance, as a natural capital, would probably be similar as the one of any other, already known, critically endangered species that has a small population. Moreover, although it represents a real scientific accomplishment and even a breakthrough, it is likely that the species will be lost in the near future and this time it will be forever, if series of conservation measures are not quickly adopted.

The authors advice that conservation efforts should be focused at ensuring the survival of as many species, as possible and if a Lazarus taxon is discovered again, this will surely be considered as an important achievement, which however, wouldn't be a fact, if we had done enough to prevent its former "extinction".

A possible extension of the current study involves narrowing the range of the application of this subject to the territory of Bulgaria. This could spark the interest towards the search of eventual presence or lack of Lazarus taxa in the animal kingdom in our country and if the answer of this riddle is a positive one, it could set up Bulgaria among the few places in the world that can be entitled "Lazarus taxa area". A possible group of organisms that can be suitable for this investigation are those belonging to the Arthropoda phylum, because of their variety, smaller dimensions and a wide range of habitats.

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# RADIOLOGICAL MONITORING OF DRINKING WATERS IN BULGARIA

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**Abstract**

**Aim:** Radiological monitoring of drinking water in Bulgaria is carried out every year. One of the main indicators under control are uranium concentration and gross alpha activity with maximum acceptable values (MAV) of 0.06 mg/l and 0.5 Bq/l, respectively until 28.11.2015 and 0.03 mg/l and 0.1 Bq/l respectively from 28.11.2015 when the last amendments in legislation entered into force.

In the context of the last amendments and variability of these indicators over time, it was of interest to analyze them for a three years period 2014 -2016. The aim was to present a summarized and updated information about the radiological status of drinking waters in Bulgaria in response to the raised public concern of possible increased radiation dose impact.

**Materials and Methods:** 793 water samples were analyzed in the period 01.01.2014 - 31.12.2016.

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A spectrophotometric method developed and validated by the Testing Laboratory of Radioecology and Radioisotopic Research at ISSAPP "N.Poushkarov" based on the principle of formation of an uranium–arsenazo-III complex was used for uranium determination. Gross alpha activity was measured by low background counting of dry water residue deposited on a planchet under method developed in accordance with ISO 9696/2010.

**Results:** The uranium concentration over the maximal acceptable values was determined in samples from the regions of Burgas, Plovdiv, Rakovski, Haskovo and Velingrad and gross alpha activity in the regions of Burgas, Plovdiv, Rakovski, Haskovo, Velingrad, Petrich and Dobrich. The data was summarized on annual basis. The percentage of samples with values exceeding MAV for the three years period was between 1 and 5 percent for natural uranium and 3 and 14 percent for gross alpha activity. An increase in the percentage of the samples over the MAV for both indicators was observed in 2016. Most of the values exceeding MAV in 2016 were in the range – 0.03 mg/l – 0.06 mg/l for uranium concentration and 0.1 – 0.6 Bq/l for gross alpha activity. After analyses in the Laboratory it was found that in most samples gross alpha activity over MAV is due to the content of natural uranium.

**Conclusion:** In the majority of the samples the values of radiological indicators under study do not exceed the maximal acceptable values. Increase in the percentage of samples with values of natural uranium and gross alpha activity over MAV is observed in 2016 when more conservative MAV have already entered into force. In most samples gross alpha activity over MAV is due to the content of natural uranium and does not impose a hazard for human health from radiological point of view. Relatively high uranium concentration and gross alpha activity are probably due to the specific hydro-geological characteristics of the regions where they were observed as well to the closed uranium mining near them.

**Keywords:** uranium, gross alpha activity, radiological monitoring

## Introduction

The consumption of safe drinking water is essential for human health.

Radiological status of water is determined by the normal presence of natural radionuclides U-238, Th-232, their daughter products and K-40, present in the soils and rocks of the aquifer, which in turn depend on the local geology of each region in the world [1]. At the same time, mining, as well the use and production of mineral fertilizers and sands can contribute to the technogenic increase in the content of natural radionuclides.

Most common way of protecting people's health from the adverse effects of drinking water contamination is the continuous or periodic monitoring of water quality indicators.

To ensure that drinking water is safe for human consumption, minimum water quality requirements are set in Directive 98/83/EC [2]. National legislation is fully harmonized with EU Directive and is in the process of implementation. The normative act regulating this area is *Ordinance No. 9 on the quality of water for drinking and household purposes* [3].

One of the main radiological indicators under control are uranium concentration and gross alpha activity with maximum acceptable values of 0.06 mg/l and 0.5 Bq/l respectively until 28.11.2015 and 0.03 mg/l and 0.1 Bq/l from 28.11.2015 when the last amendments in Ordinance No. 9 entered into force.

In the context of these amendments in legislation and the variability of these indicators over time, it was of interest to analyze them for a three years period 2014–2016. The aim

was to present a summarized and updated information about the radiological status of drinking waters in Bulgaria in response to the raised public concern of possible increased radiation dose impact.

**Materials and Methods**

Testing Laboratory of Radioecology and Radioisotopic Research at ISSAPP "N. Pushkarov " has been accredited by EA "BAS" under BDS EN ISO/IEC 17025: 2006 for determining rdionuclides in water, food, foodstuffs and soil since 2002.

793 water samples were analyzed in the period 01.01.2014 - 31.12.2016.

The spectrophotometric method developed and validated by the Laboratory based on the principle of formation of uranium–arsenazo-III complex was used for uranium determination.

Gross alpha activity was measured by low background counting of dry water residue deposited on a planchet under method developed in accordance with ISO 9696/2010 [4].

**Results and Discussion**

Uranium concentration (Fig. 1) and gross alpha activity (Fig. 2) over MAV were determined in some of the water samples.

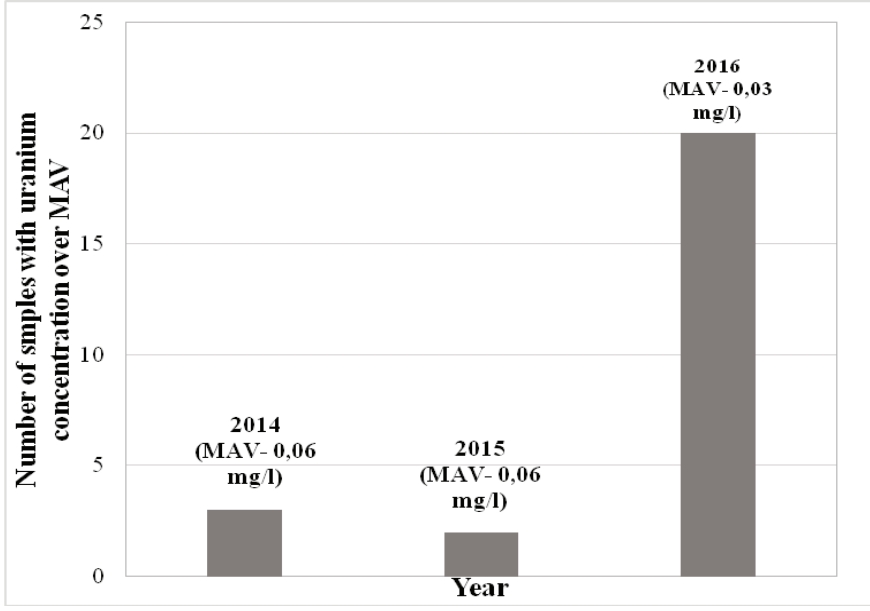


**Fig. 1. Number of samples with uranium concentration over the maximum acceptable value (MAV) per year**

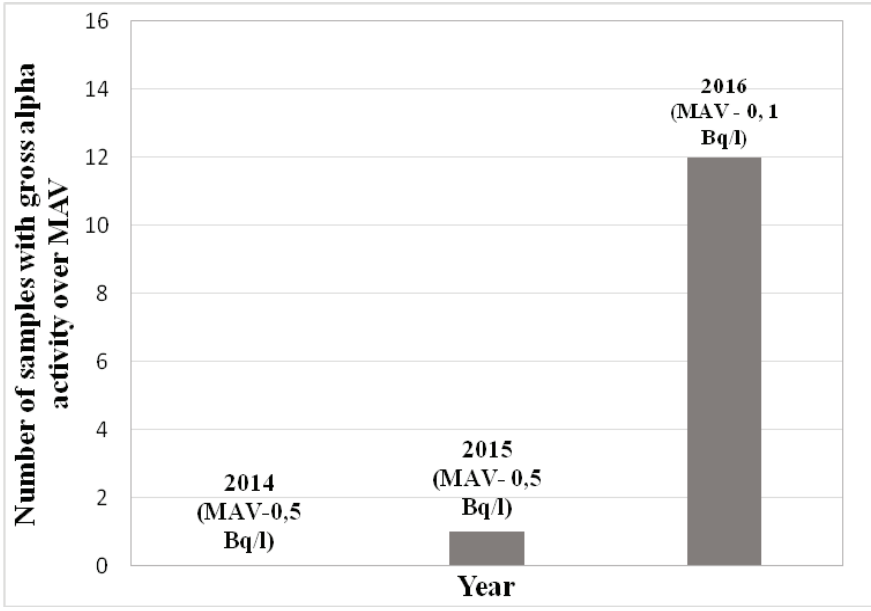


**Fig. 2. Number of samples with gross alpha activity over the maximum acceptable value (MAV) per year**

As it is shown in both figures the number of samples with values exceeding MAV increased after the adoption of more conservative maximum acceptable values for natural uranium (two times lower) and gross alpha activity (five times lower) in Ordinance 9 from 28.11.2015.

The percentage of samples with values over MAV was calculated on annual basis (Fig. 3 and Fig. 4).

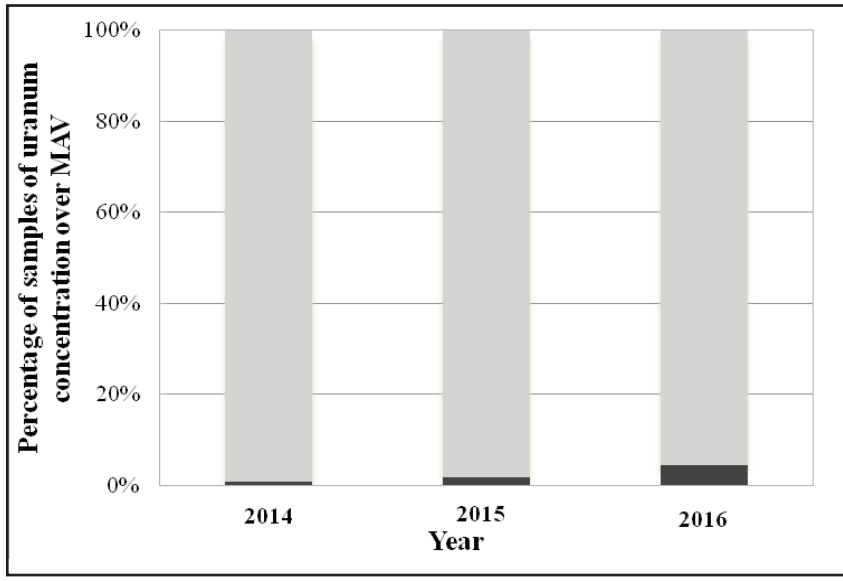


**Fig. 3. Percentage of samples with uranium concentration over MAV from the total number of samples per year**

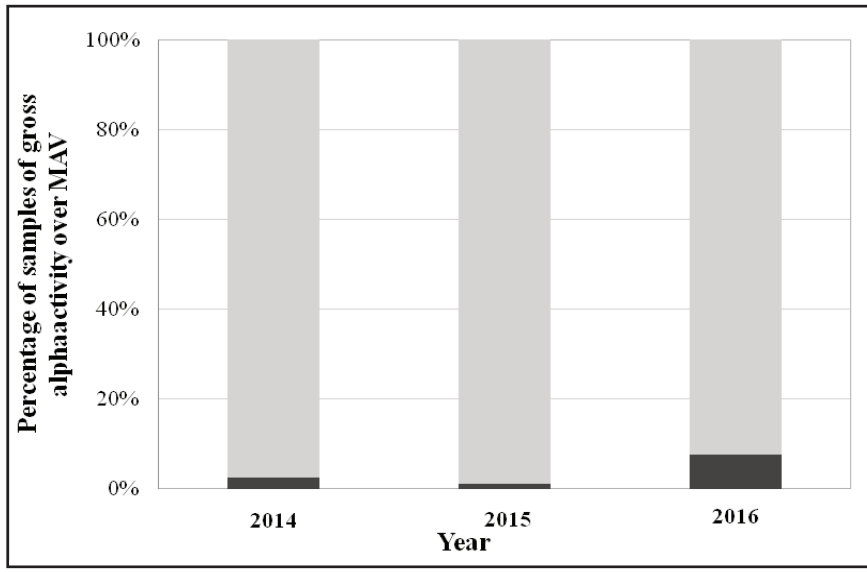


**Fig. 4. Percentage of samples with gross alpha activity MAV from the total number of samples per year**

An increase in the percentage of both indicators over the MAV was observed in 2016. Most of the values over MAV were in the range – 0.03 mg/l – 0.06 mg/l (MAV until 2016) for uranium concentration and 0.1 – 0.6 Bq/l (MAV until 2016) for gross alpha activity.

According to Ordinance No 9 when gross alpha-activity is lower than 0.1 Bq/l, the indicative dose is assumed to be lower than the maximum acceptable value of 0.1 mSv. When gross alpha activity is over 0.1 Bq/l further radiological research is required for establishing the content of certain radionuclides.

After analyses carried out in the Laboratory it was found that most of the values of gross alpha activity exceeding MAV are due to the content of natural uranium measured in them which is not hazardous for human health from radiological point of view.

Samples with relatively high uranium content are from the regions around Burgas, Plovdiv, Rakovski, Haskovo and Velingrad, and those with relatively high gross alpha activity from – Burgas, Plovdiv, Rakovski, Haskovo, Velingrad, Petrich and Dobrich.

It could be assumed that the increased content of natural uranium and gross alpha activity in these areas is due to the specific hydro-geologic structure of aquifers in these areas as well to the presence of sites associated with past uranium mining near them.

## Conclusion

1. In the majority of samples analyzed the content of radiological indicators under monitoring does not exceed the maximum acceptable values according to Ordinance 9.

2. Increase in the percentage of samples with values of natural uranium and gross alpha activity over the MAV is observed in 2016 when more conservative maximum acceptable values have already entered into force since 28 November 2015.

3. In most samples gross alpha activity over the MAV is due to the content of natural uranium and does not impose a hazard for human health from radiological point of view.

4. Relatively high uranium concentration and gross alpha activity are probably due to the specific hydro-geological characteristics of the regions where they were observed as well to the closed uranium mining near them.

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# REPORTS

## Topic:
## ECOLOGY AND EDUCATION

# SUCCESSFUL IMPLEMENTATION OF PROJECT-BASED LEARNING IN HIGHER EDUCATION: AN EXAMPLE FROM ECOLOGY

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**Abstract**

In recent years, project-based training has been successfully applied at the Faculty of Biology of Sofia University "St. Kliment Ohridski ".

The **purpose** of this article is to present a theoretically grounded and tested technology for project-based training at the higher school. The role of this training, the main types of project-based training and the requirements for its proper conduct at the higher school are structured. A specially developed Criteria Assessment System is described. A project-based training has been examined, which was conducted during the academic year 2017-2018 within the discipline Methodology and Technique of the School Biology Experiment with students of the 4th year from the specialties Biology and Chemistry, Geography and Biology and Biology and English. The methods used are theoretical analysis and synthesis, SWOT analysis, questionnaire.

The increased tendency to acquire applied knowledge and skills from students during their studies in higher schools requires the demand for practically oriented pedagogical technologies. By applying the idea of "learning by doing" specific problems for solving are posed to students, the process of learning the scientific approach is assisted in researching and mastering particular methods of analysis.

Putting pre-service teachers at the center of the learning process makes them active subjects in the process of learning and benefits their personal progress. The use of these methods is appropriate and is also applied successfully in a traditional environment. Through the project-based training, the relationship "duration of activity – durability of knowledge" is realized and a connection between subject areas and elements of real life is established.

Project-based learning motivates students by engaging them in the process of their own learning; gives them the opportunity to integrate knowledge from different fields of science and apply them to the development of a specific educational project. Thanks to this, students are active participants and partners in the learning process.

**Keywords:** project-based learning, learning by doing, higher education, pre-service teachers

The dynamic global socio-economic changes influence the priorities, objectives and paradigms of education as well as the forms and methods of learning. The training process

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recommends the forms and methods that develop the learner's personality and activity. Students learn independently, search for and discover information, formulate problems, apply their knowledge, skills and competences to solve them. In the "Strategy for the Development of Higher Education in the Republic of Bulgaria for 2014-2020" one of the problems with the quality of higher education is the lagging of the teaching methods from the innovative tendencies in the practice and the development of the students' abilities.

With the adoption and implementation of the Law on pre-primary and school education (01.08.2016), innovative fields are emerging, which are normative supports and reflections on the quality of higher pedagogical education, and hence on research approaches, methods and technologies of education in an academic environment. In defining the objectives (article 5), normative requirements important for the present article are found: acquiring competencies for applying the principles of sustainable development; the formation of sustainable attitudes and motivation for lifelong learning; acquiring skills to understand global processes, trends and their interrelations. The law regulates a system of concepts that underlie a number of future reflections on academic teaching.

Project-based learning, as part of research training, seeks to provide an answer within the limits of research, how to teach knowledge that has immediate practical application and fundamental knowledge. Through the project-based training the unity of training and research is realized i.e. learning based on up-to-date science or projects and active engagement of students in the research work. In recent years, project-based training has been successfully applied at the Faculty of Biology of Sofia University "St. Kl. Ohridski ".

This paper presents a theoretically substantiated and approbated technology for project-based training at the higher school. The role of this training, the main types of project-based training and the requirements for its proper conduct at the higher school are structured.

**Project-based learning as a personalized learning development**

Project-based learning is a technology built on the project method proposed for the first time by J. Dewey [1] and W. Kilpatrick [2]. It is based on the idea of learning by doing. As a result of a bibliographical analysis of the development of pedagogical ideas for project-based training, it is necessary to conclude that there is a wealth of well-known theoretical orientations that have long been known and preserved, which can find private-didactic interpretations and application in the teaching practice of future teachers, including biology. Pedagogical psychology has developed the questions about the content, the stages and the functions of the project activity in the training, as well as the mechanisms of management and its formation. These ideas are successfully applied in pedagogical practice [3, 4, 5, 6, 7, 8, 9]. In learning, project activity has a different place – as a learning objective and as a tool for complete personalized learning [3, 4, 10, 11]. As a research technology, it is based on the interdisciplinary competency training planned by the lecturer in order to develop skills for high integrity in the problematic situation [4, 9, 10, 11, 12, 13]. The project-based technology of research training is an activity-motivation process that is pre-planned, realized and managed by the teacher in a learning academic environment [4, 12, 13, 14, 15].

Project-based learning is a comprehensive type of personalized developmental learning based on creative acquiring of knowledge in the process of self-seeking activity and the idea of knowing, constructing, studying and evaluating the results of a completed project

[4, 9, 10, 11, 15, 16]. It is aimed at developing learning projects as a materialized product of project activity [4, 11, 12, 13, 14, 15 ].This training involves a specific design and philosophy of the educational process [4, 6, 9, 15, 16].

It is based on constructivist design – it is related to the construction and reconstruction of analytical cognitive structures (circuits) in the process of mastering the experience, activities and knowledge and adapting the varying intensity [4, 7, 11, 15]. The main "elements" of project-based learning based on constructivist design are active discovery, inclusion, engagement [3, 4 , 9 ,10 , 11, 15, 16].

Student projects can generally be classified according to the following criteria: according to the didactic goal; the content of the study material; purpose of using biological (including environmental) knowledge; result type; the degree of use of information and communication technologies in students' cognitive activity (as learning aid technologies).

**Technology of project-based learning in an academic environment**

(an empirical study)

*Conceptual design of research training*

Cognitive activity is a highly motivating factor in research training. It is almost impossible to enter the contemporary pedagogical and educational environment without it and without developed creative skills. In the sense of continuing education and self-education, the management of cognitive activity is combined with the autonomy of personality and responsibility in decision-making in the conditions of risk and dynamic search for solutions. That is why the pedagogical research approach to the organization of the learning environment is aimed at planning tasks for the development of students' creative qualities, stimulation of cognitive activity, autonomy, self-education skills and readiness for inclusion, creation and development of innovative activities and pedagogical research.

Project-based learning is a way of actively and effectively managing research activities by developing skills for working with concepts, managing learning and research processes, delivering results; a form of organization of the learning process in which students acquire skills for planning and performing practical tasks.

The current empirical study is based on actively observed and systematically tracked scientific experience in academic teaching of pre-service biology teachers.

Project-based training, which was conducted during the academic year 2017-2018 within the discipline of Methodology and Technique of the School Biology Experiment with 4$^{th}$ year students from the disciplines/specialties, Biology and Chemistry, Geography and Biology and Biology and English.

The methods used are theoretical analysis and synthesis, SWOT analysis, questionnaire.

*What are the objectives of the project-based training (including ecology)?*

There are three groups of objectives in project-based environmental education: Cognitive objectives to explore the objects of the surrounding reality, to study the ways to solve problems, to form and apply skills to work with sources of information, to set up and carry out experiments.

Objectives of the organizational activity – for applying skills in self-organizing, goal setting, activity planning, group work, and acquiring and application of discussion techniques.

Cognitive objectives to build up constructing skills, as well as modeling and design skills in the field of ecology in the pre-service biology teachers (Fig. 1).

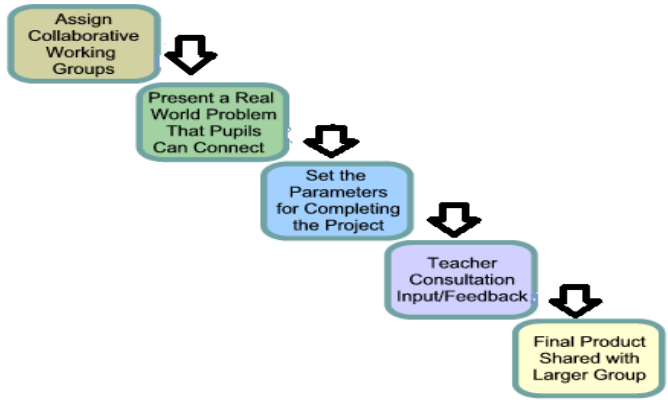


**Fig. 1. Stages of project-oriented strategy, according to the objectives of the project-based ecology training**

*What are the stages of project-based training in the field of ecology?*

Project-based education in the field of ecology education takes place in the following stages:

1. Motivation stage, which is related to creating students' interest in the upcoming activity.

2. Organizational stage during which teams are created, roles distributed within the team, the project topics are assigned. The specific case described is Mesocosm (one of the options is presented in Appendix 1).

3. An indicative stage during which students understand the objectives, tasks and basic requirements of the activity, the final product and its properties. At this stage, a system of actions to achieve the objectives is being developed.

4. Implementation stage in which the task is carried out in accordance with the action program. When describing the project-based training (with the topic "Mesocosm"), the students conduct an experiment (for 4 months) and document the results of their observations in a specially developed protocol accompanied by video or photo material.

5. Control stage in which the product's compliance with its requirements is checked.

6. The presentation of the project is a stage that includes a presentation of the result obtained, presentation of the process of activity from the origin of the project idea to the receipt of the final result, the argumentation of the decision taken and a set of used sources (self-reflection).

7. Discussion, collective argumentation, reflection, self-assessment, expert assessment and conclusions follows.

The presentation of the project is in front of the college students of the respective specialty and the coordinator-teacher. The presentation can be in different form – computer presentation with photos, video tutorial, photo album or poster.

*What is the criterion system for the projects assessment?*

The evaluation of the projects is done through an evaluation card, taking into account the achievements of each team representative in terms of knowledge, skills, competencies and value orientations. Criteria are announced in advance. They include content, technology and presentation aspects that give a quantitative value to the relevant indicator (1 to 5 points). The developed check-lists are useful not only for evaluation but also for self-assessment.

The assessment grade can be either group or individual. Project evaluation criteria are divided into the following groups:

- Argumentation of the choice of the topic, practical direction and significance
- Volume and completeness of information used, autonomy and project completion
- Argumentation of decisions and conclusions
- Originality and creativity
- Project structure
- Quality of the project design (in accordance with the preset product requirements)
- Quality of the project activity process
- Quality of process and product presentation

The results of a questionnaire conducted after the training show that the application of the project-based technology in the university education leads to: development of the research skills of the students for the management of the learning process in the application of the project method (72%), development of a pedagogical flair for the selection of training methods (68%), specialized literature and self-study of selected material (82%); organizing reviews and summaries (74%); motivation and stimulation of curiosity (84%); creating conditions for self-realization and development of students' creative potential (78%); formation and development of reflection (64%).

Project-based learning as a model for learning in an academic environment addressed in the empirical research section of the paper proves a verified and approbated system for research teaching, learning and evaluative research reflection.

**Conclusion**

Project-based training includes a specific design and philosophy of the learning process. It is related to the organization of a purposeful activity of the student, in accordance with his/her personal needs and interests. It is based on the idea of leading the educational and cognitive activities of the students in the process of obtaining results in solving practical and theoretically important problems. In the project-based training, knowledge, construction, research and evaluation of the results of a completed curriculum are realized. In it, the student becomes the leading subject of the learning process – chooses the necessary information, determines its necessity, guided by the meaning of the project.

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**Notes:** Strategy for the Development of Higher Education in the Republic of Bulgaria for 2014-2020. Law on pre-school and school education.

**Appendix 1**

A variant of the project Mesocosm

The ability of ecosystems to recover and reach a steady equilibrium can bee asily demonstrated by relatively simple experiments. Since in the eco system there is constant nutrient cycling, it only needs energy in the form of light, and so a system can be maintained practically continuously. Demonstration of these capabilities can be realized for both the aquatic and the terrestrial ecosystems. In this case, an aquarium was used in which water from a small fresh water pond was placed. The aquarium was covered so there was no additional air access. Between the lid of the aquarium and the surface of the water, there is an air layer in which the oxygen and carbon dioxide released by the organisms fall. The water contains components of the aquatic ecosystem: producers (aquaticplants), consumers (animals) and saprotrophs (microorganisms). The number of consumers (rainbowfish (*Poecilia reticulata*) - 2 pcs. and 5 snails (*Planorbis* sp.) was reduced so that no additional food is needed to influence the ecosystem from the outside. At the beginning of the

experimentall indicators of the artificial ecosystem are described. After one month, any changes that may have occurred are recorded.

Aquatic mesocosm

- 5 litre clear glass jar or aquarium tank
- Seal to prevent entry or exit of all chemical substances
- Air containing oxygen and carbondioxide
- Pond water containing autotrophs, consumers, detritivores and saprotrophs
- Mud from bed of pond
- Autotrophs are an essential component, to produce carbon compounds and regenerate oxygen used in cell respiration by organisms in the mesocosm.
- Saprotrophs are also essential, to decompose dead organic matter and recycle nutrients.
- Consumers and detritivores may not be essential, but are a normal part of ecosystems so are usually included. It is unethical to include large animals in mesocosms that cannot obtain enough food or oxygen.

Terrestrial mesocosms are set up at the same principle. They must contain soil, plants and enough water for maintaining the process of photosynthesis [17].

# THE SCHOOL EDUCATION – A CONDITION TO FORM POSITIVE ATTITUDE TOWARDS ENVIRONMENT OR ECOLOGY

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**Abstract**

Recently, in the past years, educational policy marks the outlines of real changes towards environment in the school education. The aim of the school strategy for ecological education is enriching the ecological knowledge and skills of the students. We look for the actuality of the discussed problem in the realization of the Strategy for Ecological (Ecofriendly) education in school as a new component for personal development of the learners.

**Keywords:** ecological education, environment, ecofriendly culture, ecofriendly attittude

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## Introduction

The main purpose in this writing is the problem connected with the idea to form knowledge or attitude towards environment at school. The environment is so important for good and healthy life. Clean nature is one of the most valuable resources which must be protected thinking about human's future on the planet.

During the last few years we see climate changes or global warming, which are the purposes to keep the nature and that is the way to protect the biodiversity, too.

We can declare the things that we do in this case about the fresh environment at our school Kiril Hristov, Stara Zagora town. Ecological education is similar with the responsible attitude towards the environment. At our, Kiril Hristov school the students have the responsibility towards themselves, society or nature.

Theoretical or practical importance of this problem is the main point for our work here.

Our aim is to enlarge students' ecological knowledge and ability through the school strategy for this [1].

The clear aim determines the choice of the next methods: theory, monitoring, lectures, polls, etc.

Using all of these methods, the problem to keep the environment and to learn this at school, is discussed with all the teachers work at school.

The cognitive move shows how students from 1 – 7 grade take part in the activities about the ecological education at school.

The first level unites activities connected with the idea to form the outdoor classroom. The main idea for this is that the students could learn a lot of things in a very new or attractive way in this so called Green classroom. In this way the students could be very close to nature. As the result would be heathy life and explorers [2].

Our students take part in the initiative Thousands trees for Bulgarian children, organized and ruled by Foundation 77. Till now there are more than 170 000 new planted trees in Bulgaria. The Kiril Hristov's students take care for a tree, symbol of eternity.

Students from 1 – 7 grade take part also in:

- National initiative When we became 100 000, we will plant a forest. Here we involve part parents, too. 50 white pines and 50 red oaks, given from the Forestry Stara Zagora.
- The national initiative Forestry for a day, for the day of Bulgarian forest. Students learn more about the forestry like a job and for the ecosystem. With the planting of pine oe cedar saplings they become part of the sustainable environment.
- The day of birds. This event is within the project Wild nature around us: know it and protect it financed by foundation Global library – Bulgaria. Students learn about the rare birds in Bulgaria – vultures. People in the project Light future for the black vulture, LIFE14NAT/BG/649 and from Green Balkans give more information to our students about this protected black vulture, as well as about the king eagle, Lesser Gray kestrel, usual buzzard or white stork. Because the number of these birds is lower and they are rare, that is why they need special cares and protection.

- Blue flag, international foundation for ecological education. The idea is to keep the planet's future
- Exhibitions, photos, lectures, quiz games, projects: European week of mobility – 16/22 September; International day of the Black Sea – 31st October, The world day of wetlands – 2nd February , The Earth Hour – the last Saturday of March, International Birds' day – 1st April, The forest's week – the first week of April, International Earth Day – 22 April, International day of biodiversity – 22 May. Students, who take part in all these, enlarge their ecological knowledge, form positive attitude towards the nature around them and try to keep their nature, countryside.

The second stage in the Ecological education is practical. Using all of these theoretical knowledge we tried to involve students in nature, to increase their willing to work for clean nature. For example, separately gathering the rubbish. In Kiril Hristov School there is a program about this separately rubbish bins.

1. Realise that it is good to have not too much rubbish. There is a lot of rubbish in the world so students must understand that it could be damage not only for the nature, but for themselves, too. We tried to learn students to change their mind about rubbish and its separately pick up.

2. Students promotion for savings. With the main idea to decrease throwing rubbish. It will be good not only for the nature but for their family and school.

3. Extension the subjects' life. Not to buy things which are not useful, but to enlarge their life. They learn to keep the school, to do charity, to make new ones from the old, not so useful.

4. Build a real system for separately rubbish gathering at Kiril Hristov school. Important level in this was the separately insurance of bins and the active students education to use them every day.

The program's aim:

2.1. We try to change students thinking about rubbish and its gathering in a special way at school or at home.

2.2. Build a long – lasting system for separately rule at Kiril Hristov school – Stara Zagora town.

Program's performance

There are a lot of different activities about this:

- We made a poll among the students about their interest to pick the rubbish in different bins.
- There were a lot of information lessons how to do this
- Take part in the European week for decrease rubbish ECHO, in 2016 and 2017 with the work of local people. The main idea is to know how to rule the resources and rubbish.
- There are workshops in the classrooms with the label Let's change the rubbish into something nice

- We organized charity. Subjects were made of useless materials.
- There was exhibition – flowers of recycled materials.
- Students made small ecological town of rubbish.
- There was a competition of the cleanest classroom.
- Students and teachers take part in a happening Let's clean Bulgaria together, BTV Media Group
- Practical activity for separately gathering rubbish outdoor too.
  Due to all these our students realize that they can keep the environment if they united their powers and ideas.
  The third stage of this is connected with students projects, international and national contests, competitions.
- Take part in the Green Olimpics, a huge educational event with ecological idea.
- Take part in the European mobility week. The idea is to understand the damages of the nowadays tendency for town mobility to the nature.
- 60 students take part in Nature Project – Nature – yes – informed support for European ecological net 2000; educated people teach LIFE15 – GIE/BG/000977. Made by LIFE program of the European Union, with the Bulgarian company for keeping the birds and publishing house Prosveta Libry.
- 30 students take part in a project, named The big hunt of plants. This project tried to educate students about biodiversity and its value.
- Students take part in an European project Exchange Christmas decoration, with 17 partners from European countries. Students made toys with the materials which had been used before.
- Part in XXVI International ecological forum Sreburna 2017 – Silistra town.
- Be part in XVI National contest for Nature studies or ecology in Kystendil town.

**In conclusion**, we would say that the most important for our school is towards ecological culture or thinking in our students. To have the best result it, education, must start from the very beginning classes, when pupils grow as a responsible people. The planet future depends on keeping the Earth and its nature values.

Ecological education must be connected with the personal or civil responsibility. Responsibility is the main part of development and social work.

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